Procédé de Liquéfaction, Saccharification et Fermentation Simultanée à Très Haute Gravité utilisant de la farine de manioc pour la production de l'éthanol et valorisation de son sous-produit par la Fermentation en Milieu Solide pour l'alimentation animale

Dinh Vuong Mai

UNIVERSITÉ DE BOURGOGNE FRANCHE-COMTÉ

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Simultaneous Liquefaction, Saccharification and Fermentation process at Very High Gravity using Cassava flour for ethanol production; Valorization of distillery by-product by Solid State Fermentation for animal feeds.

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Luc FILLAUDEAU	Dr, INRAE Research director - France	Reviewer
Anil KUMAR ANAL	Pr, Asian Institute of Technology - Thailand	Reviewer
LE Van Viet Man	Pr, Ho Chi Minh City University of Technology - Vietnam	Examiner
VU Nguyen Thanh	Dr, Food Industries Research Institute - Vietnam	Examiner
TO Kim Anh	Dr, Ha Noi University of Science and Technology - Vietnam	Examiner
Yves WACHÉ	Pr, AgroSup-Dijon, UBFC - France	Co-supervisor
CHU Ky Son	Dr, Ha Noi University of Science and Technology - Vietnam	Co-supervisor

ABSTRACT

A simultaneous liquefaction, saccharification and fermentation (SLSF) process of cassava flour at very high gravity was developed for ethanol production at lab (1 L) and pilot (15L and 1000L) scales. Cassava flour (CF) was mixed with tap water to obtain a concentration of 270 g/L dry matter (DM). Sequentially, the mixture of a native starch hydrolyzing enzyme containing α–amylase and glucoamylase (Stargen 002 at 2565 GAU/kg CF), glucoamylase (Amigase Mega L at 0.105% w/w), an active dry yeast Saccharomyces cerevisiae (Red Ethanol at 3.5 × 107 cells/ml), a KH_2PO_4 (9.9 mM) and urea (16.0 mM) was added into cassava slurry. The SLSF-VHG process including three steps of liquefaction, saccharification and fermentation was simultaneously carried out in the same bioreactor, at 30^{o}C. Under these conditions, the SLSF process finished after 144 h fermentation with an ethanol concentration of 14.4% v/v corresponding to the theoretical ethanol yield of 86.0%. This SLSF-VHG process was scaled up to 1000 L in order to evaluate the ethanol yield, energy consumption, production cost and environmental impact. The results showed that the ethanol concentration of 13.9% v/v corresponding to a yield of 83.1% of the theoretical ethanol yield was obtained. The greenhouse gas emissions of this process were 186.281 kg CO_2eq. By using Response Surface Methodology, the quantity of Stargen 002; Amigase Mega L and yeast inoculation cells was reduced 13.1; 26.6 and 18.0%, respectively. Under these optimized conditions, for the SLSF-VHG process at pilot scale 15 L, the ethanol content obtained was 14.1% v/v corresponding to a yield of 84.0% of the theoretical ethanol yield.

Cassava-based distillers dried grains (CDDG) obtained from SLSF-VHG process and from the ethanol plants using conventional technology in Vietnam was determined for their nutritional compositions. The results showed that the CDDG produced from the two processes had a high content of crude fiber (29.3 and 34.4%, respectively) and a low content of protein (13.2 and 11.9%, respectively). To increase value-added in this potential feed-source, solid-state fermentation (SSF) process was used. The SSF using the mold *Trichoderma harzianum* BiomaTH1 and the yeast *Yarrowia lipolytica* W29 was conducted with CDDG in 8 and 5 days of fermentation, respectively. Under optimal conditions, the crude protein fraction of CDDG fermented by *T. harzianum* BiomaTH1 or *Y. lipolytica* W29 was increased from 11.84% DM for unfermented sample to 15.29 and 14.06% DM, respectively. In addition, the total amino acids of fermented samples using *T. harzianum* and *Y. lipolytica* was increased from 11.01 % DM to 13.86 % DM and 12.39 % DM along with an increase in the essential

amino acids content which enhanced by 55% and 22%, respectively, including the limiting amino acids for pig feeds. The in vitro protein digestibility was improved significantly from 82.5% to 89.2 and 86.9% for the mold and yeast fermentation, respectively.

Keywords: *Simultaneous liquefaction, saccharification and fermentation; Very high gravity; Cassava flour; Ethanol; Cassava-based distillers dried grains; Solid state fermentation; Protein enrichment; Trichoderma harzianum; Yarrowia lipolytica; Animal feed.*

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ABBREVIATIONS

SLSF	Simultaneous liquefaction, saccharification and fermentation
Bx	Brix
CCD	Central Composite Design
CDDG	Cassava-based dried distiller grain
CDDGTH	Cassava-based dried distiller's grain fermented by *T. harzianum* BiomaTH1
CDDGYL	Cassava-based dried distiller's grain fermented by *Y. lipolytica* W29
CDS	Condensed distillers' solubles
CF	Cassava Flour
CMC	Carboxymethyl- cellulase Unit
Cps	Centipoise
DDG	Distillers dried grains
DE	Digestible Energy
DM	Dry matter
EFSA	European Food Safety Authority
FC	Formulation Cost
FGU	Fungal β-Glucanase Unit
GAU	Gluco-Amylase Unit
GHG	Greenhouse Gas Emissions
HCN	Hydrogen Cyanide
IC	Ingredient composition
ME	Metabolizable Energy
Mm	Milimole
nm	Nanometer
PDA	Potato Dextrose Agar
Rps	Revolutions per second
RSM	Response Surface Methodology
SHF	Separate saccharification and fermentation
SMF	Submerged fermentation
SSF	Simultaneous saccharification and fermentation
	Solid state fermentation
VHG	Vary high gravity

v/v	Volume/Volume
WDG	Wet distillers' grains
WDGS	Wet distillers' grains with solubles
w/v	Weigh/Volume
w/w	Weigh/Weigh
YPDA	Yeast Peptone Dextrose Agar

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GENERAL INTRODUCTION

The use of fossil fuels as the main energy resource for industrial activities caused increasingly serious issues worldwide such as environmental pollution and global warming (Demirbas 2009, Vohra, Manwar et al. 2014). One of the potential solutions is to find other environmental friendly, renewable and sustainable energy sources. Among renewable energies, bioethanol is the most widely known. It represents about 40% of the total energy consumption in the world (Tan, Lee et al. 2008, Vohra, Manwar et al. 2014). Moreover, depending on the technology, bioethanol can be produced from a variety of raw materials, including agricultural by-products and low-cost starch sources. Therefore, bioethanol brings important benefits, especially in developing countries. A variety of feedstocks, mostly from the first generation has been used for bioethanol production. The first generation bioethanol involves feedstocks rich in fermentable sugar such as sugar cane, sugar beet, sweet sorghum or in starch such as corn, wheat, rice, potato, cassava, sweet potato and barley (Lee and Lavoie 2013). Vietnam is a South-East Asian country with a strong traditional agricultural which is known for its high rank in cassava exports. According to data reported by the Vietnamese Ministry of Agriculture and Rural Development, the cassava production in Vietnam in 2019 was of 10.1 million tons, almost four times as much as in 2000. Therefore, cassava is an important driving force to realize the strategy of developing bioethanol industry of the government in Vietnam.

Recently, along with the development of biotechnology, new generations of enzyme with outstanding advantages in accelerating starch hydrolysis at low temperature have been introduced. As a result, the advanced technology of simultaneous liquefaction, saccharification and fermentation (SLSF) integrated into very high gravity (VHG) technology has been studied and applied to increase the fermentation efficiency and to reduce energy consumption (Chu-Ky, Pham et al. 2016). However, this method has not been applied successfully yet in cassava substrate.

By-products from bioethanol industry have received a great attention from researchers, manufacturers and governments in the animal feed industry. The question is how to increase value-added in this potential source, improve farm profitability, produce protein material for feed processing industries and reduce dependence on imported raw materials for feed production.

In this thesis, our studies focus on (i) the development of a SLSF-VHG process from cassava flour for ethanol production at lab and pilot scales; (ii) the evaluation of by-products from the SLSF-VHG technology and from the ethanol plants in Vietnam and (ii) protein enrichment of by-products from cassava-based ethanol plant for animal feed

CHAPTER I:

BIBLIOGRAPHIC SYNTHESIS

1. Very high gravity technology and its impact on yeast performance

1.1. Very high gravity – DefinitionAucune entrée de table d'illustration n'a été trouvée.

A higher ethanol concentration after fermentation can be obtained by increasing dissolved solids (>270 g/L) in order to achieve more than 15% (v/v) ethanol compared with 10–12% (v/v) ethanol produced by the conventional technology

Table 1: Comparison between conventional and VHG technologies for ethanol production (Zheng, Zhang et al. 2013)

Parameters	Low or normal gravity	Very high gravity
Feedstock concentration	16–20 g, max. 24 g/100 ml dissolved solids	More than 270 g/L
Ethanol content	10–12% (v/v)	More than 14 % (v/v)
Plant capacity	Fixed	Increased because of more fermentor space created through removal of insoluble matter
Plant efficiency (Labour costs; Energy costs)	Fixed/liter ethanol production	Relatively reduced (about 4% savings) due to less water in fermentor and in still to process; Avoidance of energy loss due to handling insoluble High-productivity ethanol production
Enzymes' (liquefying & saccharifying) activity	Low activity due to high dilution rate	High activity due to decreased starch-to-water ratio
Enzymes' stability	Less stable	Highly stable due to increased substrate concentration
Spoilage bacteria	Acetic and lactic acid bacteria thrive well, decreases fermentation efficiency	Spoilage bacteria cannot survive better under high osmotic conditions
Co-products/by-products	Low protein yeast	High protein yeast

1.2. Yeast stress during fermentation at very high gravity

Under very high substrate concentration (more than 27% w/v), yeast cells are subjected to various stresses during the fermentation process when the sugar level of the medium increases above their normal tolerance limits. Generally, the key factors that lead to a sluggish fermentations and to a decrease in fermentation efficiency include osmotic pressure, low water activity, high ethanol concentration at the end of fermentation and nutrient limitation (Fig.1.) (Panchal and Stewart 1980, Stewart, D'Amore et al. 1988, Jakobsen and Piper 1989, Pátková, Šmogrovičová et al. 2000).

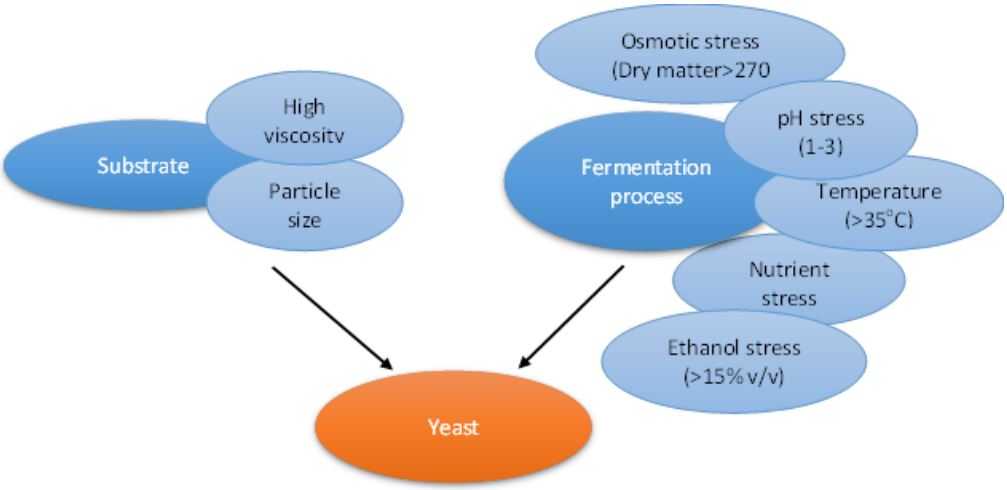


Figure 1: Impact of some prominent stresses on yeast cell under VHG conditions

There are three main possibilities to limit the drawbacks caused by VHG fermentations. The first one is the improvement of ethanol tolerance by genetic modification of strains. Only *S. cerevisiae* strains that are able to tolerate multiple stresses can be made into commercial active dry yeast for ethanol fermentation under stressful conditions (Zheng, Zhang et al. 2013). By genetic engineering, some selected strains of *S. cerevisiae* have been developed for the starch-based industrial ethanol. They possess valuable abilities that are favorable for the fermentation process such as a rapidity of fermentation and a high temperature and ethanol tolerance (up to 18% v/v) (Bellissimi and Ingledew 2005, Mukhtar, Asgher et al. 2010). The second one is the optimization of the fermentation media composition. Many research works have studied the role of the osmo-protectants or the supplementation of various nutrients for yeast growth in fermentation medium (nitrogen sources, calcium, magnesium, yeast extracts…) (Puligundla, Smogrovicova et al. 2011) Finally, the third possibility to limit yeast stress is to improve the fermentation process such as temperature, initial pH, viscosity...

1.3. Factors that could potentially influence ethanol production

1.3.1. Fermentation temperature

The growth rate of microorganisms is directly affected by the temperature. To ensure effective fermentation under VHG conditions, the fermentation temperature should not exceed 30°C (Puligundla, Smogrovicova et al. 2011). According to Liu, the free cells of *S. cerevisiae* have an optimum temperature near 30°C (Liu and Shen 2008). The high temperature is unfavorable for cells growth, becoming a stress factor for microorganism. By using wheat mashes at 36.5% dry matter (DM) for ethanol production, an optimal temperature of 27–30°C has been recommended to obtain more than 20% v/v ethanol within 55 h with the presence of 16 mM urea (Jones and Ingledew 1994).

1.3.2. Initial pH

The ethanol production is influenced directly by pH during the fermentation process. The global charge of plasma membrane can be changed by the concentrations of H^+ in the fermentation broth, which leads to changes in the permeability of some essential nutrients into the cells (Zabed, Faruq et al. 2014). It has been shown that the fermentation time is prolonged when pH is lower than 4 while the production of ethanol is reduced significantly when pH is set above 5 (Staniszewski, Kujawski et al. 2007). Therefore, pH in the range of 4 to 5 can be considered as an optimum range for the production of ethanol using *S. cerevisiae* (Wada, Kato et al. 1979, Lin, Zhang et al. 2012). In addition, the optimal pH of the raw-starch hydrolyzing enzymes which are considered key factors for no cooking process for ethanol production ranges from 3.8-4.5 (Chu-Ky, Pham et al. 2016).

1.3.3. Fermentation time

When the time of fermentation is too short, the growth of microorganisms is insufficient and fermentation is not finished. However, when fermentation time is too long, the microbial growth decreases due to the high ethanol concentration in the fermented broth, causing a waste of investment costs, a reduced economic efficiency and fermentation yield (Zabed, Faruq et al. 2014). In addition, the fermentation time varies depending on the concentration of dissolved substrates. Chu-ky et al. (2016) (Chu-Ky, Pham et al. 2016) developed a no-cooking process of broken rice in normal (180 g/L) and very high dissolved substrates (311.5 g/L) at 30^oC using raw starch hydrolyzing enzymes. Under VHG conditions, the fermentation time increased from 72 h to 122 h, respectively. At low temperature, besides the low ethanol yield obtained, more time is required to complete fermentation. For example,

at 15°C, a fermentation under VHG conditions using molasses substrate utilized only 44.0% of sugar after 240 h. The ethanol content obtained at this temperature was lower than that obtained at higher fermentation temperatures (Jones, Thomas et al. 1994).

1.3.4. Particle size of raw material

The particle size of feedstock is important for the efficiency of ethanol production. A smaller particle size of corn leads to a higher final ethanol concentration. For the conventional ethanol process, a corn particle size of 0.5 mm yielded an ethanol concentration of 12.6 % v/v, while a particle size of 5 mm yielded an ethanol concentration of only 1.62 % (v/v) (Naidu, Singh et al. 2007). For the no cooking process a smaller particle size increases the contact area between substrate and enzyme, increasing the efficiency of starch hydrolysis. This factor varies from 0.3 mm (broken rice) to 0.42 mm (pearl millet) (Gohel and Duan 2012, Chu-Ky, Pham et al. 2016).

1.3.5. Inoculation size

Inoculum rate does not have significant influence on the final ethanol concentration but significantly affects the ethanol productivity and the sugar utilization rate during fermentation (Laopaiboon, Thanonkeo et al. 2007). The fermentation time is reduced considerably when the yeast cell rate increases within a certain range. Breisha reported that increasing the yeast inoculum volume from 3% to 6% reduced fermentation time from 72 h to 48 h for ethanol production using 25% sucrose. It may be associated with the higher sugar-consumption rate of yeast cells by using a higher inoculum levels of yeast (Breisha 2010).

1.3.6. Agitation

Agitation plays an important role in getting higher yield of ethanol during fermentation by (i) satisfying the oxygen demand of a fermentation process; (ii) improving heat and mass transfer capabilities; (iii) maintaining homogeneous chemical and physical conditions in the fermentation medium; (iv) reducing the inhibition of ethanol on cells (Khongsay, Laopaiboon et al. 2012). To synthesize sterols and unsaturated fatty acids which are essential for the integrity of plasma membrane, yeast normally requires oxygen (Marquez, Millan et al. 2009, Landolfo, Zara et al. 2010). A small amount of oxygen has a positive effect on the ethanol tolerance of the yeast cells under VHG conditions, resulting in a better ethanol productivity (Alfenore, Cameleyre et al. 2004). The useful agitation rate is from 150 to 200 rpm for yeast cells in ethanol fermentation under high substrate concentrations (Khongsay, Laopaiboon et al. 2012, Zabed, Faruq et al. 2014). However, the higher agitation

rate (> 200 rpm) is not suitable for ethanol production due to the limited metabolic activities of yeast cells ((Khongsay, Laopaiboon et al. 2012, Mittal 1992).

1.3.7. Minerals

Metallic ions lead to the reprogramming of cellular metabolic network and to the yeast ethanol tolerance during fermentation. According to Yingling et al. 2011, the cassava mash used in high substrate concentration is poor in zinc (4.986 mg/100 g), magnesium (39.92 mg/100 g), and calcium (72.41 mg/100 g) (Yingling, Zongcheng et al. 2011). However, the results of their study showed that, the supplementation of these metallic ions had no significant effect on enhancing ethanol production yield. It was suggested that nutrients released from yeast cells that lost viability and were lysed, contributed to the high yield of ethanol in the absence of any added nutrients (Yingling, Zongcheng et al. 2011).

On the other hand, a high level of ion Zn^{2+} (more than 65.5 ppm) was reported to improve fermentation rates (Rees and Stewart 1999, Udeh, Kgatla et al. 2014). However, supplementing the wheat mash with phosphate, magnesium, or other trace elements (Zn, Mn, Fe, Co, Mo, Cu, B, and I) did not affect the growth rate of yeast cell (Thomas and Ingledew 1990).

1.3.8. Nitrogen sources

The yeast cells are exposed to stress conditions that include a nutrients limitation, especially for free amino nitrogen (FAN). An assimilable nitrogen sources such as urea or yeast extract is necessary for the growth of yeasts and their multiplication. It influences also the ethanol tolerance of yeasts and the rate of ethanol production in the VHG fermented media (Srichuwong, Fujiwara et al. 2009). The supplementation of an ammonium source are shown to enhance the ethanol yield (Yue, Yu et al. 2012, Pinu, Edwards et al. 2014). *S. cerevisiae* is known to be capable of utilizing ammonium ions, which can be directly assimilated into the amino acids. According to Li et al, for VHG of corn mash (340 g/L), yeast extract was found to be the most effective nitrogen source for ethanol production. The fermentation efficiency reached approximately 84.5% v/v after 72 h of fermentation when the yeast extract concentration was controlled at 2% w/v. In another work, the ethanol production from sweet sorghum juice containing 280 g/L was promoted by using 9 g/L of yeast extract (Laopaiboon, Nuanpeng et al. 2009). To reduce the ethanol production cost, yeast extract was replaced by less expensive nitrogen sources under VHG conditions (Li, Wang et al. 2017). Another nitrogen source, urea, has also been reported as able to improve the ethanol yield. *S. cerevisiae* is able to degrade urea to synthesize new complex nitrogenous molecules (Li,

Wang et al. 2017). The impact of urea on fermentation efficiency was investigated by Jones et al. (Jones and Ingledew 1994). In VHG conditions (36.5% dry matter) using wheat mashes for ethanol production, the addition of 16 mM urea was recommended to obtain more than 20% (v/v) ethanol within 55 h under an optimal temperature of 27–30°C. n another work, Li et al (Li, Wang et al. 2017) demonstrated that, under very high dissolved substrate of corn (340 g/L), the highest ethanol yield and fermentation efficiency were obtained for 150 mM urea. If the concentration of urea was less than 100 mM, the nitrogen source might be insufficient for the aerobic growth of *S. cerevisiae*. For no-cooking technologies for ethanol production from broken rice at 311.5 g/L, the addition of 16 mM urea was used along with the addition of protease to achieve the highest fermentation yield (Chu-Ky, Pham et al. 2016). Proteases were used to hydrolyze the proteins present in the grains into peptides and amino acids crucial for yeast growth (Duan, Dunn-Coleman et al. 2007, Chu-Ky, Pham et al. 2016).

1.3.9. Phosphorus sources

Inorganic phosphate, in combination with organic compounds in the cells, plays an essential role in many biochemical reactions of yeast cells. Multiplying yeast cells require phosphate to build the enzyme system necessary for alcoholic fermentation. The impact of medium constituents on ethanol production has been investigated by the Plackett-Burman statistical design using glucose 100 g/L. Izmirlioglu demonstrated that the addition of KH_2PO_4varying from 0.5 to 3 g/L had negative effects on ethanol production. The more KH_2PO_4 was added in the fermentation medium, the less ethanol concentration obtained (Izmirlioglu and Demirci 2015). However, at low phosphorus levels, the rates of assimilation of carbohydrate and subsequently of growth rate were low (Schulze 1956).

1.4. Technology for ethanol production under very high gravity

Fig.2. shows the technologies for ethanol production under VHG conditions. Today, thanks to advances in biotechnology, properties and ability of the enzyme to hydrolyze starch at sub-gelatinization temperature have improved. This allows a number of advanced methods for ethanol production under VHG condition to apply.

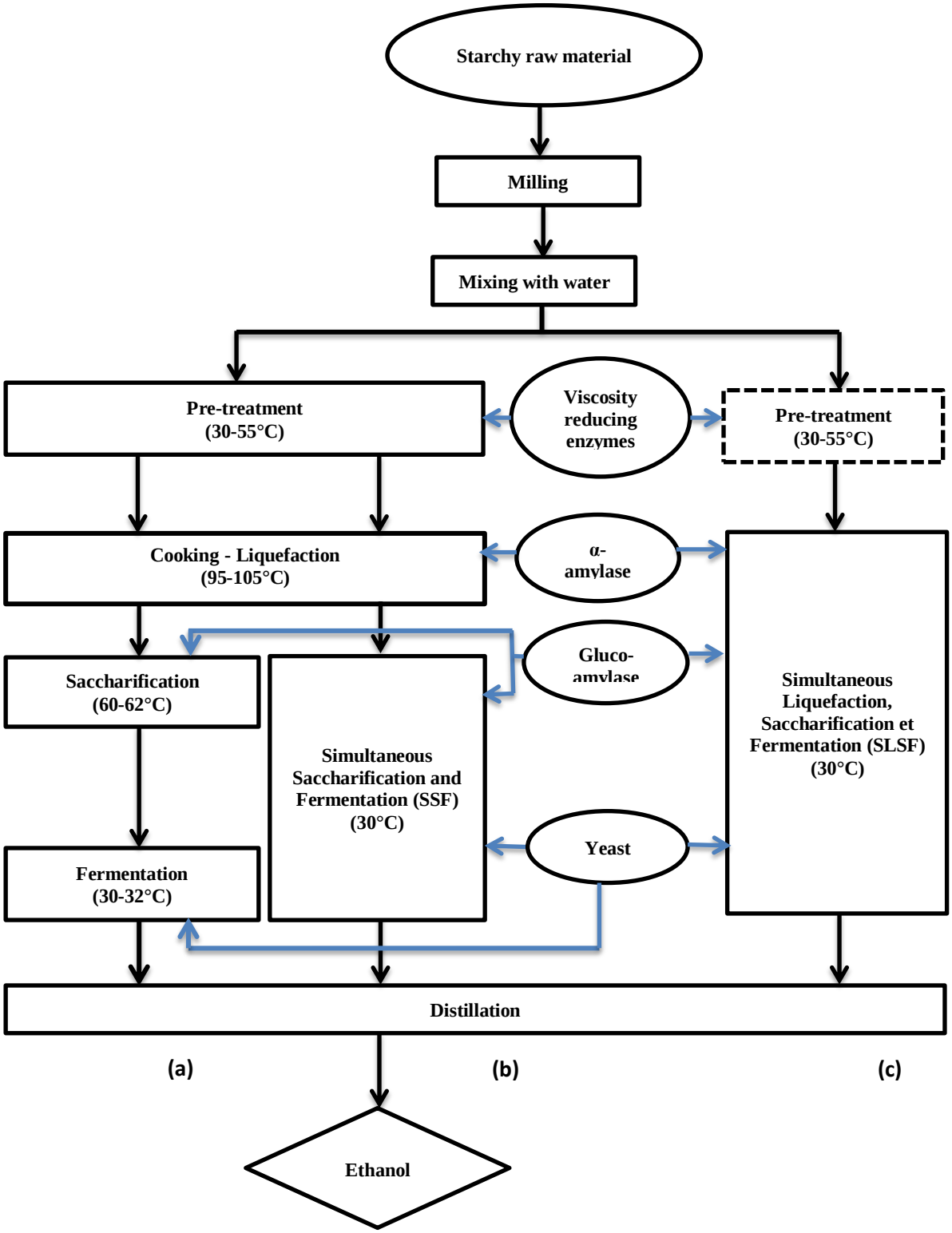


Figure 2: Technologies for ethanol production under very high gravity

(a) Conventional technology

(b) Simultaneous Saccharification and Fermentation (SSF)

(c) Simultaneous Liquefaction, Saccharification and Fermentation (SLSF)

1.4.1. Conventional process for ethanol production

The conventional separate saccharification and fermentation (SHF) consists of three separated steps for ethanol production, namely liquefaction (95–105 °C), saccharification (60–62 °C), and fermentation (30–32 °C) of starch slurry.

Liquefaction is a thermal process, applied to break intermolecular hydrogen bonds in the crystalline areas. During liquefaction, under a high temperature, starch granules are first gelatinized. They absorb water, then swell, release part of the amylose and become more susceptible to enzyme degradation. The gelatinization starts in the amorphous areas. However, the penetration of heat and moisture into the crystalline regions occurs more slowly due to its solid structure. Water weakens the amorphous regions and promotes a loss of organization in the crystallites. Under a high temperature and excess water, the movement of water and heat into the kernel occurs, causing a swelling of starch. As a result, the intermolecular hydrogen bonds are broken and water becomes bound to starch molecules. During swelling process, some amyloses are released from the granule. The granules expand to many times their original size and lose their integrity completely at a near boiling point. This increases greatly the surface area and enzyme susceptibility of the starch. The presence of free water is of critical key in starch gelatinization. Besides thermal treatment, starch gelatinization can be accelerated by mechanical treatment, chemical agents or various combinations (Lund and Lorenz 1984, Zobel 1984). Non-damaged starch caused by mechanical treatment has a low susceptibility to enzyme degradation (Pomeranz 1976). Alkali and acid promote the starch gelatinization process with or without heat treatment (Pomeranz 1976).

Then liquefied starch is converted into short chain sugars by a thermostable α-amylase. The higher efficiency of liquefaction is obtained when the temperature of α-amylase hydrolysis is close to the optimal for the enzyme activity (Pietrzak and Kawa-Rygielska 2015). According to Ebrahimi et al. (Ebrahimi, Khanahmadi et al. 2008), an increased amylase activity obtained by increasing temperature can accelerate further hydrolysis of dissolved oligosaccharides, resulting in a higher DE.

To complete starch hydrolysis of a raw starchy grain substrate, it has been reported that 2 h of liquefaction process is necessary to obtain D.E of 20-36. A shorter time of liquefaction process (0.5 or 1 h) leads to an inefficient hydrolysis into glucose polymers (Neves, Kimura et al. 2006). Under VHG condition, an agitation is absolutely necessary for

liquefaction process to enhance the interaction between starch and α-amylase and to reduce viscosity (Montesinos and Navarro 2000).

Because the process of liquefaction carries out at high temperature, heat-stable enzymes are preferred. The addition of NaCl, $CaSO_4$, $CaCl_2$, NaH_2PO_4, and Na_2HPO_4 helps activate and stabilize these enzymes. Hydrolysis caused by glucoamylase is fast at initial steps but gradually slows down as a result of the produced glucose-inhibiting effect and the diminishing of reaction substrate (Van Beynum and Roels 1985, Thomas, Hynes et al. 1996, Ebrahimi, Khanahmadi et al. 2008).

Subsequently, under the action of glucoamylase on the liquefied starch granules during **s**accharification, glucose is produced from dextrins (Zabed, Sahu et al. 2017).

Conventional technology requires a higher energy input and investment costs. The gelatinization of starch is reported for a high consumption, up to 30% of the total energy needed for ethanol production (Lee and Kim 1990). Furthermore, high initial sugar concentrations cannot be used in the fermentation step due to substrate inhibition (Shadbahr, Khan et al. 2017). These unfavorably affect an economic aspect of the conventional process, therefore leading to a high operation cost compared to other advanced technologies for ethanol production (Sriroth, Piyachomkwan et al. 2010).

1.4.2. Simultaneous saccharification and fermentation process for ethanol production

In spite of having the same liquefaction process, the simultaneous saccharification and fermentation (SSF) is more favourable than the SHF technology for bioethanol because of a high final ethanol concentration, low energy consumption, and short processing time (Nikolić, Mojović et al. 2009). The temperature used in liquefaction step in SSF varies depending on starchy substrate. According to Pejin et al. (2009) (Pejin, Mojovi et al. 2009), under VHG conditions (ratio of substrate to water was 1/3), the temperature necessary for liquefaction (60^oC) of triticale was lower than the temperature needed for wheat (65^oC). For cassava flour, this temperature was 80^oC (Nguyen, Le et al. 2014). The saccharification and fermentation are performed simultaneously in a same reactor. In this process, glucose released from starch is immediately used by yeast, and remains low during the SSF (Nguyen, Le et al. 2014). Therefore, SSF process eliminates the inhibition of saccharifying-enzymes caused by a high sugars content (Mojović, Nikolić et al. 2006, Öhgren, Bura et al. 2007, Marques, Alves et al. 2008, Nikolić, Mojović et al. 2009).

On the other hand, a same temperature is used for both saccharification and fermentation step. The difference between the optimum temperature for amyloglucosidase

activity (50-55ºC) and yeast growth (30-35ºC) is the critical problem of SSF. Pietrzak and Kawa studied ethanol fermentation of waste wheat–rye bread under VHG conditions (Pietrzak and Kawa-Rygielska 2015). An insufficient temperature of the medium led to an uncompleted hydrolysis of residual dextrins due to a loss in activity of glucoamylase (SAN Extra L). This problem could be avoided by increasing the dosage of glucoamylase at the beginning of the fermentation. Table 2 shows the studies on separate saccharification and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) technology under VHG condition using different starchy raw materials for ethanol production.

Table 2: Separate saccharification and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) technology under very high gravity condition using different starchy raw materials for ethanol production

Starchy source	Concentration (g/L)	Enzymes	Ethanol	Process	Ref.
Cassava root	350	Techzyme Q-Add (thermostable α-amylase-Bacillus licheniformis) GC147 (glucoamylase enzyme-B. licheniformis and Trichoderma reesei	104.7 ± 4.1 g/L	SHF	(Sakdaronnarong, Sraphet et al. 2020)
Cassava flour	315.4	Spezyme Alpha (containing alpha-amylase from *Bacillus licheniformis*), Optimash TBG (containing beta-glucanase from *Talaromyces emersonii*) Distillase ASP (containing glucoamylase from *Bacillus licheniformis* and *Trichoderma reesei*); Amigase Mega L (containing glucoamylase from Aspergillus niger)	17.2 %v/v (lab scale) 16.5 %v/v (pilot scale)	SSF	(Nguyen, Le et al. 2014)
Cassava flour	400	Enzyme preparations including a-amylase and glucoamylase	15.03 %v/v	SSF	(Yingling, Zongcheng et al. 2011)
Waste wheat-rye bread	350	Thermamyl 120L (thermostable α-amylase); AMG 300L - (glucoamylase)	350 g ethanol/1 kg of waste wheat bread	SHF	(Ebrahimi, Khanahmadi et al. 2008)
Broken rice and pear millet	300 and 350	Spezyme alpha; Spezyme Fred (Alpha amylase); Distillase APS; Optidex L-400 (Glucoamylase); Fermgen (Protease)	16.23-16.41 % v/v	SSF	(Gohel and Duan 2012)
Waste wheat-rye bread	300	Termamyl SC DS (Thermostable α-amylase); SAN Extra L (Glucoamylase); Neutrase 0.8 L (Protease)	411–425 g ethanol/1 kg of raw material (92–96% of practical yield)	SSF	(Pietrzak and Kawa-Rygielska 2015)
Waste wheat–rye bread	320	Ceremix 2XL (α-amylase, β-glucanase and protease); Ceremix 6X MG (α-amylase, β-glucanase, protease, pentosanase and cellulase)	366 g ethanol/kg raw material	SSF	(Kawa-Rygielska, Pietrzak et al. 2012)
Corn	350	Liquozyme SC (α-amylase) ; Spirizyme Plus (gluco-amylase)	126 g/kg raw material	SSF	(Devantier, Pedersen et al. 2005)
Corn	285.7 (1 kg/3.5L)	Termamyl SC (α-amylase); SAN Extra (glucan 1,4-α); Shearzyme 500L (endo-1,4-βxylanase), Promozyme 200L (pullulanase), Novozyme (cellobiase); multi-	The yield of ethanol: 79.59%	SHF	(Sapińska, Balcerek et al. 2013)

		enzyme complex CeluStar XL (cellulase; endo-1,3(4)-β-glucanase; endo-1,4-β-xylanase)			
Corn	333 (1:3)	Termamyl SC (heat-stable α-amylase from *Bacillus licheniformis*); SAN Extra L (glucoamylase from *Aspergillus niger*)	9.67% w/w-ethanol yield of 88.96%	SSF	(Nikolić, Mojović et al. 2010)
Wheat	350	a-amylase, Allcoholase II (glucoamylase) Neutrase (protease)	17.1 % v/v	SSF	(Thomas and Ingledew 1990)
Wheat	340	High-T^{TM} (high-temperature α-amylase) and Allcoholase^{TM} (glucoamylase)	20.4-21.5% v/v	SSF	(Thomas and Ingledew 1992)
Potato	304	Celluclast 1.5 L (cellulase) , Pectinex Ultra SP-L (pectinase), Viscozyme L (hemicellulose), Liquozyme SC (thermo-stable α-amylase) Spirizyme Fuel (glucoamylase)	16.61% v/v	SSF	(Srichuwong, Fujiwara et al. 2009)
Sweet potato	60-80g fresh/100ml	Liquozyme Supra (thermostable α-amylase); Suhong GA II (glucoamylase); xylanase	Ehanol yield of 135.1 g/kg (90.7% of the theoretical yield)	SSF	(Zhang, Chen et al. 2010)
Rye	310	Roxazyme G (cellulase and xylanase) HT α-amylase Alltech Allcoholase II Novo 348 enzyme: a complex carbohydrase mixture with β-glucanase and pentosanase Glucoamylase)	Ethanol yield of 93–94 % of theoretical	SSF	(Ingledew, Thomas et al. 1999)
				SSF	
Sweet sorghum	350	Fungal solid state fermentation Cellulase (Celluclast 1.5L) and β-glucosidase (Novozyme 188)	Ethanol yield was 85.6%		(Molaverdi, Karimi et al. 2013)
Sorghum	300	Liquozyme SC DC (heat-stable α-amylase) Spirizyme Fuel (gluco-amylase)	Fermentation efficiencies of 86.0–92.2%	SSF	(Yan, Wu et al. 2011)
Sorghum	300	Liquozyme SC DS (heat-stable α-amylase) Spirizyme (gluco-amylase)	Fermentation efficiencies of 85.2-87.9%		(Wu, Zhao et al. 2007)
Oats	336; 423	High-T TM (high-temperature a-amylase); Allcoholase II TM (glucoamylase) and Beta-glucanase from	16% v/v	SSF	(Thomas and Ingledew 1995)

		Aspergillus niger			
Rye	357	GC 626 (acid-stable α-amylase); Optimash™VR (blend of xylanase and cellulase); Stargen 002; Promozyme 200L (pullulanase)	More than 92% of the theoretical yield	SSF	(Pielech-Przybylska, Balcerek et al. 2019)
Triticale	333 (1:3)	Ultrasound pretreatment at 60 °C using available enzymes present in triticale grain for Schharification	Maximum bioethanol content of 9.55% (w/v) or theoretical bioethanol yield of 84.56%	SHF	(Pejin, Mojović et al. 2012)
Winter Barley	300	Spezyme Xtra (thermostable αamylase) and Optimash TBG (β-glucanase) Fermenzyme L-400 (glucoamylase/protease mix)	15% v/v	SSF	(Nghiem, Taylor et al. 2011)

1.4.3. Simultaneous Liquefaction, Saccharification, and Fermentation process for ethanol production

Simultaneous Liquefaction, Saccharification, and Fermentation process (SLSF) or no-cook process has been researched with the aim of increasing ethanol yield and saving energy and investment cost (Kelsall and Piggot 2009, Gohel and Duan 2012, Chu-Ky, Pham et al. 2016). In this technology, three separated steps consist of liquefaction, saccharification and fermentation are integrated into only one step, in a unique bioreactor, at a unique pH and at ambient temperature. α-amylase; gluco-amylase are added simultaneously to the slurry with the presence of yeast. SLSF process minimizes the sugar accumulation in the vessel, potentially increasing yield and concentration of final ethanol (Robertson, Wong et al. 2006, Xu and Duan 2010).

With biotechnological advances in recent years, cold starch hydrolysis for bioethanol production and food research has received a great attention (Wang, Singh et al. 2007, Gibreel, Sandercock et al. 2009, Li, Vasanthan et al. 2012). Enzymes used in this process are known as granular starch hydrolyzing enzymes which are able to directly hydrolyze raw starch granules below the starch gelatinization temperature. Only approximately 10 % of amylolytic enzymes which include α-amylases, gluco-amylases, α-glucosidases, β-amylase and maltogenic β-amylase are able to digest raw starch (Xu, Yan et al. 2016). Several types of the raw starch-digesting enzymes have been produced by microorganisms, including fungi and bacteria (Sun, Zhao et al. 2010). Actually, they are commercially available in the form of enzyme cocktails. A well-known example is Stargen 002. This enzyme cocktail consists of α-amylases and glucoamylases synthetized by *Aspergillus niger* and *Aspergillus kawachi* that work synergistically to hydrolyze granular starch. These enzymes are adsorbed on the surface of starch grain and form holes on this surface where glucose is released (Chu-Ky, Pham et al. 2016). It is noted that, the liquefaction and saccharification require high energy consumption which represents about 30–40 % of the total energy used during starch-based ethanol production (Lee and Kim 1990). By using raw starch-digesting enzymes, this technology reduces the equipment requirements and simplifies the manufacturing process for ethanol production (Sun, Zhao et al. 2010, Robertson, Wong et al. 2006).

Depending on the substrate used for the fermentation, auxiliary enzymes must be used appropriately to increase the fermentation efficiency. In fact, Pietrzak and Kawa (Pietrzak and Kawa-Rygielska 2014) studied a no-cooking process using granular starch hydrolyzing

enzyme for ethanol production from waste wheat–rye bread at 30% w/w. The results showed that the direct conversion process without enzymatic pretreatment of bread waste was less efficient than the SHF process. On the contrary, pretreatment of raw material by a multi-directional enzymatic preparation (Ceremix 6X MG-a mixing enzyme includes α-amylase, β-glucanase, pentosanase, cellulase, protease) showed a higher ethanol yield in comparison to the SHF process. In general, during the no-cooking process, starch is present mainly in the insoluble and non-gelatinized form. This process shows a lower viscosity than that of thermal one (Poonsrisawat, Wanlapatit et al. 2014). Indeed, the previous study of no-cooking process using broken rice at 311.5 g/L showed that no significant change in viscosity was recognized by adding Optimash (β-glucanase). This result could be explained by the fact that, without gelatinization, beta-glucanase did not reduce significantly the viscosity of the mash (Chu-Ky, Pham et al. 2016). Some studies on SLSF process at VHG condition are summarized in table 3.

Table 3: Simultaneous Liquefaction, Saccharification, and Fermentation process (SLSF) under VHG conditions using different starchy raw materials for ethanol production

Starchy source	Concentration (g/L)	Ethanol production	Enzyme used	Ref.
Cassava	60 ml H2O+120 chopped cassava roots	18.0-18.9 g/L	*Aspergillus awamori* NRRL 31 12 and *Aspergillus niger* (cultivated on wheat bran and used as Koji enzymes for xylan and cellulase enzymes)	(Ueda, Zenin et al. 1981)
Barley	300	14.3 % v/v	Stargen 001 (an enzyme cocktail containing *Aspergillus kawachi* α-amylase expressed in *Trichoderma reesei* and a glucoamylase that work synergistically to hydrolyze granular starch to glucose), optimash TBG (beta-glucanase, xylanase and cellulase enzyme complex); Fermgen (protease) Viscozyme barley and viscozyme wheat (viscosity reducing), Liquozyme SC (α-amylase), and Spirizyme fuel (glucoamylase)	(Gibreel, Sandercock et al. 2009)
Triticale and wheat	280-300	13.3-15.0 % v/v	Stargen 001; Optimash TBG; Fermgen Viscozyme Barley and Viscozyme Wheat	(Gibreel, Sandercock et al. 2011)
Cassava roots and cassava chips	320	17.54 % v/v	Enzyme prepared by Solid State Fermentation using *Aspergillus aculeatus* , a mixture containing endoglucanase, FPase, xylanase, polygalacturonase, β-glucanase, and mannanase	(Poonsrisawat, Wanlapatit et al. 2014)
Waste wheat-rye bread	354.36	80.00% ethanol yield	Stargen 002 (an enzyme cocktail containing α-amylases and glucoamylases synthetized by *Aspergillus niger* and *Aspergillus kawachi)*; Neutrase 0.8 L (a bacterial protease form *Bacillus amyloliquefaciensis*); Ceremix 6X MG (a preparation displaying multidirectional substrate specificity: α-amylase, β-glucanase, pentosanase, cellulase, protease)	(Pietrzak and Kawa-Rygielska 2014)
Broken rice	311.5	17.0-17.6 % v/v	Stargen 002, Amigase MegaL (gluco-amylase); Fermgen	(Chu-Ky, Pham et al. 2016)
Rye starch	280.0	94.6 g/L	Stargen 002; GC 626 (acid α-amylase)	(Strąk-Graczyk and Balcerek 2020)

2. Enzymes in Very High Gravity technology for ethanol production: potentials and applications

2.1. Substrates used for ethanol production under very high gravity conditions

The cost of the fermentation substrate significantly influences the economics of the ethanol production. Thanks to the advantages related to the lower investment and operation costs as well as the reduced energy requirement, the use of cost-effective feedstock for VHG ethanol production is important to reduce costs of final ethanol product. Therefore, a wide range of medium substrates, including saccharine and starchy materials, have been utilized in VHG condition (Puligundla, Smogrovicova et al. 2011, Zheng, Zhang et al. 2013).

Different types of biomass are used as raw materials for ethanol production. They are mostly classified into three groups depending on their carbohydrate sources (fig.3): (i) sugar-containing raw materials such as sugar beet, sugarcane, molasses, whey, sweet sorghum, (ii) starch-containing feedstocks such as corn, wheat, rice cassava, potato and (iii) lignocellulosic biomass such as straw, agricultural waste, crop and wood residues (Mussatto, Dragone et al. 2010). The group (i) and (ii) are defined as "the first generation" while the group (iii) is "the second generation". About 60% of the bioethanol production worldwide are produced from starch-containing feedstocks and about 40% are produced from sugar cane and sugar beet (Platform 2010).

Depending on the type of feedstock, the cost of raw material has a considerable impact on the bioethanol production cost, representing from 40 to 75% of the total costs (Li, Liu et al. 2014). The cost levels of bioethanol produced from different energy crops varies significantly. For example, the production cost of bioethanol from sugar cane in Brazil ranges from 0.20 to 0.30 USD/L. In the USA and the European Union where the bioethanol produced mainly from sugar beet and corn, the lowest production costs are in the range of 0.30 and 0.53 USD/L, respectively (Balat and Balat 2009, Festel, Würmseher et al. 2014). In China where the main raw materials for ethanol production are wheat, sweet sorghum or cassava, the costs change from 0.28 to 0.46 USD/L depending on the feedstock costs. In India, the cost of bioethanol production from the sugar-containing and lignocellulose-containing raw materials is around 0.44 USD/L and 1.0 USD/L, respectively (Festel, Würmseher et al. 2014, Zhao, Zhang et al. 2015).

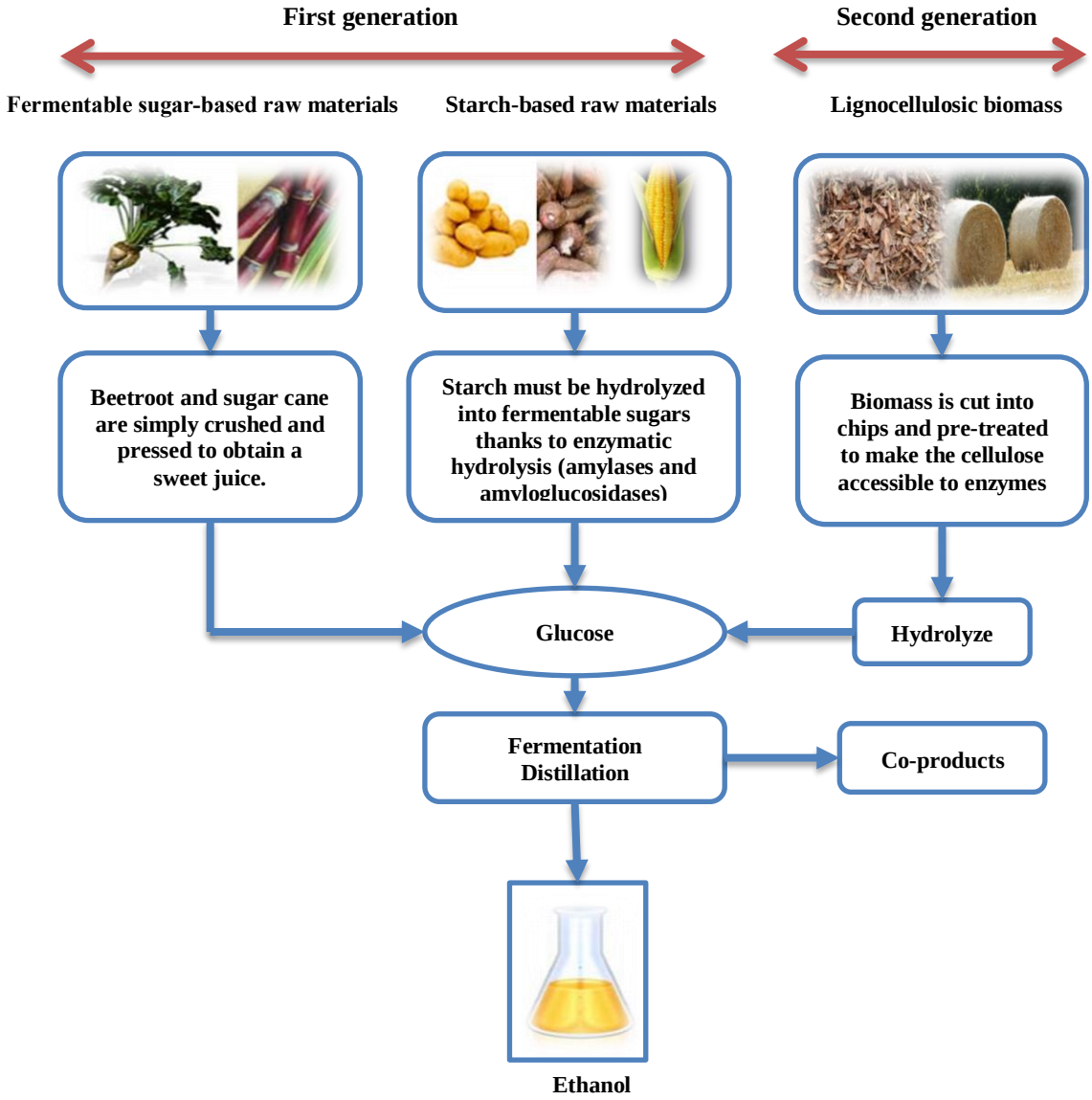


Figure 3: Three generations of biomass used for ethanol production

Sugar cane and sugar beet are the most important sugar crops in the world. They require only a milling process to extract sugars for juice or molasses which are used directly as fermentation medium for ethanol production (İçöz, Tuğrul et al. 2009). Therefore, no pretreatment is required for bioethanol production from these sugar-containing plants, which simplifies significantly the process compared to that from starch-containing feedstock (Linoj, Prabha et al. 2006). The VHG technology has been applied for sugarcane juice of 30 and 35 °Bx and for sugar beet of 300 g/L (Joannis-Cassan, Riess et al. 2014, Monteiro, Ferraz et al. 2018).

The grain and root/tuber crops contain large quantities of starch (Jobling 2004). In the USA, corn is the primary feed grain, accounting for more than 95% of bioethanol production. Besides, sorghum has received an increasing attention from scientists, governments and

farmers in the USA for bioethanol production (Solomon, Barnes et al. 2007). Other starch-containing sources used for bioethanol production in Asia countries are rice and cassava, especially in Thailand, China and Vietnam. Cassava tubers contain nearly 80% starch and below 1.5% protein content. In order to improve handling and transport, fresh cassava is chipped and dried to obtain dried cassava chips which are used more widely for bioethanol production (Khanal 2011). Cassava materials have also been used for ethanol production under very high substrate concentration (Yong, Sixin et al. 2011, Nguyen, Le et al. 2014, Puligundla, Obulam et al. 2014). Broken rice is a by-product from rice processing, consisting in fragments of rice grains broken in the field, during drying, transport, or milling. The broken rice contains a high content of starch (68-81%) and protein (8-9.5%). Therefore, this cheap starchy material has been also used for ethanol production at VHG conditions (Gohel and Duan 2012, Chu-Ky, Pham et al. 2016).

The ethanol produced from starch requires enzymes and yeast strains with a higher ethanol tolerance than catalysts for fermentable sugar-containing feedstock (Schubert 2006). To produce ethanol from starch-containing feedstock, the principal steps are: (i) starch hydrolysis in which starch is converted into glucose by α-amylase and glucoamylase; (ii) fermentation process in which glucose is converted into ethanol by the yeast *Saccharomyces cerevisiae*.

Other unconventional substrates have been used under VHG conditions for ethanol production. According to Pietrzak and Kawa-Rygielska, a high ethanol concentration was obtained at low temperatures of gelatinization (59°C) by using wheat-rye bread at 300 g/L (Pietrzak and Kawa-Rygielska 2015). In another study, the by-products of dates were used as the fermentation substrate. The ethanol content obtained from the high concentration of the syrup of dates (360 g/L) reached 92 g/L (Djelal, Chniti et al. 2017). The use of sweet potato at very high substrate concentration for ethanol production have also been investigated (Shen, Zhang et al. 2011, Shen, Guo et al. 2012, Srichuwong, Orikasa et al. 2012).

Lignocellulose-containing materials consist of six main groups: crop residues (cane and sweet sorghum bagasse, corn stover, different straw types, rice hulls, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (waste paper and recycled paper sludge), herbaceous biomass (alfalfa hay, switchgrass and other types of grasses) and municipal solid wastes (Pandey 2011). The hydrolysis of lignocellulosic biomass to the monomeric sugars is the key step. It allows microorganisms to metabolize the monomeric sugars into ethanol. Generally, this process is performed by acids, alkaline or

enzyme methods (Kamm, Gruber et al. 2006, Pandey 2011). However, within the scope of this review, we focus only on ethanol production from starch-containing feedstock for ethanol production.

2.2. Starch - structure and classification

Starch consists of two types of molecules: the linear (amylose) and the branched (amylopectin). The key enzyme used in hydrolyzing starch is α-amylase which is active on α-1, 4 linkages in amylose (Mousdale 2008) .

Table 4 shows the properties of some main starch sources (grains and tubers). The starch granules from different botanical sources are characterized differently by their shapes, sizes and morphology. The sizes of starch granules vary from submicron to more than 100 microns in diameter. The cereal starches have amylose of smaller molecular sizes than tuber and root starches. The shapes of starch granules are diverse, including spherical, disk, oval, polygonal, dome shape and elongated rod shape. Most starch granules are composed of amylose and amylopectin (Jane, Kasemsuwan et al. 1994). The studies on structure of starch and its properties play an important role in determining the starch hydrolysis and its interaction with enzyme, especially for raw starch-digesting enzymes.

The starch granule is organized in amorphous areas made up by amylose and crystalline area which is constituted of linear fractions of amylopectin. During enzymatic hydrolysis, the less organized amorphous rings are more attacked than the crystalline lamella which offer higher resistance to enzymatic erosion (Oates 1997).

Based on the amylopectin branch chain-length, the starch granules are classified as different types of crystalline, displaying A-, B- and C-type X-ray patterns which have the different unit cells. The A-type polymorphic starch has a monoclinic unit cell while the B-type polymorphic starch has a hexagonal unit cell. The C type polymorphic starch has a mixture of the A-type and the B-type polymorphic unit cells. The A type is typical of most starches of cereal origin such as corn, wheat, and rice while the B type is found in potatoes, other root starches. The C type is commonly found in legume granular starches such as smooth pea and various bean starches.

Table 4: Properties of some starch sources (grains and tubers) (Eggum 1979, Zhai, Lu et al. 2001, Rolland-Sabaté, Sánchez et al. 2012, Bajaj, Singh et al. 2018, Silva, Peres et al. 2019, Sts k-Graczyk, Balcerek et al. 2019)

	Corn	Wheat	Barley	Rye	Sorghum	Potato	Cassava	Rice	Oat
Polymorphic patterns type	A	A	A	A	A	B	A	A	A
Shape	Polyhedral	Spherical and flat circular (lens)	Round or elliptical	Spherical	Oval or semi-spherical	Oval	Round	Polyhedral	Polyhedral but sometimes ovoid or hemispherical
Granule size	15-18	20-25	20-25	31.0	15.5	35	15.6	3-8	3-10
Amylose	22.4-34.5	18-30	22	20-30	20-30	20.1-31	16.8-21.5	17-23	23-24
Starch (%)	65-72	62-70	52-64	52-65	72-75	60-75	65-82	77.2	44-61
Sugar (%)	2.2	-	-	-	-	0.3-5.5	0.25	-	1.2
Protein (%)	9-12	12-14	10-11	10-15	11-12	6-11	2.3	7-13	9-11
Fat (%)	4.5	3.0	2.5-3	2-3	3.6	0.5-1.1	0.8	0.7-2.9	5.7-10.1
Fiber (%)	-	-	-	-	-	-	4.6	1.2-1.9	-
Ash (%)	1.5	2.0	2.3	2.0	1.7	2.5-5.4	2-5	0.9-1.5	3.0

2.3. Starch hydrolyzing enzyme - key factors for ethanol production

To produce ethanol, a variety of sources of enzymes from various microorganisms with different optimal pH and temperatures has been used for the different purposes of treatment, especially α-amylase and glucoamylase. The digestion of starch granules is a complex process. It involves a diffusion of the enzymes toward the granules, an adsorption of the enzymes onto the granule surface and a hydrolysis of the starch chains (Lehmann and Robin 2007). For starch hydrolysis, the key condition is the adsorption of enzymes onto starch (Slaughter, Ellis et al. 2001, Higuchi, Ohashi et al. 2005). In other words, without adsorption, enzyme can not hydrolyze the starch granules. Some factors have a significant impact on the enzyme absorption, especially the temperature; the pH; the length of the polysaccharide chain or the area of the starch granule (Walker and Hope 1963). Another key factor is the state (solution and in colloidal form) of the enzyme in the reaction mixture (Tomasik and Horton 2012). The thermal stabilization of enzyme is the result of its complexation onto the starch or

the modified starches (Chandorkar and Badenhuizen 1966, Kazuo, Masaru et al. 1998). The enzymatic digestion leads to a liquefaction of starch along with the formation of reducing sugars. The susceptibility of the starch to liquefaction depends largely on the nature starch, its state (native granular or gelatinized), and the enzyme used (Masłyk, Leszczyński et al. 2003). The final products obtained thus depend on the origin of the starch and the enzyme, the temperature, the pH, and also the amount of enzyme used. In addition, the enzymes isolated from various bacteria or fungi may play a different role in starch digestion (Dalmia and Nikolov 1991).

For native starch hydrolyzing digestion, according to Blazek, J (2010) (Blazek 2010), have observed in granules the different susceptibility of semicrystalline and amorphous growth rings toward enzymatic digestion. Levels of degradation were more clearly found in large granules than in small ones. The internal structures seemed more susceptible to digestion than the outer layers. The enzymatic digestion led to an alteration on the granule surface and a degradation of the external part by exocorrosion. Under exocorrosion, the internal part of the granule is corroded, forming small pores in which enzymes penetrate into the granule (Oates 1997). As a result, some granules become hollow with only more resistant external layers remaining (Blazek 2010). Besides starch hydrolyzing enzyme, other auxiliary enzymes also play an important role in increasing fermentation efficiency (Table 5).

Table 5: Enzymes used in ethanol production at VHG condition

Enzyme	Reactions	Application	Products	Commercial enzymes	Source
α-amylase	Starch hydrolysis	- Conversion of starch into shorter glucose chains -Reduced viscosity	Dextris/ oligosaccharides/ glucose	Stargen 002; Termamyl Spezyme Fred Spezyme alpha GC 626 Liquozyme Supra	*Bacillus* species
Glucoamylase	Dextrin hydrolysis	- Conversion of dextrins into glucose -Reduced viscosity	Glucose	Amigase Mega L Optidex L-400 SAN Extra L AMG 300L Distillase APS	*Aspergillus* species
Proteases	Protein digestion	-Conversion of proteins into peptides and amino acids crucial for yeast growth - Release starch by breaking down complex starch-protein matrix -Reduced viscosity	Peptides and amino acids	Fermgen Fermgen 2.5X Alcalase 2.4L Neutrase 0.8 L	*Aspergillus niger*, *Kluyveromyces lactis*
Pullulanases	Hydrolyzing α-1,6 branch points of starch	-Reduced viscosity - Increase the amount of fermentable sugars	Dextrins and oligosaccharides	Promozyme 200L	*Bacillus* species *Klebsiella planticola*, *Geobacillus stearothermophilus*
β-glucanase	Hydrolyzing (1,3)(1,4)-β-D-glucans	-Reduced viscosity		Optimash TBG	*Trichoderma reesei*
Pectinase	Degrading pectins	-Reduced viscosity -Remove pectin which can form complexes with starch/protein	Pectic acid	Pectinex Ultra SP-L	*A. niger*

Cellulase (β-1,4-endoglucanase, cellobiohydrolase, and β-glucosidase)	Degrading cellulose	Reduced viscosity Can be produce glucose for fermentation	Glucose		*A. niger*, *A. nidulans*, and *A. oryzae*
Hemicellulase	Degrading hemicellulose	Reduced viscosity	Glucose		
Xylanase (endoxylanases, exoxylanases and β-xylosidases)	Enhanced digestibility of starch; breakdown of xylan	Reduced viscosity	Glucose	Optimash VR	*Aspergillus sp.*; *Trichoderma sp.*; *Bacillus sp* *Streptomyces sp.*, *Pseudomonas sp*
β-Glucosidase	Hydrolysis of oligosaccharides	Reduced viscosity	Simple sugars or glucose		A. niger
Mix enzyme cocktail	Multiple effects due to the combination of enzymes		Ceremix 6X MG (α-amylase, β-glucanase, protease, pentosanase and cellulase) Ceremix 2XL (α-amylase, β-glucanase and protease) Viscozyme (carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase)		

2.4. Factors affecting the efficiency of enzymatic hydrolysis in VHG conditions

2.4.1. Nature of starch

Starch with a low amylose content is typically easier to enzymatically digest than that with a higher amylose content. With high amylose contents, the granular starch presents longer chains in amylopectin, having the ability to form more stable double helices and stronger crystallites, reducing the enzyme susceptibility (Blazek 2010, Lacerda, Leite et al. 2019). According to Blazek, using α-amylase for enzymatic digestion, after 24 h, the most resistant starch granules was high-amylose maize (amylose content-AM 70%), whereas other regular starches were more readily digestible for tapioca (AM 17%) and for rice starch (AM 10%) (Blazek 2010). Study of Jane (2006) (Jane 2006) reported that amylose is mainly found in the granule periphery. The amylose and amylopectin molecules would be less susceptible to enzymatic attack due to their strong association in the periphery of the granules. That explains why starches with high proportions of amylose are more resistant to enzymes in both raw and cooked forms

The enzyme digestibility depends largely on a polymorphism of starch. The study of Hoover and Zhou (2003) reported that the legume granular starches (C-type) are more susceptible to pancreatic α-amylase than the granular starches of potato or corn (B-type), but less susceptible than the cereal or cassava granular starches (A- type) (Hoover and Zhou 2003).

The starches that naturally present a **porous surface** are degraded more easily than those with a smooth surface. The pores of starch granules from different origin have different sizes, from micro to macropores (diameters from 2 to 50 nm). Holes are observed on over the entire granule surface (maize, sorghum, millet) or all along the equatorial groove of large granules (wheat, rye, and barley) of cereal starch. The granules of tapioca, rice, oat, canna, and arrowroot maybe have no pores (Fannon, Hauber et al. 1992). These pores on the surface of starch may be initially attacked by enzymes, allowing enzymes direct access to the granule interior (Franco 1992). The study of Sujka and Jamroz showed that the starches of corn, wheat, and rice were some irregularities and holes (cavities or channels) in the sections of granules but the structure of potato starch granule was tightly packed. This means cereals in generals are more susceptible to enzyme α-amylase (Sujka and Jamróz 2010).

The shape and size of starch granules vary depending on the botanical source and the environmental condition under which a crop was grown. The starch granules with a lower

diameter are more susceptible to enzymes compared to those of higher diameter due to their higher contact area (Franco 1992, Tester, Qi et al. 2006). The observation of Franco and Ciacco about enzymatic hydrolysis on starch indicated that the enzymatic attack on the large granules of cassava and corn starches (> 16 μm) was characterized by the considerable corrosion of granule surface, mainly in the radial direction. For small granules, the enzymatic action was characterized, mainly by the surface erosion with a partial or total solubilization of the granules (rice, oat) (Franco 1992).

2.4.2. Viscosity

In general, the substrate concentration is proportional to the viscosity. The substrate concentration is defined as the amount of substrate per total solution. The viscosity is the quantity that describes a fluid's resistance to flow. Generally, increasing the concentration of the dissolved substance leads to an increase in viscosity. The viscosity is mainly caused by the presence (apart from starch) of the non-starch polysaccharides such as pentosans, β-glucans, cellulose and hemicelluloses in cereal grains (Aastrup 1979, Uribe and Sampedro 2003). These polymers are found in plant cell walls and form the viscous colloidal solutions or the suspensions in water. They can not be hydrolyzed by amylolytic enzymes into simple sugars. Indeed, under VHG conditions, during gelatinization step, the viscosity of process using raw wheat flour and raw wheat flour with brans (mainly composed of pentosans and cellulose in wheat brans) was 300 and 1500 cps, respectively (Montesinos and Navarro 2000).

In addition, proteins could lead to a high viscosity of the mash, depending also on the nature of the protein and its solubility. For example, the primary cause of viscosity of wheat mash appears to be gluten (Taylor and Cluskey 1962). In the case of root and tuber mashes at VHG condition, the high viscosity comes from their high viscous nature due to a high content of pectin (30–50%); cellulose; hemicelluloses and lignocellulose in cell walls of dicotyledonous plants (Sørensen, Pauly et al. 2000, Srichuwong, Fujiwara et al. 2009).

In general, the viscosity limits the interactions enzyme-substrate. Wee et al. (2011) (Wee, Annuar et al. 2011) studied the effect of a high substrate concentration towards the enzymatic hydrolysis by using glucoamylase. The results showed that, the yield of reducing sugar obtained was low when the substrate concentration increased. Furthermore, in ather study, Uribe and Sampedro (2003) (Uribe and Sampedro 2003) indicated that friction between a high solvent viscosity and proteins in solution resulted in decreasing motion and inhibiting catalysis.

Therefore, a high viscosity causes some inconveniences as follows: (i) the handling difficulties during process; (ii) the resistance to solid–liquid separation; (iii) the incomplete hydrolysis of starch to fermentable sugars and (iv) the lower fermentation efficiency (Srikanta, Jaleel et al. 1992, Ingledew, Thomas et al. 1999).

Mechanical pretreatment reduces grain size and crystallinity degree. It increases content of the amorphous phase, therefore slightly reduces the viscosity and improves the susceptibility of starch to hydrolysis enzyme (Tumuluru, Conner et al. 2016, González, Loubes et al. 2018).

2.4.3. Interaction between starch and protein

Starch exists inside the endosperm of cereals, enmeshed in a protein matrix (Rooney and Pflugfelder 1986). In normal condition, some of the starches are tightly trapped within the web-like protein matrix. Therefore, the swelling of the protein could increase accessibility of amylolytic enzymes to starch by a formation of large extended web-like protein structures during cooking process (Hamaker and Bugusu 2003). The changes related to protein structures during mashing could potentially impact on an incomplete gelatinization and hydrolysis of starch as well as a fermentation efficiency (Wu, Zhao et al. 2007).

2.4.4. Interaction between starch and lipid

In cereal starches, a small quantity of natural lipids is found in the form of lysophospho-lipids and free fatty acids. These lipids form complexes with amylolytic enzymes and amylose (Rooney and Pflugfelder 1986). The amylose-lipid complexes have a high melting temperature, about 85-105°C (Sievert and Pomeranz 1990). Those complexes reduce water binding and swelling of starch granules. Thus, they limit the access of amylolytic enzymes, decreasing fermentation efficiency (Matser and Steeneken 1998). According to Moorthy (Moorthy 2002), the root and tuber starches are characterized by a low lipid content (1%), which has a less effect on the enzymatic accessibility on starch than the cereal starch.

In other studies on waste wheat–rye bread at very high solids loading for ethanol production, during bread baking and staling, starch was partially gelatinized, depolymerized and interacted with other ingredients of flour (gluten and lipids), leading to a decrease in susceptibility of the waste wheat–rye bread to enzymatic hydrolysis (Kawa-Rygielska, Pietrzak et al. 2012, Pietrzak and Kawa-Rygielska 2015)

2.4.5. Tannins

Tannins are found in the pigmented testa of some sorghum cultivars which use for bioethanol in some countries such as USA, China, India and Belgium (Wu, Li et al. 2012, Suryaningsih and Irhas 2014). The tannins are well known as an adverse agent on starch digestibility due to their ability to interact with proteins (including enzymes) and polysaccharides by the hydrogen bonding coupled with the hydrophobic interactions (Hosny 1979, Sathe, Deshpande et al. 1982, Rooney and Pflugfelder 1986, Schofield, Mbugua et al. 2001). Besides increasing viscosity, a high amount of tannins in grains limits accessibility of enzyme to the starch, thus leading to an incomplete hydrolysis during liquefaction process. Moreover, the protein cross-linking is formed during heating or cooking. It prevents starch granules from absorbing water, and from enzymatic degradation. Therefore, tannins can reduce fermentation efficiency (Duodu, Taylor et al. 2003). Wu, Xiaorong, et al. 2007 showed that, high tannin content samples had a lower fermentation efficiency (85.2%) compared to that obtained by the low tannin content samples (more than 87.9%) ($P < 0.05$) (Wu, Zhao et al. 2007). According to this study, the slow liquefaction process was not improved even adding 10–20 times more the thermo-stable α-amylase due to a high viscosity caused by the high tannin sorghums. This was explained by the complex formation between tannins in the sorghums and the extra added α-amylase (Wu, Zhao et al. 2007).

The viscosity of the mash during liquefaction can be reduced by removing the tannins present in the pericarp and the testa of the sorghum seeds. Using this method, the fermentation efficiencies of high-tannin sorghum samples was increased to 87% (Corredor, Bean et al. 2006). In another study, for ethanol production under VHG conditions using a corn starch, the content of tannins decreased from 7.4% to 1.5% w/w by extraction process using 40% v/v ethanol-water solution as the solvent. The final ethanol concentration reached 86.4 g/L (Chao, Liu et al. 2017).

2.4.6. Pectin and cellulose

The limitation of using starchy materials, especially root and tuber mashes at very high dissolved solids are from their high viscous nature. Pectin - the gelling polysaccharides is found in the cell walls of dicotyledonous plants, accounting for 30–50% of this structure (Sørensen, Pauly et al. 2000). Pectin may also contain rhamnose, xylose, arabinose, and galactose (Mohnen 2008). Its content varies depending on the type of tubers, roots and their varieties, from 0.31 to 0.61% in cassava (Potter and McComb 2008); 0.21 to 0.33% in potato

(BenitoInfante, OmarEGarcía et al. 2013) or 0.61 to 0.78% in sweet potato (Baker 1997). Besides increasing the viscosity, pectin could hold the sugar molecules in fermentation medium. This pectin-sugar complex is not broken by simple water extraction (Reddy, Reddy et al. 2011). Compared to cereals, enzymatic hydrolysis of starch from tuber crops is more difficult due to the latex and trapping of starch granules in the pectin-hemicellulose matrix. During liquefaction process, lignin and hemicellulose and cellulose form a physical barrier limiting access of the hydrolytic enzymes to the starch due to their resistance (Snow and O'dea 1981).

2.4.7. β-glucan and pentosan

Maize, wheat, sorghum, millet have similar levels of β-glucan (0.1%-0.5%) while this content in goat and barley, rye and oats is higher, ranging from 2.5-4.2% w/w (Henry 1987, Owuama and Okafor 1990, Thomas and Ingledew 1992, Thomas and Ingledew 2005). Pentosan is commonly found in cereal grains. Maize, sorghum and millet have same levels of pentosan (about 3.0% w/w). This content is lower than other cereal grains, such as wheat (6.6%); barley (6.6%); oats (5.8%) or rye (9.0%) (Henry 1987). The pentosan and β-glucans are well known to be the primary cause of viscosity (Boros, Marquardt et al. 1993, Agu, Bringhurst et al. 2006). Using cereal grains as fermentation substrate for ethanol production, some practical difficulties are also observed due to a viscosity development during mashing through the solubilization of β-glucan and pentosan and the subsequent their formation of gels.

2.4.8. Phytic acid

Cereals generally contain phytic acid which can form complexes with starch and protein, thereby limiting their susceptibility to enzymatic hydrolysis. It can bind with starch via hydrogen bonds, or via starch-associated proteins (Lehrfeld 1989, Pejin, Mojovi et al. 2009).

2.4.9. Thermal treatment of starch

Generally, without thermal treatment, the native starch (in the form of non-gelatinized granules) is more resistant to enzymatic hydrolysis by amylolytic enzymes than gelatinized starch (Das and Kayastha 2019). Native starch granules are resistant to the penetration by both water and starch-hydrolyzing-enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. However, during gelatinization, these

hydrogen bonds are weakened and broken down by a high temperature in the presence of water. Thanks to this thermal treatment, the starch initially in insoluble state changes into soluble state. This step leads to a disruption of the starch granules and exposes it easily to enzyme attack (Uthumporn, Shariffa et al. 2011).

High temperature facilitates the adsorption ability of enzyme and its penetration into starch granules by increasing holes sizes and expanding internal cavities, which naturally present in granules (Shariffa, Karim et al. 2009). According to Tien Cuong et al. (Nguyen, Chu-ky et al. 2020), at sub-gelatinization hydrolysis of cassava flour under VHG conditions using Stargen 002 (α-amylase and glucoamylase), the highest glucose concentration (87 g/L) was found after 48 h hydrolysis by the pretreatment at 50 °C whereas the lowest glucose concentration was obtained by pretreatment at 30 °C (66 g/L). The thermal pretreatment at 50 °C in 30 mins for corn starch could cause the irreversible swelling of granules (mostly in the amorphous region) and provide more access for the enzyme to attack starch granule (Li, Cai et al. 2014). This study also found that the higher temperature for pretreatment (50 °C) increased the adsorption of amylase 5-fold over the untreated substrate (at 30 °C) (Nguyen, Chu-ky et al. 2020).

2.4.10. Condition usage of enzyme

Each enzyme is determined by its own properties related to the optimal substrate hydrolysis and optimal operating conditions such as temperature, pH, reaction duration….Starch hydrolysis efficiency increases when a high amount of amylase is used (Tomasik and Horton 2012). However, overdose of amylase may inhibit hydrolysis of starch. In fact, during the hydrolysis of starch, the cleavage of each glycosidic bond consumes one water molecule (Kamerling 2007). In concentrated solutions of enzyme, the water molecules are eliminated by those reactions, leading to a decrease in hydrolysis. The reaction stops after reaching the decomposition limit of starch. Regardless of whether the substrate is from tuber or cereal starch, the products formed are dextrins (Marchal and Tramper 1999).

2.5. Application of enzymes for ethanol production using VHG technology

2.5.1. Reduction of viscosity

High concentration of dissolved substrates results in a high viscosity during mash preparation. The presence of non-starch polysaccharides has been responsible for a high viscosity in some starchy substrates as described above. This can lead to some handling

difficulties including: (i) resistance to solid–liquid separation; (ii) incomplete hydrolysis of starch to fermentable sugars (Puligundla, Smogrovicova et al. 2011). Therefore, the high viscosity causes a low fermentation efficiency (Zhang, Chen et al. 2010). To deal with this problem, enzymatic treatment has been recommended to reduce mash viscosity under VHG conditions. Those enzymes could increase the water-to-grain ratio by releasing water bound from mash components (Puligundla, Smogrovicova et al. 2011).

To reduce starch paste's viscosity under VHG conditions, starchy substrate can be pretreated by using the cell-wall degrading enzymes such as cellulases, pectinase, hemi-cellulases and xylanase. According to Srichuwong et al. (2009), under VHG process of sweet potato at 28% dry matter for ethanol production (Srichuwong, Fujiwara et al. 2009), the enzymatic pretreatment at 50 °C for 50 min was applied. The cell-wall degrading enzymes (cellulase, hemi-cellulase and pectinase) have been used to decrease mash viscosity. As a result, the mash viscosity was reduced from 300 to approximately 50 cp. After 61.5 h of fermentation, ethanol concentration reached 16.6% v/v which was equivalent to 89.7% of theoretical yield.

In another work, to reduce mash viscosity of liquefied sweet potato mash at VHG conditions, the xylanase was used (Zhang, Chen et al. 2010). The enzymatic pretreatment was carried out at 30°C for 90 min, leading to a decrease of mash viscosity from 9,863.2 to 498.1 cp. Ethanol concentration was of 16.3% v/v corresponding to 91.4% of the theoretical ethanol yield. When scaled up, the use of xylanase decreased the viscosity from approximately 30000 to 500 cp. The ethanol yield obtained was of 91.27% (Zhang, Zhao et al. 2011).

In another study on conventional and no-cooking processes for ethanol production at 32% dry matter, Poonsrisawat et al. investigated the viscosity reduction of cassava mash by using cell wall degrading enzymes (endoglucanase, FPase, xylanase, polygalacturonase, β-glucanase, and mannanase) from *Aspergillus aculeatus* (Poonsrisawat, Wanlapatit et al. 2014). The viscosity was reduced to the operating level of <500 mPas for both processes. The ethanol content for thermal and non-thermal process reached 19.65 and 17.54% (v/v), respectively.

According to Nguyen et al, for SSF process, beta-glucanase had a positive impact on reducing viscosity from 340 to 270 cps of cassava mash at 31,5 % initial solid (Nguyen, Le et al. 2014). The ethanol content obtained was 17.2% v/v corresponding to 86.1% of the theoretical ethanol yield at lab scale and 16.5% v/v corresponding to 83.6% of the theoretical ethanol yield at pilot scale.

Viscosity of potato mash at 30.4% initial substrate was reduced by a pretreatment with mixed enzyme preparations of pectinase, cellulase and hemicellulase. Under optimized condition, ethanol yield of 16.61% (v/v) was achieved corresponding to 89.7% of the theoretical yield (Srichuwong, Fujiwara et al. 2009). Application of the preparation CeluStar XL containing cellulase enzyme complex had the most significant improvement in ethanol productivity from corn mash prepared at 28.6% solid with an increase of 10% compared to samples control without enzymatic treatment (Sapińska, Balcerek et al. 2013).

The study of Balcerek and Pielech-Przybylska (Balcerek and Pielech-Przybylska 2013) showed that a positive effect of supportive enzymes (xylanase and pullulanase) on reducing viscosity of rye mashes viscosity and on increasing significantly ethanol yield efficiency was observed.

It should be noted that viscosity can be reduced slightly by α-amylase. This enzyme acts on the starch molecules breaking α-(1-4) linkages, producing dextrin, therefore resulting in a lower swelling during gelatinization. According to Rocha et al (2010) (Rocha, Carneiro et al. 2010), α-amylase treatment caused a reduction of intrinsic viscosities of 9.8, 8.7, 4.9, and 1.3% for cassava, sweet potato, peruvian carrot, and potato starches, respectively.

2.5.2. Support for starch hydrolysis

Phytase application has been applied for ethanol production under VHG conditions for ethanol production. This enzyme supports in converting the long chain branches of α-1,6 glycosidelinkages into the linear dextrins during SSF process (Gantelet and Duchiron 1999). Therefore, it further enhances fermentable sugar generation by glucoamylase reaction, resulting in a shortened hydrolysis time by as much as 37% for ethanol production from wheat flour at a starch concentration of 23% w/v (or substrate concentration of 32.8% w/v) (Gantelet and Duchiron 1999).

In another study, the corn mashes supplemented with phytase (50 mL phystase/ton of raw material, at 55 °C, pH 5.5) either before or after the starch hydrolysis showed a higher final ethanol concentration (1.0 and 0.6% v/v, respectively) compared to controls samples. In addition, the phytate hydrolysis increases significantly initial fermenting sugar concentrations from substrate, leading to a higher availability of starch for α-amylase hydrolysis (Mikulski, Kłosowski et al. 2014).

According to Dawid Mikulski, the phytate hydrolysis using microbial phytase has recommended as an alternative solution to supplementation of high gravity mashes for ethanol fermentation (Mikulski, Rolbiecka et al. 2017).

Pentosanase has been used in pretreatment of waste wheat-rye bread mashes at VHG condition. Indeed, rye flour contains about 9% of pentosans which form crosslinked structures with other ingredients of wheat-rye bread. By treating with Ceremix 6X MG preparation (containing pentosanase), a higher carbohydrate content and protein soluble were obtained from wheat-rye bread mashes, contributing to a higher fermentation efficiency (Kawa-Rygielska, Pietrzak et al. 2012).

An increased ration of gluco-amylase over α-amylase significantly enhances glucose production and thereby, enhances ethanol yield (Fujii and Kawamura 1985). Indeed, α-amylase is responsible for the production of oligosaccharides from starch molecules while gluco-amylase converts oligosaccharides into glucose. Gluco-amylase plays an important role in peeling the molecule from the surface and in revealing new glucoside bonds on the next layer of the granule, which are reacted by a-amylase. The cooperation of both enzymes is the synergism, helping increased ethanol concentration and fermentation productivity (Fujii and Kawamura 1985). According to Sakdaronnarong et al. (2018), the synergistic action between α-amylase (Techzyme Q-Add enzyme) at 93°C, pH 5.6 for liquefaction and Gluco-amylase (GC 147) at 61.5°C pH 4.2 for saccharification was the suitable enzymatic synergistic among all enzyme-matching designs for SSF process from cassava at 35% solid. The highest ethanol obtained was 27.3 g/L from 72 h (Sakdaronnarong, Srimarut et al. 2018). For SLSF process of broken rice at 31.15% solid, the reduction in process duration for ethanol production was observed by an addition of gluco-amylase (Amigase Mega L) which hydrolyzes dextrins into fermentable glucose. Therefore a higher content of sugar was released and available for fermentation (Chu-Ky, Pham et al. 2016).

2.5.3. Support for yeast nutrition/breaking down complex starch-protein matrix

Protease has been also used widely for pretreatment of starchy substrate under VHG conditions. This enzyme shows some benefit as follows: (i) hydrolyzing protein into amino acids, peptides, and FAN which support yeast growth during fermentation; (ii) breaking down complex starch-protein matrix (Duan, Dunn-Coleman et al. 2009, Gohel and Duan 2012, Kawa-Rygielska, Pietrzak et al. 2012, Chu-Ky, Pham et al. 2016). Indeed, the study on waste wheat-rye bread mashes at concentration of 32% (w/w) showed that the addition of Ceremix (commercial mixing enzyme with protease) caused disruption of the gluten network in bread, releasing a higher maltose and maltotriose content. This resulted in a significant increase in ethanol yield (approx. 36.0 g ethanol per 100 g of bread DM) in comparison to control sample (35.24 g ethanol per 100 g of bread DM) (Kawa-Rygielska, Pietrzak et al. 2012). For no-

cooking process using broken rice at 311.5 g/L, the protease was reported to hydrolyze the protein matrices in the kernel that binds the various fractions and to degrade the "hard-to-hydrolyze" starch. Therefore, by using protease for rice-based ethanol production, the ethanol yield and ethanol content increased significantly (Chu-Ky, Pham et al. 2016).

2.5.4. Ultrasound – improvement of starch sensitivity to enzyme

An ultrasound pretreatment is also an interesting solution to increase the conversion of starch materials into glucose and the accessibility of starch granules to enzyme (Khanal, Montalbo et al. 2007).

Normally, ultrasound has been used at a low frequency (from 16 to 100 kHz). When it propagates in a medium such as a liquid or slurry, it produces a cavitation and acoustic streaming. During ultrasonic treatment, a formation of microbubbles, its growth and collapse are observed (Li, Ma et al. 2009, Manchun, Nunthanid et al. 2012). In addition, an increase in both pressure and temperature affects the depolymerisation of polysaccharides due to a mechanical breakage of macromolecular C-C bonds and a chemical degradation of these polymers (Jambrak, Herceg et al. 2010).

Ultrasonic pretreatment of starch granules has been reported to reduce the molecular weight of amylose and amylopectin, as well as to improve starch solubility. It facilitates the disintegration of starch granules by exposing a much larger surface area of starch to enzymes. Therefore, it enhances enzymatic hydrolysis of starch (Li, Ma et al. 2009, Nikolić, Mojović et al. 2009, Jambrak, Herceg et al. 2010).

Pielech-Przybylska, K (2019) studied the effects of ultrasound pretreatment, pullulanase digestion and hop α-acids preparation on the efficiency of SSF at high gravity mashes (28.5% w/w) prepared from rye starch for ethnaol production (Pielech-Przybylska, Balcerek et al. 2019). The results showed that ultrasonic pretreatment alone or such treatment combined with pullulanase digestion leads to a decrease in viscosity of 60-69 and 85%, respectively. By pretreating rye starch by ultrasound for 10 min, a higher fermentation efficiency (over 21%) was observed compared to the control mash ($p < 0.05$). A combination of pullulanase digestion; ultrasound pretreatment and the antimicrobial action of hop α-acid preparation increased the fermentation efficiency (about 30%), in comparison to the control sample ($p < 0.05$).

3. Solid State Fermentation process and its application in protein enrichment from cassava byproducts

3.1. Cassava-based bioethanol and its byproduct

During bioethanol production from cassava, wastes are generated. Their quantity and quality depend significantly on the raw materials quality and on the processing types (Fig.4). Similar to the dry milling process for bioethanol production of corn grains, the fermentation beer is distilled and ethanol is recovered and left a remaining phase, so-called whole stillage, containing both solid (wet distillers' grains) and liquid waste (thin stillage). The remaining thin stillage is concentrated through evaporator system to produce a syrup called condensed distiller solubles (CDS) which are mixed with wet distillers' grains to become distillers wet grains with solubles (DWGS) and then dried into DDGS in order to greatly lengthen their shelf-life (Monceaux 2009).

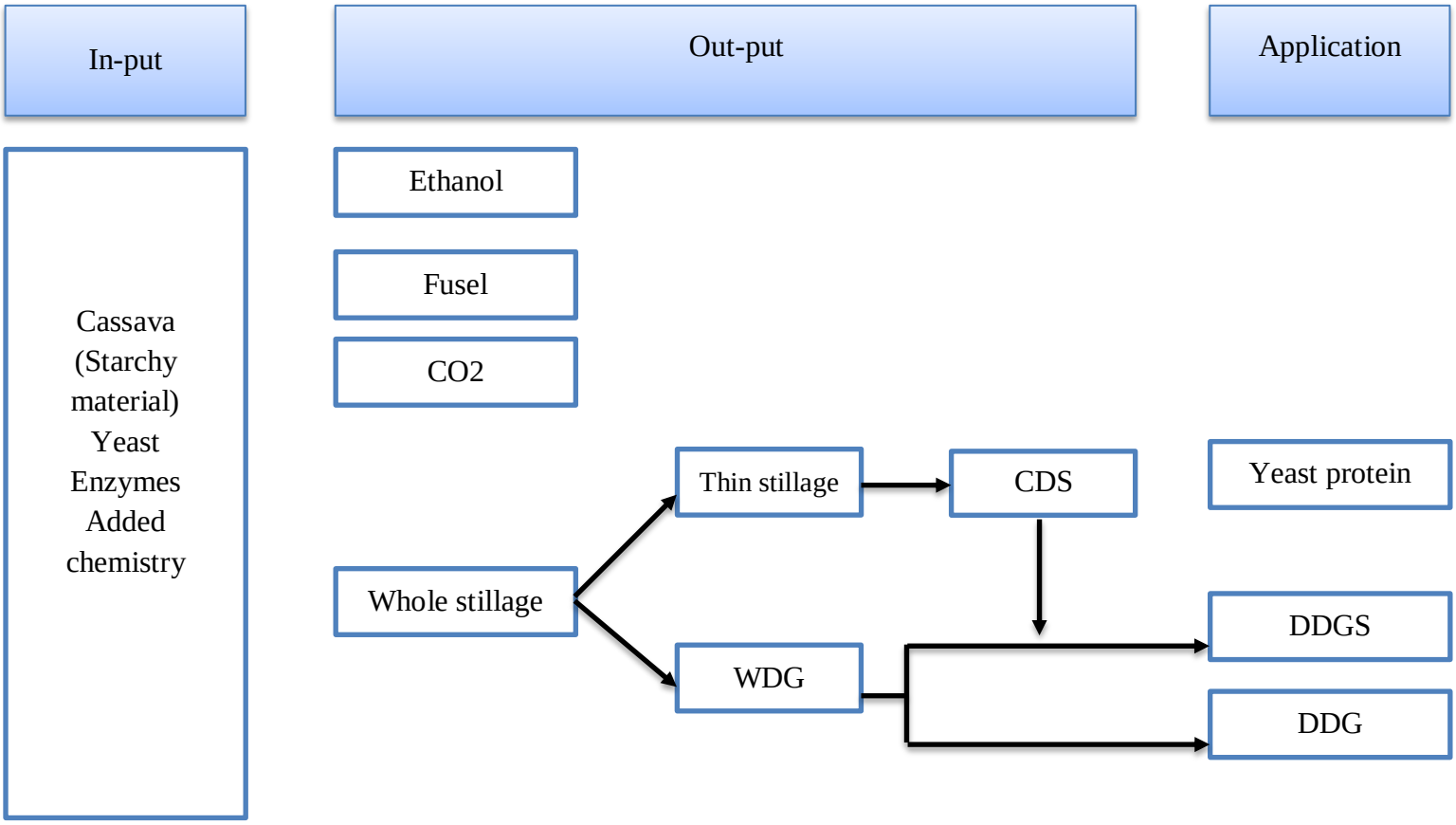


Figure 4: In-put; Out-put of bioethanol from cassava and application of its added chemistry

WDG: Wet distillers' grains

CDS: Condensed distillers' solubles

DDGS: Dried distillers' grains with solubles

DDG: Dried distillers' grains

Since only starch and sugars are converted into ethanol, non-fermentable components in cereal grains are concentrated by a factor of more than two in DDGS (Monceaux 2009). However, the nutritional composition of DDGS varies depending on the raw materials and the ethanol production technology, including distillation process. The composition of DDG and DDGS have been of great interest to researchers in the area of animal science, ethanol producers, and especially to the animal feeding industry as the majority of this has been sold as feed ingredients for livestock (Liu 2011).

3.2. Composition of cassava-based DDG and its potential application as fermentation medium

Cassava is used largely for bioethanol production in Vietnam. The WDG from cassava-based bioethanol plants has been used for a long time ago for purpose of feeding in Vietnam (Nguyen et al. 2002). However, there has been little work elucidating the nutrient composition of cassava-based DDG, except little data on cassava byproducts from ethanol plants in Vietnam, Thailand and Korea. In these works, the cassava DDG had a low content of protein (11-14%) (Taranu et al. 2019) (Sriroth et al. 2006), and a high content of crude fiber (32%) (Taranu, Nguyen et al. 2019). In addition, hydrogen cyanide (HCN) – an antinutrient is found in cassava DDG (Ofuya et al. 1994). The concentration of HCN in fresh cassava tuber and cassava by-products is much higher than the threshold recommended by the EFSA (European Food Safety Authority), which is less than 50 mg kg^{-1} feed, to prevent acute toxicity in animals (Coursey 1973, Vuong et al. 2020).

Therefore, a biological process is needed to reduce anti-nutritional levels and enrich protein content in order to consume cassava tuber and its byproducts safely for human and animal.

3.3. Solid state fermentation for protein enrichment

One of the most popular ways to enhance protein content in agriculture wastes is the solid-state fermentation (SSF) technology. The SSF is a process whereby an insoluble substrate containing sufficient moisture but without free water allows the microorganism to grow and metabolize (Godoy et al. 2018). The differences between solid state fermentation and submerged fermentation are shown in table 6.

Table 6: Comparison between Solid-State Fermentation (SSF) and Submerged Fermentation (SMF) (Chen 2013)

	SSF	SMF
Water	No free water. Low water consumption	Water is the main component of the culture High water consumption
Substrate	Insoluble Low cost	Soluble Higher cost
Culture system	Three phases (gas, liquid and solid) and gas is the continuous phase	Mainly liquid; the liquid is the continuous phase
Inoculation	Inoculation size is large, more than 10% Batch process	Inoculation size is small, less than 10% Continuous process
Oxygen requirement	From the gas phase	From dissolved oxygen
Energy consumption	Low	High
Equipment cost/ investment	Low Simple fermentation bioreactor	High High-tech design fermentation bioreactor
Equipment volumes	Low	High
Industrial level	Small scale	High scale
Development of microorganisms	Microorganisms adhere and penetrate into the solid substrate	Microorganisms uniformly distribute in the culture system
Product concentration	High	Low
Mixing or agitation	Difficult due to sensitivity of microorganisms, especially filamentous fungi	Easy
Temperature and pH control	Difficult	Easy
Detection of fermentation parameters	Difficult	Easy
Medium fermentation	Heterogeneity	Homogeneity
Product extraction	Extraction process is simple and controllable	Extraction process is complex; A large amount of waste water
Pollution (effluents)	Little volume of effluents	High volume of effluents

The SSF possesses some advantage as follows: (i) use of various agro-industrial wastes as fermentation medium; (ii) minimization of contamination due to a low water demand; (iii) higher productivity due to a higher volume of the substrate loading; (iv) low investment and operating cost; (v) low recovery cost in downstream processing due to less liquid waste produced. Therefore, SSF has been used in many fields, including protein enrichment for animal feed production (Singhania et al. 2009).

Even though SSF offers certain unique advantages compared to the liquid fermentation, it has also some disadvantages as follows: (i) engineering problems during fermentation due to the build-up of temperature, pH control, oxygen transfer, mass and heat transfer, substrate and moisture gradients; (ii) inhomogeneous distribution of the cell mass,

nutrients, temperature, pH, moisture content in substrate; (iii) difficulty in steady aeration for the whole substrate; (iv) difficulty in biomass measurement of microbial growth (Singhania, Patel et al. 2009, Chen 2013).

3.4. Microorganisms used in SSF process for traditional food application

Table 7 shows the main groups of microorganism cultivated in Solid State Fermentation and used in food production. Fungi remain the most widely used group of microorganisms for SSF process because the fermentation conditions are close to their habitat in nature. The fungi have a wide variety of applications in SSF process thanks to their physiological, enzymological and biochemical properties (Mitchell et al. 2006, Carboué et al. 2017), consisting of yeasts; filamentous and macroscopic fungi and mushrooms. The *Aspergillus*, *Trichoderma*, and *Mucour* are well-known fungal species for the SSF process for laboratory and pilot scale (Sangsurasak et al. 1996). Besides the fungi, bacteria are also used widely in SSF process, essentially gram-positive bacteria, traditionally for the production and maturation of cheese.

Table 7: Main groups of microorganism cultivated in Solid State Fermentation and used in food production

(Blandino et al. 2003, Longo et al. 2008, Bourdichon et al. 2012, Montet et al. 2016)

	Genus/species	Example of application	Fermentation medium (substrate)
Bacterium	Brevibacterium	Cheese production	Cheese
	Micrococcus	Cheese and sausage production	Cheese and meat
	Lactobacillus	Various fermented foods	Maturing cheese and cereals
	Streptococcus	Cheese production	Rice and cereals
	Bacillus	Fermented foods	Corn and soybeans
Yeast	Saccharomyces	Bread production and various fermented products	Corn, wheat, millet and other cereals
	Endomicopsis burtonii	Fermented foods	Rice
	Debaryomyces hansenii	Production of bread, cheese and sausages	Wheat, maturing cheese and meat
	Yarrowia lipolytica	Cheese and sausage production	Maturing cheese and meat
	Candida	Various fermented products	Rice and cereals
	Pichia	Various fermented products	Rice and cereals
Filamentous fungi	Aspergillus oryzae	Fermented foods (miso), sake, soy sauce and other condiments	Rice, corn, wheat, barley, soybeans and other cereals
	Aspergillus sojae	Fermented foods (miso), soy sauce and other condiments	Rice, wheat, corn and soy
	Aspergillus niger	Fermented foods, sake and condiments	Rice, wheat, maize, sorghum and cassava
	Aspergillus flavus	Fermented foods	Cassava

	Aspergillus awamori	Fermented foods, liquors	Rice and soybeans
	Rhizopus oligosporus	Fermented foods (tempeh)	Rice and soybeans, cereals and legumes
	Rhizopus oryzae	Fermented foods (tempeh)	Rice and soybeans
	Fusarium solani	Cheese production and fermented foods	Corn and wheat
	Penicillium	Cheese production	Maturing cheese
	Monascus	Fermented foods (red rice)	Rice
	Geotrichum candidum	Cheese production	Maturing cheese
	Mucor	Fermented foods (natto, tempeh, sufu), cheese production	Soybeans, rice and black beans
	Neurospora	Fermented foods	Soybeans
Mushrooms	Agaricus bisporus	Edible and medicinal mushroom	Rice straw and wheat
	Lentinus edodes	Edible and medicinal mushroom	Sawdust, corn cob and pulp of coffee
	Pleurotus ostreatus (oyster mushroom)	Edible and medicinal mushroom	Rice straw, wheat, beans, cotton, corn cob
	Volvaria volvacea (volvaria)	Edible and medicinal mushroom	Rice straw, wheat, water hyacinth and palm residue
	Flammulina velutipes (enoki)	Edible and medicinal mushroom	Rice bran and sawdust
	Grifola frondosa (maitake)	Edible and medicinal mushroom	Sawdust
	Ganoderma lucidum (reishi)	Edible and medicinal mushroom	Sawdust
	Coprinus comatus	Edible and medicinal mushroom	Sawdust and rice bran

3.5. Microorganisms used in SSF process for agricultural residues

The nature of wastes generated from agricultural and food manufacturing practices varies depending on the type of crops and the processing technology. The straw and fruits from agricultural sector; the starch residues generated by starch industries and the spent media from fermentation industries are the most abundant wastes. Despite reusing of these wastes significantly brings numerous values in term of economic and environmental impacts, their valorization has been limited by the available technology (Pandey et al. 2000, Pandey et al. 2000).

The agricultural wastes are characterized by a high content of fiber and a low content of protein, vitamin, oil and other nutrients. However, these wastes may be applied for animal feed industries following protein enrichment by biological process (Pandey, Soccol et al. 2000).

Selection of microorganism for protein enrichment by SSF process using lignocellulosic wastes depends largely on the target of final product; technology used and nature of lignocellulosic substrate. The protein enrichment performed by SSF process is

potentially associated with the metabolic activities and biomass development of microorganisms. These have been proved to increase the crude protein content along with a higher protein digestibility of fermented lignocellulosic substrate (Villas-Bôas et al. 2002).

3.6. Microorganisms used in SSF process for protein enrichment from cassava substrate

Besides some bacterial species, yeast genera such as *Saccharomyces, Yarrowia, Candida,* and filamentous fungi of *Aspergillus, Chaetomium, Paecilomyces, Penicillium,* and *Trichoderma* genera have been used for protein enrichment (table 8). *Trichoderma* species are considered useful and not harmful to humans and animals (Şişman et al. 2013). They have been known for producing many extracellular enzymes and are mostly used in food and textile industries to degrade complex polysaccharides (Ezekiel and Aworh 2013). For instance, *Trichoderma harzianum* actively takes part in the decomposition of plant residues in the soil. Its efficiency to enrich the protein content in various cellulosic agricultural by-products was reported in previous studies such as peels of mango, orange, apple, banana and tomato wastes; rice polishing; sunflower lignocellulosic fraction; and cassava root meal (Parrado et al. 1993, Ahmed et al. 2017, Siada et al. 2018).

Table 8: Some microorganisms used in SSF process for protein enrichment from cassava residues

Genus/species	Result	Substrate	Year	Ref.
Trichoderma harzianum BiomaTH1 *Yarrowia lipolytica* W29	Increase in crude protein from 11.01 (% DM) to 13.86 (% DM) and 12.39 (% DM) along with an increase in the essential amino acids content which enhanced by 55% and 22%, respectively,	Cassava-based DDG	2020	(Vuong, Thanh et al. 2020)
Saccharomyces cerevisiae	Reduced tannin content Increase in crude protein from 10.08 to 14.11–16.07 %	Cassava leave	2019	(Hawashi et al. 2019)
Rhizopus oligosporus	Increase in crude protein (15.2%) Reduced cyanide content	Mixture of cassava leaves and babassu mesocarp	2016	(Morales et al. 2018)

Trichoderma pseudokoningii	Protein content was increased from 8.4 to12.5 % with the presence of urea	Residue of cassava peel mixed with cassava trimmings	2015	(Bayitse et al. 2015)
Lactobacillus plantarum *Saccharomyces cerevisiae* *Rhizopus oryzae*	Levels of protein increased from 1.92% to 8.58%, 2.29%, and 4.72% using *L. plantarum*, *S. cerevisiae*, and *R. oryzae* respectively.	Cassava tuber	2015	(Gunawan et al. 2014)
Trichoderma viride	Crude protein increased from 4.21% in unfermented cassava peel samples to 10.43 % in fermented samples	Cassava peel	2013	(Ezekiel et al. 2013)

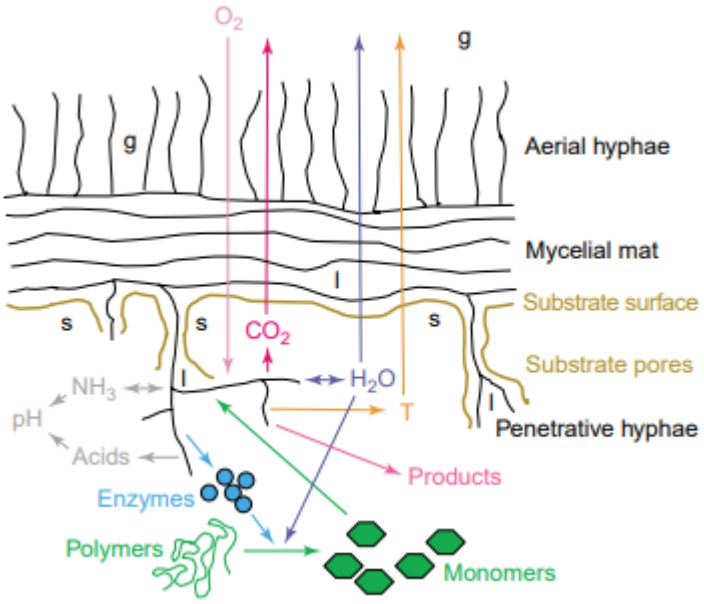
On the other hand, the non-conventional yeast *Yarrowia lipolytica* was certified as GRAS (Generally Recognized As Safe) from FDA (American Food and Drug Administration) for use in food and pharmaceutical industries. Recently, it has received a great attention as a potential source of single cell protein (SCP). This obligate aerobic yeast is well known for its ability to produce proteases, peptidases and lipases, increasing the nutritional value of the agricultural substrate (Ritala et al. 2017). However, *Y. lipolytica* does not produce cellulases and hemicellulases so it cannot reduce the content of fiber in agricultural residues (Ritala, Häkkinen et al. 2017).

Many previous studies have shown that SSF processes using molds and yeasts not only increase the crude protein content but also enhance significantly the profile of essential amino acids in agricultural substrates at very low cost (Ritala, Häkkinen et al. 2017). The essential amino acids, especially lysine, leucine, methionine, valine, isoleucine, threonine have been commonly used in animal feeds, since they tend to be deficient in natural feedstuffs and cannot be synthesized by animals (Richardson et al. 1978).

3.7. Material transfers at the microscopic scale

Fig. 5 shows the biological and physicochemical reactions of fungi during SSF process. Usually, fungi used for SSF process are inoculated into the solid substrate as spores. Once germinated, the mycelium is formed. They consist of long, branching filaments

(hyphae) which were found in the gaseous space, on the surface and inside solid particles (pores). The metabolic activities are mainly occurred near the substrate surface and within the pores. Hydrolytic enzymes which are produced by the mycelium diffuse to the solid matrix and catalyze the degradation of macromolecules into smaller units before to be absorbed by the fungi. During SSF process, carbon sources provided from byproduct substrate along with other nutrients are aerobically transformed into microbial biomass, metabolites, CO_2 and H_2O. These transformations are exothermic, generating heat inside the substrate and the bioreactor (Hölker et al. 2005).



l: Liquid
g: Gas
s: Solid
T: Temperature

Figure 5: Biological and physicochemical reactions of fungi on a microscopic scale (Hölker and Lenz 2005)

Besides the difficulties caused by heterogeneity of fermentation medium, the generation of heat is the most uncontrollable factor during solid fermentaion, especially for a high volume scale (Ritala, Häkkinen et al. 2017).

Besides some bacterial species, yeast genera such as Saccharomyces, Yarrowia, Candida, and filamentous fungi of Aspergillus, Chaetomium, Paecilomyces, Penicillium, and Trichoderma genera have been used for protein enrichment and detoxification (Oboh et al. 2003, Bayitse, Hou et al. 2015).

3.8. SSF process - general steps

The different stages performed before and during the SSF process are shown in Figure 6. Depending on the substrate and microorganism used, pretreatments applied to the substrate

are adapted and the parameters during fermentation process are determined (temperature, humidity, aeration and pH) (Mitchell, Berovič et al. 2006).

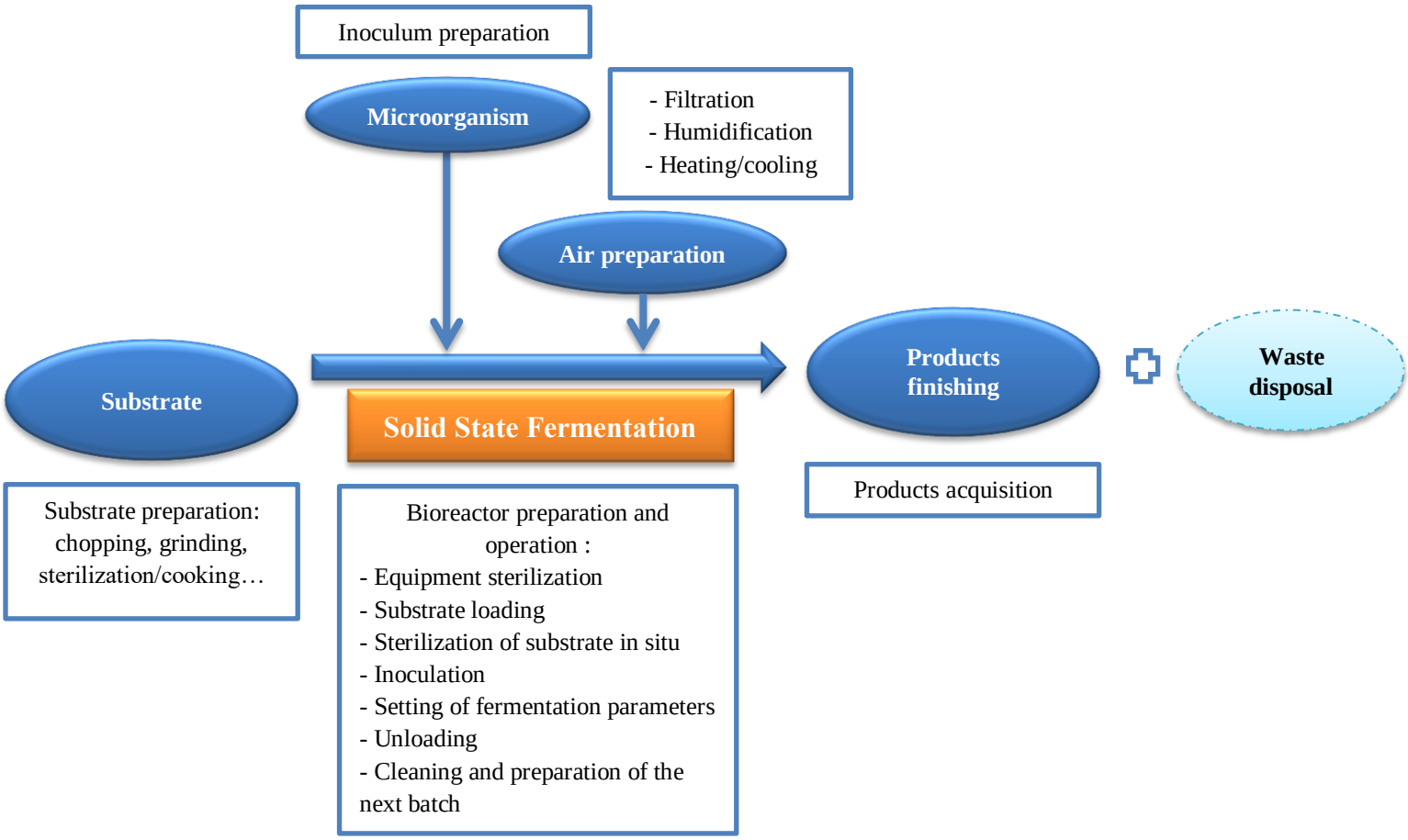


Figure 6: General steps of an SSF process

(Mitchell, Berovič et al. 2006)

The major processing steps of an SSF process include:

- Inoculum preparation
- Substrate preparation
- Bioreactor preparation
- Inoculation and loading
- Bioreactor operation
- Unloading
- Downstream processing
- Waste disposal

These steps during SSF process can be varied, depending largely on the microorganism, nature of substrate, targeted products and equipment used (Erlenmeyer flask fitted with a cotton stopper or forced aeration fermenter) as well as possibilities of sampling for monitoring microorganism's growth (Durand 1998).

3.9. Factors that influence SSF process

The SSF process has a number of interesting advantages which come from its simplicity and its closeness to the natural habitat of many microorganisms. Each microorganism requires the different conditions for its growth and metabolism. Therefore, solid substrate and bioreactor system play a major role in the success of SSF process. The productivity of this process can be affected by various factors which can be divided into three major categories, namely:

Biological factors and Physico-chemical factors

3.9.1. Biological factors

3.9.1.1.Type of microorganism:

A suitable microorganism using for SSF process plays a key role in degrading and utilizing the solid substrate in order to produce the targeted products. The selection of microorganism depends largely on the type of solid substrate, growth requirement and targeted final product (Gawande et al. 1999). The microorganism used in SSF process is mainly fungi, bacteria, yeast. Among them, filamentous fungi are dominated as an ideal microorganism for SSF process due to their good tolerance to low water activity and high osmotic pressure conditions (Krishna 2005).

The use of mono- or co-culture of selected microorganism is usually dependent on the nature of solid substrate, growth requirement and targeted final product. The advantage of using a single microorganism is to improve the rate of substrate utilization and to control the product formation (Nigam et al. 2009).

Inoculation of the culture medium is most often used under the form of spores or yeast cells suspension. The optimum amount of spores or cells used varies depending on the specific conditions (substrate for example), particularly for the production enzymes (Sella et al. 2009, Carboué, Perraud-Gaime et al. 2017). Overdose of spore or yeast cells for inoculation can also inhibit the synthesis of metabolites due to the rapid depletion of nutrients after germination. It has also been shown that the age and the physiological state of the spores have a significant impact on the development of microorganisms (Ritala, Häkkinen et al. 2017).

3.9.1.2.Nature of substrates

The substrates used in solid fermentation come mainly from organic residues or agriculture by-product (Villas-Bôas, Esposito et al. 2002, Krishna 2005). The matrix of solid

substrate must have a good capacity of absorption and must contain the nutrients necessary for the development of the microorganism (source nitrogen, carbon and mineral). Mechanical treatment of substrate (grinding, cutting...) aims at reducing particle size as well as increasing porosity and homogeneity of fermentation substrates. Chemical (addition of acid/akaline or nutrients) and thermal treatment (heating, sterilization) facilitate the growth of microorganisms through appropriate fermentation conditions along with limited contamination risk (pH, moisture, nutrient...) (Assamoi et al. 2009, Jao et al. 2011).

3.9.2. Physico-chemical factors

3.9.2.1.Moisture content

The SSF process is characterized by almost no free water in the substrate. Fungi and yeast can grow depending upon the water activity of the substrate (Pandey, Soccol et al. 2000). To ensure the normal physiological requirements for fungi and yeast during fermentation, they require a water activity value between 0.6 and 0.7 (Gervais et al. 2003). The water content level also plays an important role in the variation of three phase structure: water retention, permeability and thermal conductivity. Insufficient quantity of water does not allow a good diffusion of solutes and gases, leading to a cellular inhibition because of a lack of substrates or through too high concentration of inhibitive metabolites in or near the cells (Ugalde et al. 2002). On the contrary, the higher level of humidity results in changes in structure substrates. It reduces porosity of the interparticle space and consequent interference with oxygen transfer, leading the reduction of microorganism growth (Mahanta et al. 2008, Mustafa et al. 2016). A proper moisture content varies between 35 and 80% w/w, and especially, more important for cellulosic substrates which are known for their high water absorption capacity (Raimbault 1998).

3.9.2.2.Particle sizes

For SSF process, the substrate's particle size plays an important role in the adhesion of microorganisms, the transfer of heat and nutrients as well as the circulation of oxygen. Those factors directly affect the microorganism growth and the formation of targeted products. Indeed, small particle sizes decrease interparticle space leading to reduction in substrate porosity (Camacho-Ruiz et al. 2003) which results in problem of aeration. In contrast, larger particles provide better aeration but lesser surface area which limits the adhesion of the microorganisms, especially the mold (Pandey, Soccol et al. 2000). Therefore, the appropriate particle size is extremely important to address the aeration and surface area problems and

improve the permeability conditions of the media, the contact surface between the substrate and the microorganisms.

3.9.2.3.pH

Along with initial moisture content, an appropriate pH is also a key factor for facilitating microorganism growth through the synthesis of biomass and the degradation of available nutrient sources. The pH changes significantly during fermentation due to the microbial metabolism including consumption of nutrients and synthesis of metabolic products. The heterogeneity of the solid media along with a lack of an effective measurement method does not allow to control accurately and reliably pH variations. In addition, the mycelium can grow in a large pH range of 2-9 with an optimal range of 3-6 while pH range of bacteria is fairly neutral values (Assamoi, Destain et al. 2009). The initial pH of solid fermentation medium can be changed by the addition of acids or bases. It is noted that, for microorganism, pH influences directly the transport of various components across the cell membrane, thereby affecting their growth. An accessibility to certain carbohydrates such as hemicellulose can be significantly reduced when the pH is unfavorable (Pandya et al. 2012).

3.9.2.4.Temperature

The overall temperature of the medium varies considerably during fermentation process due to the heat generated from the metabolic activities of microorganism and accumulated in the system (nee'Nigam and Pandey 2009). It leads to some difficulties as following: (i) disturbing/stopping the growth of microorganisms and the formation of products (Pandey 1991, Pandey et al. 2008); (ii) decreasing rapidly moisture content (Khanahmadi et al. 2006) and (iii) forming a condensation which increases heterogeneity in the solid substrate (Khanahmadi et al. 2004, Khanahmadi, Roostaazad et al. 2006). The heat generated can be dissipated by aeration and/or agitation of the substrate medium as well as an addition of water by direct injection or humidified air during fermentation. An air cooling system can also facilitate heat removal. Thin layer reactors are more efficient at removing heat generation compared to thick layer ones (Shoja et al. 2007).

3.9.2.5.Aeration

Oxygen requirements vary depending on microorganisms. It is not a limiting factor in solid fermentation when the substrate is well studied. In general, the aeration plays an important role in: (i) meeting the oxygen demand for aerobic microorganism during fermentation; (ii) regulating heat and moisture content in the inter-particle solid substrates. It was considered to be a positive effect on microbial growth and product formation (Zhang et

al. 2003, Assamoi et al. 2007). In addition, the aeration using saturated air helps control the temperature and the moisture gradients of the solid medium (Umsza-Guez et al. 2011).

3.9.2.6.Ratio C/N

Among the fermenting medium parameters, the carbon/nitrogen ratio is one of the most important factors for the microorganism growth and product formation (enzymes, microbial biomass...). Nitrogen content in the fermenting medium can be a limiting factor for SSF process. Agricultural and agro-industrial co-products used as solid substrates in SSF process have variable nitrogen content. The nitrogen content of agricultural residues varied significantly, from 0.63-1.47% for straw to 1.90-4.84% for grains and seeds (Praspaliauskas et al. 2020). An appropriate ratio of C/N has been proven to facilitate microbial biomass in SSF process (Yang et al. 1986, Vuong, Thanh et al. 2020). Nitrogen or carbon source supplementation is not always necessary. It depends largely on the availability of nitrogen content in substrate as well as the nutrient requirement of the microorganism.

3.10. Bioreactor design

The design of bioreactors must take into account some important aspects, including temperature, oxygen transfer, moisture content of solid matrix, mixing or agitation, aeration and heat transfer. The strategies may be used based on the requirement of micro-organisms as well as the final products. However, the SSF bioreactors must respond to have an effective oxygen transfer, an effective heat removal, a good water distribution and solid matrix mixing/agitation during fermentation. In general, based on the mixing system used, SSF bioreactors can be classified as static bioreactors (fixed bed, perforated trays) or stirred bioreactor (horizontal drum or stirred drum). SSF bioreactors also classified according to the type of aeration (without forced aeration-Tray chamber or with forced aeration - Packed bed; horizontal drum and fluidized bed) (Durand 2003, Bhattacharyya et al. 2008, Spier et al. 2011) or employed mixing system (Singhania, Patel et al. 2009). For the laboratory-scale studies, SSF process is generally carried out using petri dishes, jars, wide-mouth Erlenmeyer flasks, Roux bottles and roller bottles (Durand et al. 1997, Pandey et al. 2001, Durand 2003). These bioreactors at laboratory-scale usually do not need forced aeration and agitation. Therefore, it is difficult to scale up or transfer to pilot scale. For pilot and industrial-scale, the various bioreactors are used in SSF such as a tray fermenters used in Koji process, plafractor bioreactor, packed-bed bioreactor.

4. Bibliography conclusion

The very high gravity technology has received a great attention from researchers and producers in the ethanol industry. Besides its advantages in increasing plant productivity, reducing risk of bacterial contamination and energy consumption, VHG technology reveals some drawbacks mainly from increased stress on the yeast cells during the fermentation and from high viscosity of mash. To deal with those problems, new technologies along with advanced enzymes are introduced and applied under the VHG conditions. Among them, SLSF process or no-cook process has been researched by using granular starch hydrolyzing enzymes to increase ethanol yield, to save energy consumption and investment cost. The SLSF has been used widely for ethanol production, mostly for cereal starch. Its application in root or tuber starch, especially cassava is still limited due to their special starch structure. Therefore, enzymes could be a potential solution to overcome the restrictions from SLSF-VHG technology using cassava starch from researchers and producers in the ethanol industry.

Cassava is used largely for bioethanol production, especially in Vietnam. Annually, a large amount of ethanol distiller spent is generated. However, the content of cassava-based DDG was characterized as high in crude fiber; low in protein and amino acids with limited interest for animal feeding. According to the previous studies, SSF processes using molds and yeasts not only increase the crude protein content but also enhance significantly the profile of essential amino acids in agricultural substrates at very low cost. Therefore, the SSF process could be potentially applied to increase protein-value and reduce anti-nutrients in cassava-based DDG for animal feeds.

CHAPTER II

SIMULTANEOUS LIQUEFACTION, SACCHARIFICATION AND FERMENTATION AT VERY HIGH GRAVITY FOR ETHANOL PRODUCTION FROM CASSAVA FLOUR

1. Introduction

In recent years, bio-based alternatives to conventional petroleum-derived fuels have attracted much attention due to the concerns over environmental pollution issues and the exhaustion of fossil resources (Moser 2011). In particular, bioethanol has been considered as one of the most promising alternative fuels from renewable sources, especially in developing countries. Therefore, significant socioeconomic benefits could be brought by this alternative source of energy, such as foreign-exchange savings for rural sectors of all countries in the world (Demirbas 2009). In addition, in comparison with fossil fuels, bioethanol has been known as environmental friendly, non-toxic and sustainable fuels along with very low CO_2 emissions (Sivakumar et al. 2010). However, bioethanol is currently more expensive than gasoline in terms of production cost (Zaldivar et al. 2001). This reason has motivated researchers to optimize and improve the bioethanol process in order to enhance its competitiveness. Along with classic biomass such as corn, wheat, and sugar beet, other agricultural raw materials rich in fermentable carbohydrates, cassava has received special attention for biological transformation into ethanol (Kosugi et al. 2009). This tuber is a major crops food in developing countries, providing the basic diet for a large part of the world's human population, especially in Asia. In addition to being consumed as a food, especially in Vietnam and South-East Asia, cassava has been regarded as an ideal raw material for bio-ethanol production thanks to the following advantages: (i) low price; (ii) high starch-containing raw materials; (iii) "all year round" availability; (iv) easily hydrolyzable nature (Sriroth et al. 2007). Indeed, the government also adapted the policy to improve the beverage ethanol industry in Vietnam. By the Development strategy of ethanol production in Vietnam from 2007-2025, bioethanol production would achieve 1.8 million tons in 2025, which should account for 5% of the country’s demand (Ministry-of-Industry). Recently, according to the roadmap approved by the Prime Minister, E5 biofuel made from cassava will be used as a substitute for traditional fuels on land-road vehicles and officially used nationwide from 1.12.2015 in Vietnam. Therefore, in the future, the bio-ethanol industry has a great potential in Vietnam.

The conventional process for bioethanol production in Vietnam involves an energy-consuming liquefaction (95–105 ◦C), separate saccharification (60–62 ◦C), and fermentation (30–32 ◦C) of starch slurry (Gohel and Duan 2012). The high-temperature used in the liquefaction step completely sterilizes harmful microorganisms and extensively gelatinizes starch granules, thereby increasing the efficiency of saccharification for high ethanol yield.

Consequently, this results in high energy consumption and adds costs to the equipment used in the whole process thus increasing the overall production cost.

With biotechnological advances in recent years, cold starch hydrolysis for bioethanol production and food research has received great attention (Wang et al. 2007, Gibreel et al. 2009, Li et al. 2012). The enzymes utilized for this process are known as granular starch hydrolyzing (GSH) enzymes. They are commercially available in the form of enzyme cocktails. One typical and widely known example is Stargen 002. This enzyme cocktail consists of α-amylases and glucoamylases synthetized by *Aspergillus niger* and *Aspergillus kawachi* that work synergistically to hydrolyze granular starch. These enzymes are adsorbed on the surface of starch grain and form holes on this surface where glucose is released (Shetty et al. 2006). Thus, the application of native starch hydrolyzing enzymes saves energy due to the elimination of the high temperature treatment in liquefaction process.

Simultaneous Liquefaction, Saccharification, and Fermentation process (SLSF) or no-cook process has been researched by application of GSH enzymes to increase ethanol yield, to save energy consumption and investment cost (Kelsall and Piggot 2009, Gohel and Duan 2012). In this technology, three separated steps involving liquefaction, saccharification and fermentation are integrated into only one step, in a unique bioreactor, at a unique pH and at ambient temperature. Alpha-amylase and gluco-amylase are added simultaneously to the slurry, in the presence of yeast. SLSF process minimizes the sugar accumulation in the vessel, potentially increasing yields and concentrations of ethanol ((Robertson et al. 2006, Xu and Duan 2010)).

Very high gravity (VHG) fermentation technology is a promising strategy for increasing the volumetric productivity and the cost effectiveness of the SLSF. In VHG technology, the substrates are prepared at high dissolved solid content, at least 270 g/ L. This technology leads to an increase in plant capacity, saving in process water, a reduced risk of bacterial contamination, and reduction in capital costs and energy consumption (Puligundla et al. 2011). Currently, SLSF- VHG fermentation has been successfully carried out on different substrates, mostly from cereal grains including broken rice (Chu-Ky et al. 2016), rye (Strąk-Graczyk and Balcerek 2020), triticale; wheat (Gibreel et al. 2011) and barley (Gibreel et al. 2009) while its application in root or tuber mash, especially cassava is still limited. This is mainly due to the fact that granules of raw cassava starch resist enzymatic hydrolysis more than cereal starch (Piyachomkwan et al. 2007). The cassava's granular starch is trapped in cell walls matrix which contains high contents of cellulose, hemicelluloses and lignocellulose.

This matrix forms a natural barrier against enzyme attack from raw cassava starch, especially in VHG conditions. According to Shanavas et al. (Shanavas et al. 2011), at low solid content (10%) of cassava starch, a high fermentation efficiency (up to 95%) was obtained. In contrast, the fermentation efficiencies obtained at higher solid contents (20 and 30%) were of only 52 and 42%, respectively. A too high substrate concentration leads, to an increase in the cassava flour-interparticle interactions and in the suspension viscosity, which limits the mass and heat transfer, as well as the catalyst diffusion in suspension (Nguyen et al. 2020). Consequently, it results in a decrease in the adsorption of enzyme on starch granule and a lower fermentation yield.

Currently, using in-house auxiliary enzymes by solid state fermentation in combination with thermal pre-treatment of slurry has been researched on SLSF in VHG conditions of cassava substrate to improve enzymatic hydrolysis and ethanol yield (Puligundla et al. 2011). Ethanol production by a non-thermal process from raw cassava flour (18.2% dried solid) using a cocktail enzyme from *Aspergillus kawachii* in pilot scale was also reported with high efficiency (Sugimoto et al. 2012). In another study, Poonsrisawat et al. (Poonsrisawat et al. 2014) investigated the viscosity reduction of cassava for ethanol fermentation at very high gravity by using cell wall degrading enzymes from *Aspergillus aculeatus*. Cassava root mash was adjusted to 32% (w/w) dry matter and was pretreated with 0.5% (w/w) viscosity reducing enzyme preparation and incubated at 45 ◦C, pH 5.0 for 2 h. Low-temp amylase (in-house enzyme prepared from *Aspergillus aculeatus*) was added at 0.35 IU/g of raw starch and fermented by *Saccharomyces cerevisiae* at pH 4.5, 32 ◦C for 96 h. The ethanol content reached 17.54% v/v corresponding to 75.33% for this non-thermal process. However, the thermal pre-treatment led to an increase in the production cost, carbon dioxide emission, and investment equipment cost. Therefore, the environmentally friendly property and energy-saving advantages of no-cooking process for ethanol production have been reduced.

Another approach is to use a higher amount of key enzymes for hydrolyzing native cassava starch. The enzymatic hydrolysis of native cassava granules at low temperature could be increased significantly by increasing the quantity of Stargen 002. According to Hargono et *al.*, the highest concentration of reducing sugar was obtained at different dissolved cassava concentration (100; 200 and 300 g/L) when a higher amount of Stargen 002 was used (1.5% w/w compared to 0.5 and 1% w/w) (Hargono et al. 2018).

At very high gravity conditions, the high osmotic pressure, nutrient limitations and the formation of high levels of toxic fermentation products, especially ethanol, can inhibit yeast performance and decrease the fermentation yield (Pátková et al. 2000). Consequently, for enhancing ethanol productivity, most studies have focused on the supplementation of nutrients, mixed auxiliary enzymes, increasing inoculation rates and enzyme used, as well as on the optimization of fermentation conditions (temperature, pH, agitation and aeration strategy). Response-surface methodology (RSM) has been introduced to optimize the ethanol production factors in a small number of experimental runs with cost-saving statistical experimental design (Box and Draper 1959, Meyer et al. 1996, Myers et al. 2004).

In this work, we aimed at developing and optimizing a no-cooking ethanol process based on: (i) decreasing energy consumption by utilizing enzymes able to degrade the complex cell-wall matrix in cassava roots and to hydrolyze raw starch at lower temperatures; (ii) evaluating the efficiency of different cocktails of enzyme used for SLSF process, for the purpose of selecting the most efficient one; (iii) scaling up the SLSF process of cassava flour at pilot scale (1000 L) and evaluating the SLSF process in terms of ethanol yield, quality, reduced energy use, reduced production cost and environmental impact; (iv) optimizing the SLSF process by using the RMS method in order to reduce quantity of key-factors used in process and (v) recovering cassava-based distillers dried grains (DDG) obtained from ethanol by-products for animal feeding usage.

2. Material and methods

2.1. Microorganism

Commercial active dry yeast *Saccharomyces cerevisiae* (Ethanol Red), kindly provided by Fermentis (France), was used in this study. Dry yeast was hydrated in tap water at 38 ◦C for 20 min prior to addition to the mash of cassava flour.

2.2. Materials

Cassava flour was obtained in Tuyen Quang province (Northern Vietnam). After a thorough drying, cassava chips were ground into cassava flour at a size lower than 0.3 mm, and stored at a dry and cool place in the lab. The starch content of the cassava flour used in this work was 76.3 ± 0.97%. The crude fiber content was 3.7±0.15% and the humidity was 11.4 ± 0.7%.

Different kinds of commercial enzymes kindly provided by Dupont (previously known as Genencor - A Danisco Division) were used in this work including Stargen 002 (containing *Aspergillus kawachi* α-amylase expressed in *Tricoderma reesei* and a glucoamylase from *T. reesei*), Accellerase 1500 (containing cellulase from *T. reesei*), Fermgen (containing protease from *T. reesei*). Amigase Mega L (containing glucoamylase from *A. niger*) was provided by DSM – Food Specialties – Beverage Ingredients. Viscozyme cassava R (containing beta glucanase) was provided by Novozymes. Properties of these enzymes are presented in Table 9.

Prop-aide was provided by Leaf by Lesaffre. Prop-aide is a yeast nutrient complex consisting of organic and mineral nitrogen and vitamins to enhance yeast growth during fermentation (Lesaffre).

Table 9: Characteristics of the enzymes used in this work

No	Enzyme	Nature	Op. pH	Op. temperature (^oC)	Activity
1	Stargen 002	Glucoamylase and α-amylase	4.0-4.5	20-40	570 GAU/g[a]
2	Acellerase 1500	Cellulase	4.0-5.0	50.0-65.0	2800 CMC U/g[b]
3	Viscozyme Cassava R	β-glucanase	4.0-5.0	40.0-50.0	100 FBG/g[c]
4	Amigase Mega L	Glucoamylase	3.5-5.0	50.0-65.0	-

[a] GAU: Gluco-Amylase Unit defined by Dupont (one Gluco-amylase Unit (GAU) is the amount of enzyme that liberates one gram of reducing sugars calculated as glucose per hour from soluble starch substrate under the conditions of the assay).

[b] CMC: Carboxymethyl- cellulase Unit defied by Dupont (one unit of CMC is the amount of enzyme that released 1 μmol of glucose per min under the conditions of assay)

[c] FBG: Fungal β-Glucanase Unit defined by Novozymmes (one FBG is the amount of enzyme that produces reducing carbohydrate equivalent to 1 μmol of glucose per minute under the conditions of the assay).

2.3. Simultaneous liquefaction, saccharification and fermentation (SLSF) at laboratory scale

The five SLSF processes developed in this work are shown in Table 10 and Fig.7. Briefly, the cassava flour (CF) was mixed with tap water in a 1-liter reactor to achieve a concentration of 270 g/L dry solid in a final volume of 1 L. Depending on investigated processes (SLSF1, 2, 3), α-amylase, glucoamylase, β-glucanase, cellulase; active dry yeast Red Ethanol (3.5×10^7 cells/mL), and urea (16 mM), KH_2PO_4 (9.9 mM) were added simultaneously into the mixture. The SLSF was conducted with stirring at 56 rpm during the first 8h.

- SLSF1: α-amylase (Stargen 002) at the dosage of 855 GAU/kg cassava flour (CF) and glucoamylase (Amigase Mega L) at the dosage of 0.035% w/w were added
- SLSF2 was similar to SLSF1 with a twice higher amount of enzyme used (Stargen 002: 1710 GAU/kg CF and Amigase Mega L: 0.07% w/w)
- SLSF3 was similar to SLSF1 with a thrice higher amount of enzyme used (Stargen 002: 2565 GAU/kg CF and Amigase Mega L: 0.105% w/w)
- SLSF4 was similar to SLSF1 with an additional cellulase (Acellerase 1500) and β-glucanase (Viscozyme cassava R) at the dosage of 812 CMC U/g cellulose and 30.25 FBG/kg CF, respectively. The cassava flour was pretreated by Accellerase 1500 and Viscozyme cassava R with stirring at 50 rpm for 2 h at 30^0C. The α-amylase and glucoamylase were added sequentially at 50 rpm for 1 h at 30^0C.
- SLSF5 was similar to SLSF1 with an additional Pro-paide (0.33 g/L) into cassava slurry.

Table 10: Differentiation for the SLSF processes used in this study at lab scale

Ingredients used	**SLSF1**	**SLSF2**	**SLSF3**	**SLSF4**	**SLSF5**
Cassava flour (g/L dry solid)			270		
Stargen 002 (GAU/kg)	855	1710	2565	855	855
Amigase Mega L (% w/w)	0.035	0.07	0.105	0.035	0.035
Viscozyme cassava R (FBG/kg)	-	-	-	-	30.25
Accellerase (CMC U/kg)	-	-	-	-	812
Pro-paide (g/L)	-	-	-	0.33	-
Yeast (cells/ml)			3.5×10^{7}		
Urea (mM)			16.0		
KH_2PO_4 (mM)			9.9		

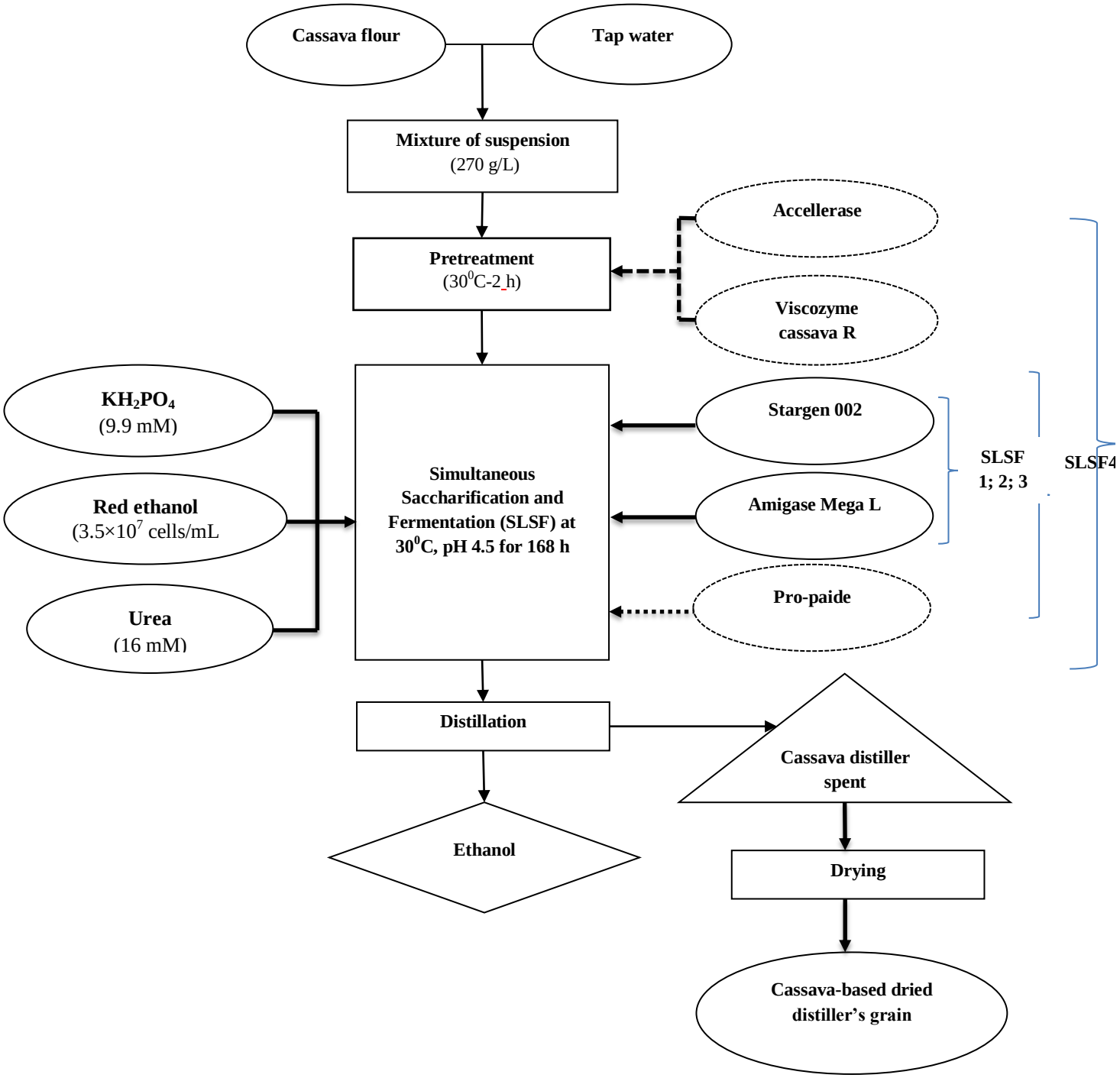


Figure 7: The different processes of SLSF used in this study for ethanol production at lab scale

2.4. Simultaneous Liquefaction, Saccharification and Fermentation at Very high Gravity at pilot scale 1000 L

The SLSF at 270 g/L carried out in Minh Lam (Phu Xuyen-Hanoi-Vietnam) for the pilot scale (1000 L) based on the results obtained by SLSF3 at lab scale as described in 2.3. Table 11 and 12 show the main steps and the ingredients used for SLSF at pilot scale.

Table 11: Ingredients used in SLSF process at 270 g/L of cassava flour for ethanol production at pilot scale 1000 L

	Dose	Unit	Quantity for 1000L	Unit
Raw material	270	g/L	303.37	kg
Stargen 002	4.56	ml/kg	1384.73	ml
Amigase Mega L	0.93	ml/kg	282.12	ml
KH_2PO_4	9.97	mM	1.35	kg
Red ethanol	$3.5*10^7$	cells/ml	0.13	kg
Urea	16	mM	0.96	kg

Table 12: Description of SLSF process at 270 g/L of cassava flour for ethanol production at pilot scale 1000 L

No	Steps	Description
0	Propagation - production of "seed yeast".	One-tenth of total fermentation volume (100 L) along with the same dose of ingredients (enzyme, yeast, yeast nutrients) is used to produce the "seed yeast". The propagation process was performed for 8 h to get the yeast cells concentration of $3.5*10^8$ cells/ml.
1	Mixing	Mixing the raw material with tap water in a mixing-tank at room temperature (about 30 ° C) to reach 270 g/L dry matter under stirring condition (56-60 rpm)
2	pH adjustment	Checking the initial pH and adjusting the pH with H2SO4 20% acid (or/and Citric acid) to get pH value = 4.5.
3	Enzyme; yeast and nutrients supplement	Adding commercial enzymes under stirring condition: Stargen 002 (1384.73 ml); Amigase Mega L (284.12 ml); and nutrients (KH_2PO_4: 1.35 kg; Urea 0.96 kg). The beer fermentation obtained in propagation process was poured into the remaining volume (900 L). Continuous stirring at 56-60 rpm for the first 6-10 hours after supplementation
4	Fermentation	SLSF process at 270 g/L was performed at 28 - 32 ° C for 168 h (for cassava flour).

2.5.Energy measurements

During the experiments, the consumptions of electrical energy and water were evaluated. Electricity consumption was measured and calculated by electricity meter. The whole quantity of water used in this study was measured by water meter.

2.6.Greenhouse gas (GHG) emissions

The SLSF process requires a lower temperature for liquefaction and saccharification of substrate, at 30-35°C, compared to conventional technologies. In addition, in this study, the energy used for SLSF process came entirely from electricity thanks to its advantageous characteristics a same reactor was utilized for all three processes of liquefaction, saccharification and fermentation and tropical climatic conditions in Vietnam. Consequently SLSF is expected to use less energy, which should have a positive effect on the reduction of greenhouse gas (GHG) emissions of cassava-based ethanol. GHG emissions from energy use during SLSF from cassava flour were calculated using emission factors of the ReCiPe method (H and World weightings, 100-year). The electricity specific factor ($kgCO_2/kWh$) is 0.467 (Brander, 2011).

2.7.Preparation of cassava-based distillers dried grains

After fermentation, the distillers wet grains (DWG) were collected from the whole stillage by using plate filter. Cassava-based DWG (with a moisture of 62%) were then dried in a chamber dryer at 60 ◦C for 5 h to obtain cassava-based distillers dried grains (DDG) which were ready for compositional analysis.

2.8.Preparation of cassava-based distillers dried grains

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2.9. Optimization process

The Doehlert design allows description of a region around an optimal response. The number of experiments required is given by k^2+k+n (experiments), where k is the number of variables and n is the number of center points. In our case, the n value was 2. It allows the uniform distribution of the experiments in a three-dimensional space. For three variables (X_1;

X_2 and X_3), a set of 14 experiments in which a central experiment (duplicate) has the coded values (0,0,0), are distributed on a sphere with a radius of 1.

In this study, the ethanol concentration was estimated under the influence of three variables: Stargen 002 (X_1), Amigase (X_2) and yeast cell (X_3). Each experiment could be set with its three coded values. The original values and the corresponding coded values (Table 3) were used for setting up the experiments and the model. Table 13 shows the experimental matrix obtained by applying the Doehlert design methodology for three factors (X_1; X_2; X_3) and experimental values for ethanol concentration Y_{Eth} (% v/v) after 144 h fermentation.

Table 13: Levels of the factors tested in the central composite design (CCD)-Response surface methodology (RSM)

Independent variables	Codes	Levels		
		-1	0	+1
Stargen 002 (ml/kg NL)	X_1	1.52	3.04	4.56
Amigase mega L (ml/kg NL)	X_2	0.33	0.66	0.99
Yeast cell (*10^7cells/ml)	X_3	0.88	2.19	3.50

Table 14: Experimental matrix set up by applying the Doehlert design methodology for three factors and experimental values for ethanol concentration Y_{Eth} (% v/v) obtained after 144 h fermentation

No.	Coded values			Experimental values			Mean
	X_1	X_2	X_3	X_1	X_2	X_3	Y_{Eth}
1	1	0	0	4.56	0.66	0.75	14.4
2	-1	0	0	1.52	0.66	0.75	13.7
3	0.5	0.87	0	3.80	0.95	0.75	14.4
4	-0.5	-0.87	0	2.28	0.37	0.75	13.7
5	0.5	-0.87	0	3.80	0.37	0.75	13.7
6	-0.5	0.87	0	2.28	0.95	0.75	13.9
7	0.5	0.29	0.82	3.8	0.76	1.12	14.4
8	-0.5	-0.29	-0.82	2.28	0.56	0.38	13.5
9	0.5	-0.29	-0.82	3.8	0.56	0.38	13.7
10	0	0.58	-0.82	3.04	0.85	0.38	13.9
11	-0.5	0.29	0.82	2.28	0.76	1.12	14.1
12	0	-0.58	0.82	3.04	0.47	1.12	14.1
13	0	0	0	3.04	0.66	0.75	14.2
14	0	0	0	3.04	0.66	0.75	14.2

By using Nemrod software (LPRAI, Marseille, France), multiple regression analysis based on the least square method which concerned the linear and quadratic effects of the three

factors and their interactions were investigated. Thus, the equation giving Y_{Eth} was a second-order polynomial model including 10 coefficients (b_0, b_1, b_{12}…b_{23})

$$Y_{Eth} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{12} + b_{22}X_{22} + b_{33}X_{32} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{31}X_3X_1$$

2.10. Analytical procedures.

The fermentation beer was centrifuged and filtered to measure reducing sugar by the DNS (3,5 dinitrosalicylic acid) method (Miller 1959). Residual sugar was measured with the same method after the hydrolyzation of fermentation beer by HCl 2% for 120 min at 100°C (Le Thanh 2007). The fermentation beer was distilled to obtain ethanol and the ethanol concentration was determined by an ethanol ebulliometer (Dujardin-Salleron, France). To measure total acidity, the filtered fermentation beer was neutralized by NaOH 0.1N using phenolphthalein and given in gH_2SO_4/l.

The volumetric ethanol production (Q_P) was calculated by the following equation:

$$Q_P = \frac{P}{t}$$

Where P is the actual ethanol concentration produced (g/l), t is the fermentation time (h) giving the highest ethanol concentration.

The fermentation yield was calculated by following equation:

$$\text{Fermentation yield (\%)} = \frac{\text{Practical ethanol yield (\% v/v)}}{\text{Theoretical ethanol yield (\% v/v)}} * 100$$

$$\text{Where theoretical ethanol yield (\% v/v)} = A * B * \frac{180}{162} * \frac{92}{180} * \frac{1}{0.789}$$

A: Weigh of cassava flour used (g)

B: Percentage of starch in cassava flour (%)

Cassava-based DDG was analyzed for moisture (AOAC 927.05), protein (AOAC 991.20), crude fibers (AOAC 993.21), fats (AOAC 991.36), and ash (AOAC 930.30).

3. Results and discussion

3.1. Impact of a supplementation of auxiliary enzymes and yeast nutrient on ethanol production

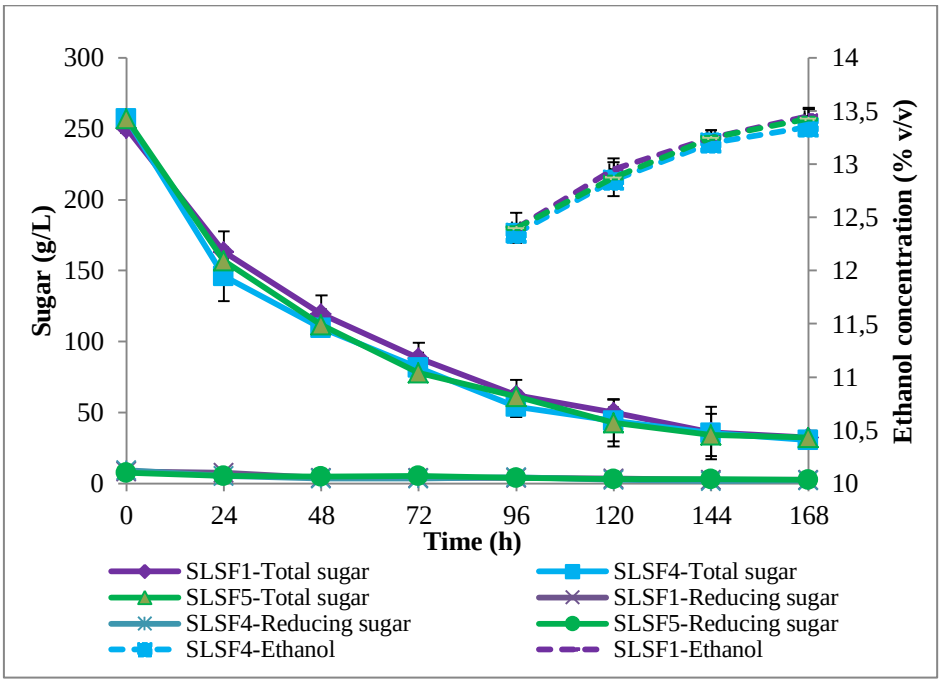


Figure 8: Evolution of ethanol, total and reducing sugar during SLSF at 270 g/L for SLSF 1;4 and 5 processes

The figure 8 shows the impact of a supplementation of auxiliary enzymes and yeast nutrient on ethanol production. After 168 h of fermentation, SLSF1 achieved an ethanol content of 13.4±0.07% v/v equivalent to an ethanol yield of 80.05 %. The high content of total sugar (32.61 g/L) along with the long fermentation time (168 h) indicated the relatively low fermentation efficiency. This maybe is due to the viscosity and the special structure of cassava starch which is trapped in a complex cell-wall matrix consisting of cellulose fixed in a polysaccharide and protein matrix (Poonsrisawat et al. 2014). In addition, the yeast performance was maybe inhibited at a high dissolved solid content (270 g/L) which could have decreased the ethanol content at the end of the fermentation. Therefore, the cellulase (Accellerase 1500) and β-glucanase (Viscozyme cassava R) were added simultaneously for SLSF4 process while the addition of prop-aid was used for SLSF5 process. Our expectation was that, the supplementation of auxiliary enzymes or yeast nutrient helps increase ethanol content and shorten the fermentation duration. After 168 h of fermentation, the results showed that, the ethanol content of SLSF4 and SLSF5 process was 13.35±0.07 and 13.43±0.11 %v/v, respectively equivalent to an ethanol yield around of 80.0%. In other words, no significant

effect of auxiliary enzymes and yeast nutrient on ethanol performance was observed. In addition, the viscosity of cassava slurry in SLSF4 process supplemented with the auxiliary enzymes was not significantly different compared to that of SLSF1 process (108.2 cps).

That could be explained by the resistance of cassava starch to enzyme hydrolysis without thermal treatment. Indeed, the combination of auxiliary enzymes and thermal treatment facilitates the adsorption and penetration ability of α-amylase into starch granules by increasing holes sizes and expanding internal cavities, which naturally presented in granules (Shariffa et al. 2009, Li et al. 2012). According to Oate (Oates 1997), by increasing the incubation temperature approximately to 60^{o}C, a higher hydrolysis of native starch can be achieved. The previous research of Tien Cuong et *al.* showed that the highest glucose concentration (87 g/L) was found after 48 h hydrolysis by the pretreatment of cassava native starch using β-glucanase at 50°C whereas the lowest glucose concentration was obtained by pretreatment at 30 °C (66 g/L) (Nguyen et al. 2020). Thermal pretreatment at 50 °C in 30 min for corn starch could cause partial breakage of the starch structure with the irreversible swelling of granules (mostly in the amorphous region) and provide more access for the enzyme to attack starch granule. This study also found that the higher temperature for pretreatment (50 °C) increased the adsorption of amylase 5-fold over the untreated substrate (at 30 °C) (Li et al. 2014).

3.2.Impact of an amount of Stargen 002 and Amigase on ethanol production

Without thermal treatment, the impact of a higher amount of Stargen 002 and Amigase Mega L on ethanol production was investigated. The figure 9 shows the impact of the amount of enzymes used on the fermentation efficiency. Three different doses of Stargen 0002 and Amigase Mega L were used. For SLSF1 process, the amount of Stargen 002 and Amigase Megase L was 855 GAU/kg RM and 0.035% w/w according to the provider recommendation, respectively. For the SLSF2 and SLSF3 process, the amount of enzyme used (Stargen 002 and Amigase Mega L) was doubled and tripled. The results showed that, for three processes, the reducing sugar concentration remained at low levels during fermentation. It demonstrated the advantages of the SLSF process in decreasing the osmotic pressure on yeast and in reducing the contamination risk thanks to a low concentration of reducing sugar in fermentation broth. The SLSF3 process was finished after 144 h of fermentation with an ethanol concentration of 14.4%v/v corresponding to a fermentation yield of 86.0%.

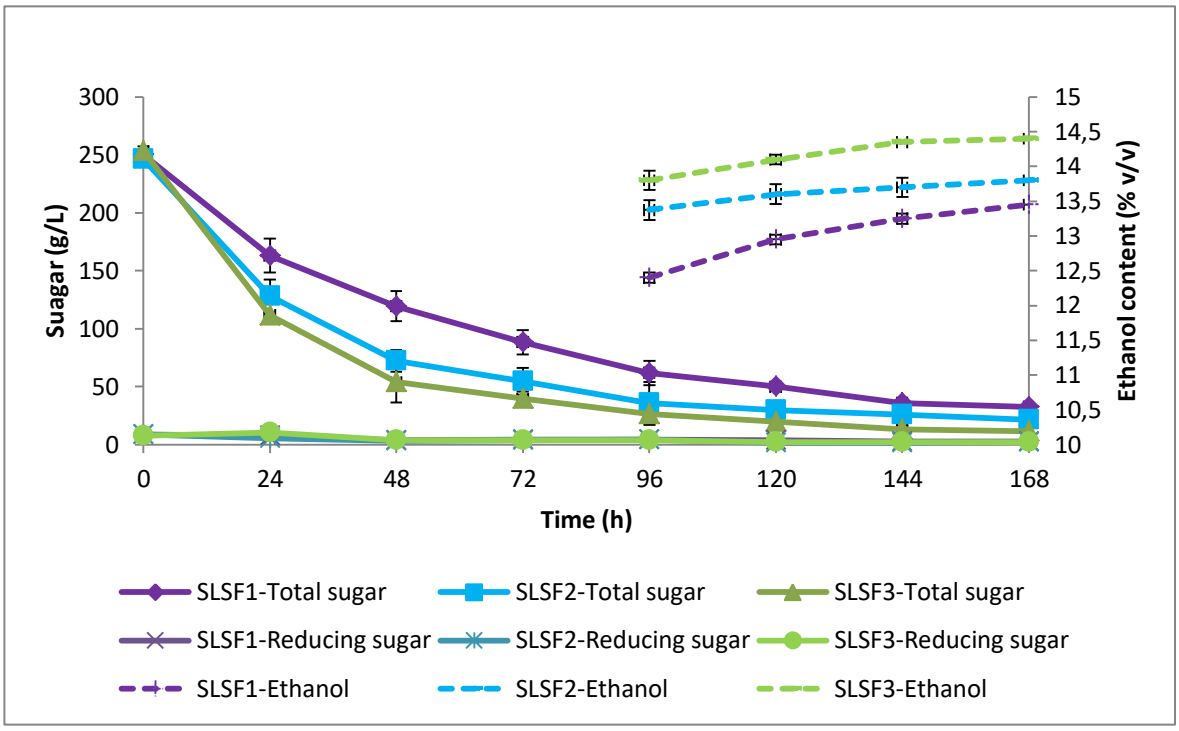


Figure 9: Evolution of ethanol, total and reducing sugar during SLSF at 270 g/L for SLSF 1; 2 and 3 process with different amount of Stargen 002 and Amigase

For the SLSF1 and SLSF2 process, the ethanol content was 13.4 and 13.8 % v/v equivalent to a fermentation yield of 80.0 and 82.4%, respectively after 168 h fermentation. Along with the longer duration of fermentation, the residual sugar content of SLSF1 and SLSF2 process was 35.9 and 25.7 g/L, respectively which were higher than that of SLSF3 process (13.2 g/L). According to Hargono et al., for enzymatic hydrolysis, the highest concentration of reducing sugar was obtained at very high gravity (300 g/L) of cassava substrate when the higher amount of Stargen 002 used was 1.5% w/w compared to that of 0.5 and 1% w/w (Hargono et al. 2018). In another study on non-thermal process of high dissolved cassava starch (200 g/L) for ethanol production, the process used the higher concentration of Stargen 002 (1 and 2% w/w) showed the significant increase in final ethanol concentration which was 57.62 and 59.65 g/L, respectively after 72 h fermentation. With the lower Stargen 002 concentration (0.5% w/w), the ethanol content was only 46.39 g/L (Hargono et al. 2015). A synergistic influence of both Stargen 002 (α-amylase and glucoamylase) and Amigase Mega L (glucoamylase) on the final concentration of ethanol after 144 h was responsible. Indeed, α-amylase is responsible for the production of oligosaccharides from starch molecules, while glucoamylase converts oligosaccharides into glucose (Åkerberg, Zacchi et al. 2000). Gluco-amylase plays an important role in peeling the molecule from the granule

surface and revealing new glucoside bonds on the next layer of the granule, which is catalyzed by α-amylase. The cooperation of both enzymes is the synergism, increasing the ethanol concentration and the fermentation productivity (Fujii, Homma et al. 1988). Therefore, the use of a higher dosage of Stargen 002 and Amigase Mega L has a positive effect on the fermentation process, increasing significantly the final ethanol content and reducing duration of fermentation.

3.3. Evolution of ethanol; total and residual sugar during SLSF process at pilot scale 1000 L

Table 15: Change of pH and temperature during SLSF process at pilot scale 1000 L

Time (h)	0	10	24	34	54	72	96	103	120	127	139	144	151	170
pH	4.62	3.99	4.08	4.1	4.19	4.22	4.28	4.27	4.29	4.25	4.27	4.28	4.29	4.29
Temp. (^{o}C)	32.0	34.5	33.5	33.0	33.5	33.0	33.0	33.0	32.5	32.5	32.5	32.5	32.5	33.0

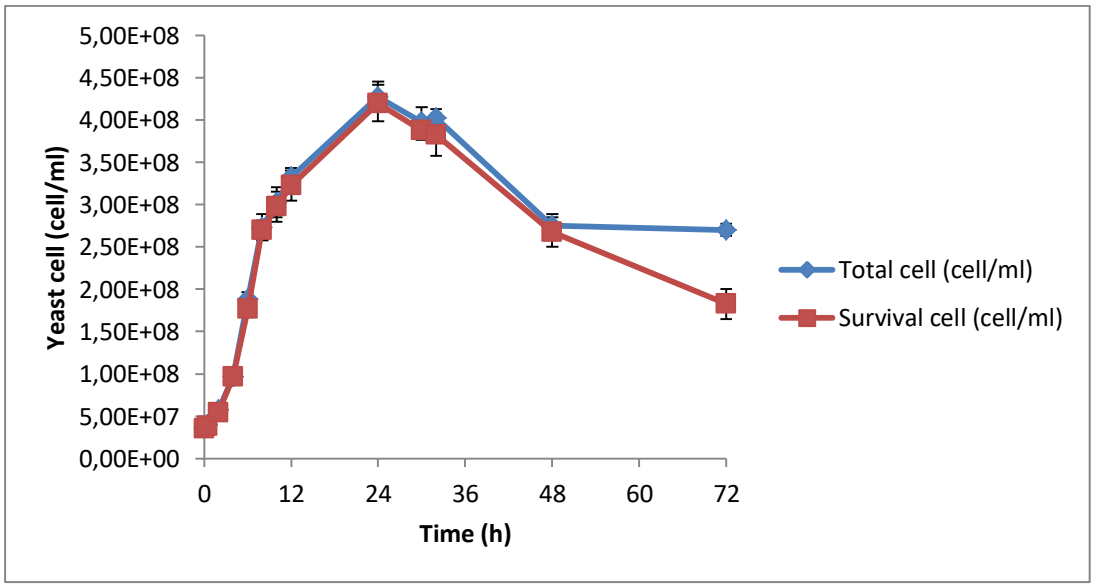


Figure 10: Growth of yeast during SLSF process at pilot scale 1000 L

Table 15 and figure 10 show the change of pH; temperature and yeast cell during fermentation. Fermentation temperature was kept at a stable level thanks to the cooling system. pH decreased during the first 10 h of fermentation, then gradually increased and stabilized after 54 hours of fermentation. The maximum yeast biomass was observed after 24 h of fermentation, reaching $4.2*10^{8}$ cells/ml. During the growth phase, the carbon source is maily used for yeast biomass development. After this phase, the yeast uses glucose mainly to product ethanol and other chemical by-products.

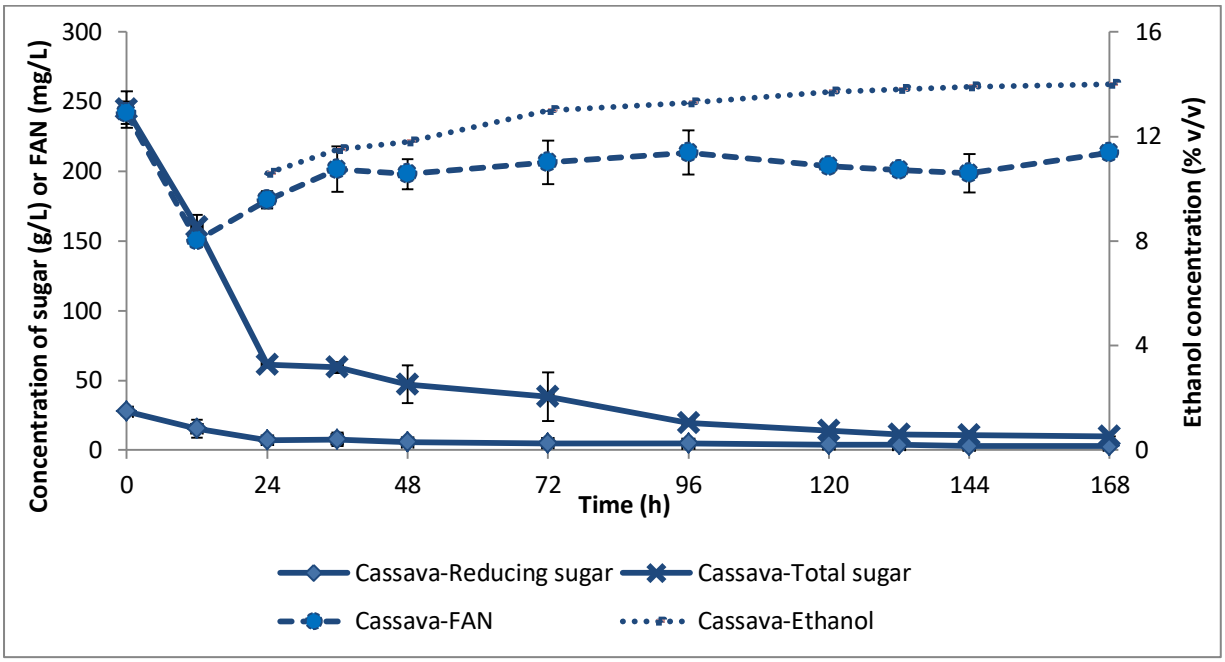


Figure 11: Evolution of ethanol, free amino nitrogen and residual sugars during SLSF process at pilot scale 1000 L

Figure 11 shows the evolution of ethanol concentration, the residual sugars during SLSF process of cassava flour. The protease was not used for SLSF using cassava flour due to the low protein content in cassava substrate (2.23±0.15%). The ethanol content obtained was 13.9 and 14.0%, equivalent to an ethanol yield of 83.06 and 83.66% after 144 and 168h, respectively. The volumetric ethanol production of SLSF at 270 g/L using cassava flour was of 0.122 g/L.h after 144 h. The concentration of reducing sugar and total acidity were of 2.9 g/L and 1.3 g H_2SO_4/L respectively. The concentrations of reducing sugar and total acidity remained low at the end of fermentation, indicating that the fermentation deviation and/or bacterial contamination did not occur.

The FAN is an important source of N for yeast growth. The amount of FAN decreased over the first 12 hours due to FAN consumption by the yeast. The autolysis of yeast cells may be the reason for the increase in FAN concentration for the next fermentation hours. This result is consistent with the research on fermentation of rye and triticale substrate at a concentration of 285 g/L using a traditional process (liquefaction, saccharification and fermentation separately) with urea supplementation (Wang et al. 1998). According to this study, during the first 24 hours, FAN levels decreased significantly due to yeast consumption, and then increased due to self-fertilization

For evaluating the ethanol yiled, the ethanol at 96% v/v was calculated with a theorical distillation performance of 98%. In summary, the totoal of ethanol 96% v/v obtained from SSF process using cassava flour was 141.9 L, respectively (Table 16).

Table 16: Synthesis of yield and total volume of ethanol 96% v/v produced using SLSF process at pilot scale 1000 L

Parameters	Unit	SLSF at 270 g/L
		Cassava flour
Fermentation volume	L	1000
Fermentation time (h)	h	144
Ethanol concentration of beer	%v/v	13.9
Yield	%	83.06
Volume of ethanol 96% v/v produced	L	141.9

3.4. Energy use, water use and production costs of SLSF

Table 17: Electricity and water used for SLSF processes at pilot scale 1000 L

Parameters	Unit	SLSF at 270 g/L
		Cassava flour
Electricity	kWh	329.7
Water	m^3	2.32

Table 17 shows the electricity and the water used for SLSF process of cassava. Water used was 2.32 m^3/1000 L. The water was utilized mostly for mixing raw-material; cooling during fermentation in which temperature was slightly increased by yeast activities and other washing. In general, no-cooking process at high initial substrate saved significantly the use of water for mixing raw-material as well as for cooling steps (liquefaction and saccharification steps) compared to conventional process (Puligundla et al. 2011). On the other hand, SLSF process used high amount of electricity. The total electricity used for SLSF of cassava flour was 329.7 kWh. The cassava chips were milled more finely (size lower than 0.3 mm), resulting in a high electricity use (9.7 kWh). In addition, SLSF of cassava flour required stirring in the first 8 hours after mixing raw materials with water in order to avoid their sedimentation. Besides stirring, the distillation step consumed a lot of electricity. Other additional activities required also electricity such as pumps, lamps, etc.

It can be noted that the higher contribution to the cost came from the raw materials (table 18). The cassava flour accounted for 38.3% of the total production cost. The raw material cost was reduced significantly due to the low price of cassava in Vietnam. However,

this raw material cost can vary considerably depending on the harvest season and the availability of raw materials in the market. The net profits of cassava were of 87.943 USD/1000 L.

Table 18: Production costs for SLSF process of cassava flour. Quantities are expressed for 1000 L of total fermentation volume

	Unit price (USD)	Quantity	Price (USD)
Raw material (kg)	0.241	303.4	73.307
Electricity (KWh)	0.13	329.7	22.760
Water (m^3)	0.20	2.32	0.464
Enzyme, yeast, urea, acid			29.204
Labor (day) [a]	6.903	2	13.806
Depreciation and other expenses (day) [b]	8.629	6	51.775
Total production cost			191.316
Value of ethanol 96% production (m^3)	1.968 (USD/L)	141.9	279.259
Theoretical profits	87.943		

[a] The labor day requirement (need only one worker) was 2 days, for the first and the last day of fermentation for SLSF of cassava flour.

[b] The "Depreciation and other expenses" was based on the duration of fermentation. This value of SLSF of cassava flour was of 6 days (144 h).

3.5. Greenhouse Gas Emissions generated by Electricity

Greenhouse gases are defined as the gases trap heat in the atmosphere. These gases are released during the combustion of fossil fuels, such as coal, oil, and natural gas, to produce electricity. Table 8 shows the GHG emissions generated by electricity of SLSF process using cassava flour. The GHG emissions of cassava based SLSF process was 153.969 kg CO_2eq.

Table 19: Greenhouse Gas Emissions of electricity use for SLSF process at pilot scale 1000 L

	Unit	SLSF at 270 g/L
		Cassava flour
Electricity	kg CO_2eq/total raw-material	153.969
	kg CO_2eq/L ethanol	1.085

3.6. Cassava-based distillers dried grains compositions

After SLSF at very high gravity at pilot scale (1000 L), the distillers wet grains (DWG) were separated from the whole stillage by using a plate filter. Then, cassava-based DWG were dried in a chamber dryer to obtain cassava-based distillers dried grains (DDG). The main compositions of cassava-based DDG were analyzed (Table 20). The cassava-based DDG had a higher contents fiber (29.32 ± 1.72%) and a lower protein (13.22 ± 0.29%) in comparison with those of corn-based DDGS (Kingsly et al. 2010).

Table 20: Main composition of cassava-based distillers dried grains

Composition	Value (% MS)
Moisture	9.02±0.12
Protein	13.22±0.29%
Fiber	29.32±1.72%
Ash	4,37±0,05
Lipid	2,51 0.04

3.7. Optimization of SLSF process

To optimize the selected SLSF-VHG process at lab and pilot scale as described, the Doehlert design was used.

Table 21: Model coefficients obtained with 14 experiments

Response means	Coefficient	Value	Signif. %
	$\mathbf{b_0}$	14.2	$<0.01^{***}$
Star	$\mathbf{b_1}$	0.300	0.0801^{***}
Amig	$\mathbf{b_2}$	0.260	0.112^{**}
Yeas.	$\mathbf{b_3}$	0.306	0.0765^{***}
Star²	$\mathbf{b_{11}}$	-0.150	4.05^{*}
Amig²	$\mathbf{b_{22}}$	-0.317	0.430^{**}
Yeas²	$\mathbf{b_{33}}$	-0.258	0.694^{**}
Star . Amig	$\mathbf{b_{12}}$	0.289	0.876^{**}
Star . Yeas	$\mathbf{b_{13}}$	-0.041	56.5^{NS}
Amig . Yeas	$\mathbf{b_{23}}$	-0.165	6.3^{NS}
	$\mathbf{R^2}$	0.992	

*Significant (P<0.05); ** Significant (P<0.01); *** Significant (P<0.001)

^{NS} Not Significant

Table 21 shows the estimate of the model coefficients calculated by the multiple regression analysis. The regression coefficient, R^2, was equal to 0.992 for Ethanol Concentration (Y_{Eth}). In other words, the models explained about 99.2% of the fraction of the

variation about the mean (Gunst 1996). The b_1, b_2, b_3 showed their positive values which were 0.300; 0.260 and 0.306, respectively. That means the quantity of enzyme (Stargen 002 and Amigase Mega L) and the yeast cells had significant influence on the final ethanol production ($p \leq 0.01$). They had a linear positive effect on the final ethanol concentration after 168 h of fermentation. In other words, the higher quantity of enzyme and yeast inoculation used, the higher ethanol concentration obtained. However, the influence of b_3 (yeast cells) and b_1 (Stargen 002) were greater than the influence of b_2 (Amigase Mega L).

The quadratic coefficients b_{11}, b_{22}, b_{33} were all negative values ($p \leq 0.05$). This means that, when the above factors (Stargen 002; Amigase Mega L and yeast cells) increased to a certain extent, the ethanol content decreased. That could be explained by the high concentration of dissolved substrates generated by both Stargen 002 (α-amylase and glucoamylase) and Amigase Mega L (Glucoamylase). The high dissolved sugars reduced significantly the yeast performance due to an increased osmotic stress during fermentation process at VHG condition (Puligundla, Smogrovicova et al. 2011).

On the one hand, a significant interaction between two enzymes, Stargen 002 and Amigase Mega L was demonstrated ($p \leq 0.01$). The positive values for b_{12} suggested a synergistic influence of both variables on the final concentration of ethanol after 168 h of fermentation. Indeed, α-amylase is responsible for the production of oligosaccharides from starch molecules, while glucoamylase converts oligosaccharides into glucose (Åkerberg, Zacchi et al. 2000). Gluco-amylase plays an important role in peeling the molecule from the granule surface and revealing new glucoside bonds on the next layer of the granule, which is catalyzed by α-amylase. The cooperation of both enzymes is the synergism, increasing the ethanol concentration and the fermentation productivity (Fujii, Homma et al. 1988). On the other hand, no significant interaction between the inoculation yeast cells with Stargen 002 or with Amigase Mega L (b_{23} and b_{13}) ($P > 0.05$).

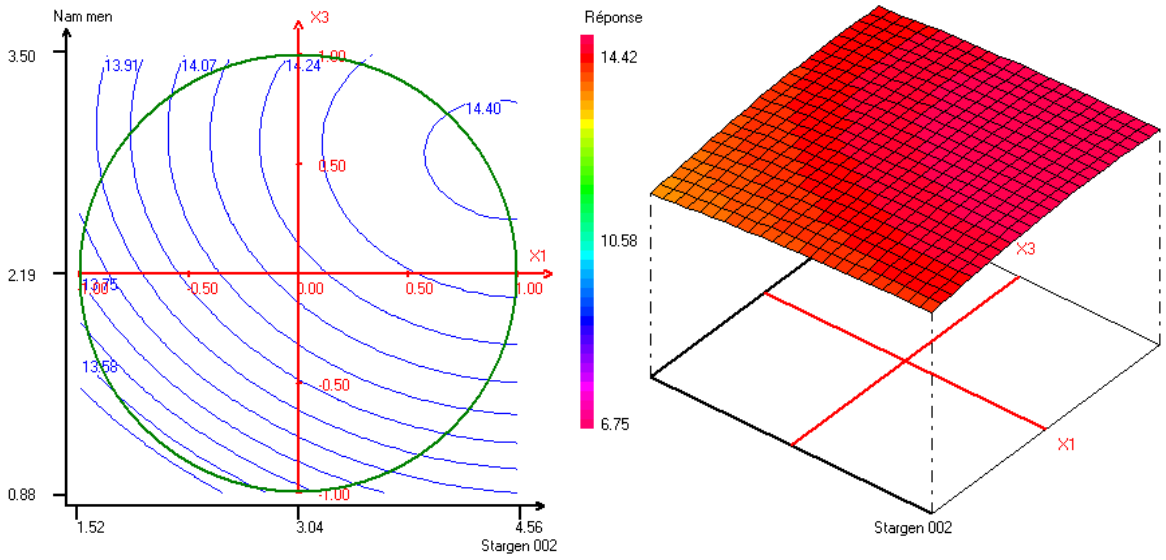


Figure 12: Contour plots of the influence of Stargen 002 and inoculation yeast cells on final ethanol concentration

In order to determine the optimum conditions for ethanol production, surface response contour plots were drawn. By fixing arbitrarily one factor at the center of the matrix, while the two other factors varied. The fixed factor should be the one with the lowest influence on the final ethanol content compared to two other factors. In our case, the fixed factor was Amigase Mega L. By fixing Amigase Mega L at 0.66 ml/kg cassava flour, the effect of Stargen 002 (X_1) and inoculation yeast cells (X_2) on the final ethanol content is shown in figure 12. The optimum conditions are schematized as a parabola. The area bounded by the parabola and the circle border represents the optimum conditions of Stargen 002 and inoculation yeast cells. The optimized point was selected based on the highest ethanol concentration (14.4% v/v) along with the lowest quantity of Stargen 002 and inoculation yeast cells. Therefore, the optimized point with Stargen 002 of 3.91 ml/kg cassava flour and the inoculation yeast cells of 2.87 cells/ml was selected as showed in table 22.

Table 22: Optimized amount of enzymes and inoculation yeast cells

Variable	Original process	Optimized process	Reduction (%)
Stargen (ml/kg NL)	4.5	3.91	13.1
Amigase (ml/kg NL)	0.9	0.66	26.6
Yeast cells (cells/ml)	3.5	2.87	18.0

3.8. Application of optimized SLSF process for ethanol production at pilot scale (15L)

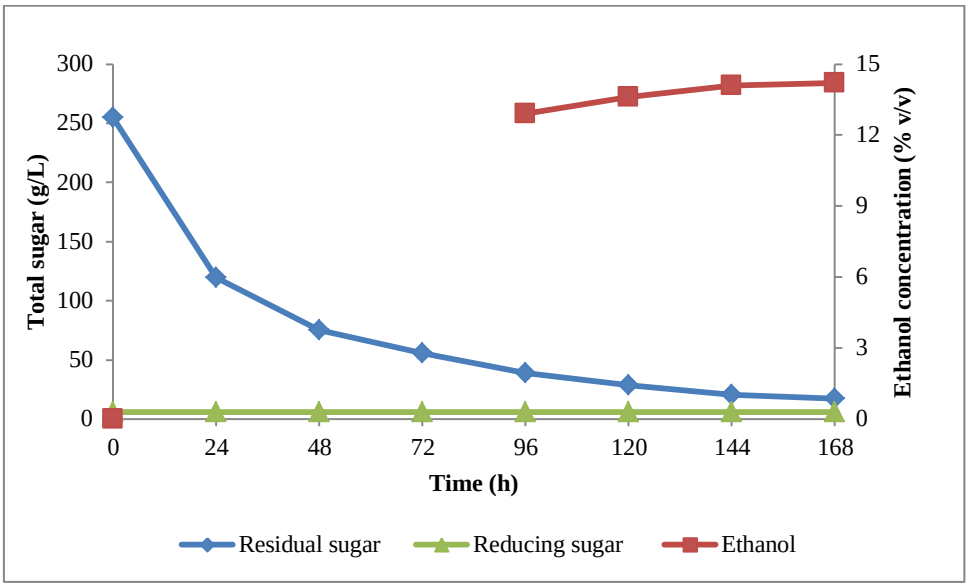


Figure 13: Evolutions of residual sugar and ethanol concentration of optimized SLSF process at pilot scale

The main goal of the pilot experiment was to verify the ethanol yield using optimized SLSF process and check problems that were not significantly noticed at the laboratory scale. According to the results of SLSF at lab scale at 270 g/L as described above, optimized SLSF was scaled up to the pilot scale (15 l/batch). Figure 13 shows the evolutions of the residual, reducing sugar and ethanol concentration at pilot scales. After 144 h fermentation, the ethanol content obtained was 14.1% v/v equivalent an ethanol yield of 84.0%. The ethanol yield was lower than that obtained at laboratory scale (14.4% v/v) ($p<0.05$). During SLSF at the pilot scale, the content of glucose (<7 g/l) and the total acidity (<1.3 g H_2SO_4/l) remained lower than the minimal inhibitory concentrations of those metabolites.

To sum up, the optimized SLSF process of cassava flour was applied successfully by using a combination of Stargen 002 (α-amylase and glucoamylase) and Amigase Mega L (glucoamylase). The duration of the process was reduced significantly when using appropriate enzymes while the final ethanol concentration was guaranteed at high level.

4. Conclusion

There was no significant effect of auxiliary enzymes and yeast nutrient on ethanol performance at the no-cooking process without thermal treatment. A higher dosage of α-amylase (Stargen 002: 2565 GAU/kg CF) and glucoamylase (Amigase Mega L: 0.105% w/w) increased significantly the final ethanol content and decreased the duration of fermentation

At lab scale, by using a higher dosage of Stargen 002 (2565 GAU/kg cassava flour) and Amigase Mega L (0.105% w/w), the SLSF process finished after 144 h of fermentation with an ethanol content of 14.4% v/v corresponding to a fermentation yield of 86.0%. At pilot scale 1000 L using these dosages of enzyme, the ethanol content of 13.9% v/v corresponding to a yield of 83.1% of the theoretical ethanol yield was obtained along with the positive economic and environmental aspects.

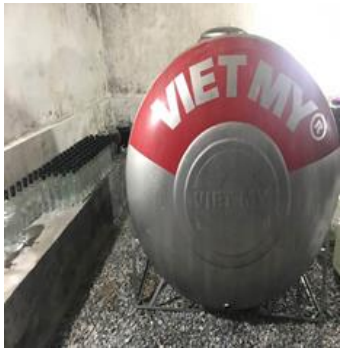
After the optimization of SLSF process by Response Surface Methodology, the quantity of Stargen 002; Amigase Mega L and yeast inoculation cells was reduced 13.1; 26.6 and 18.0%, respectively while the fermentation efficiency remained high at pilot scale 15 L (84.0% of the theoretical ethanol yield). Cassava-based distillers dried grains obtained from SLSF-VHG process had a high content of crude fiber (29.3%) and a low content of protein (13.2 %).

These studies on SLSF-VHG using cassava flour for ethanol production have a high applicability and a great potential for the ethanol and animal feeding industry in Vietnam.

Unique tank for Liquefaction, Saccharification and Fermentation-with an agitator

Distillation “pot”

Container - alcohol condensation using water



Storage tank

CHAPTER III:

ASSESSMENT OF BIOCHEMICAL AND NUTRITIONAL VALUES OF RICE- AND CASSAVA-BASED WET DISTILLER SPENT FROM SOME ETHANOL PLANTS IN VIETNAM FOR ANIMAL FEEDING

1. Introduction

DDGS, short name of dried distiller's grain with solubles, has been known as a co-product from bioethanol industry from corn or wheat (REF). In dry-grind processes, the fermentation beer is distilled and ethanol is recovered and left a remaining phase so-called whole stillage containing most of non-volatile components. The whole stillage composes of soluble and insoluble matter contains the fiber, fats, protein, other unfermented components of the grain, and yeast cells. Whole stillage is usually centrifuged to produce a liquid fraction (thin stillage) and a solids fraction (wet distillers' grains). The remaining thin stillage is concentrated through multiple effect evaporators to produce syrup called condensed distillers' solubles (CDS) (Monceaux and Kuehner 2009). While wet distillers' grains, syrup, or the combination of both (wet distillers' grains with solubles, WDGS) can be sold as animal feed, the combination of wet distillers' grains and syrup is often dried to produce dried distillers' grains with solubles (DDGS) in order to greatly lengthen its shelf-life. Since only starch and sugars are converted into ethanol, non-fermentable components in cereal grains are concentrated by a factor of more than two in DDGS (Monceaux and Kuehner 2009). Indeed, the composition of DDGS has been of great interest to researchers in the area of animal science, ethanol producers, and especially to the animal feeding industry as the majority of this has been sold as feed ingredients for livestock (Liu 2011). For example, the amount of sold DDGS from the US to all over the world was around 12 million tons in 2016, in which Vietnam at third position imported approximately 1.2 million tons DDGS (Council 2017). Indeed, the Vietnamese Ministry of Industry and Trade declared that bio-fuel production will achieve 1.8 million tons in 2025, which accounts for 5% of country's demand (Ministry-of-Industry 2007). Moreover, the government also adapted the policy to improve the beverage ethanol industry in Vietnam. By the Development strategy of beverage ethanol production in Vietnam from 2007-2025 (Ministry-of-Industry 2007), ethanol industry will produce 188 million liters ethanol for food industry in 2025. Overall, the beverage and bio-ethanol industry has a great potential in Vietnam in the future. More recently, the Ministry of Industry and Trade is not extending the January 1, 2018 deadline for petrol wholesalers to switch to E5 biofuel made from cassava. The main raw materials for bioethanol production are rice and cassava. Regardingless raw materials, it is estimated that to gain 1 liters of ethanol, 2.3 kg of material are used and about 0.15 kg (6.5% of raw material) of distiller spent produced. This means that annually, approximately 0.72 million tons of ethanol distiller spent are generated in Vietnam. In contrast, the wet by-product from ethanol factory has been used since long time ago for purpose of feeding in Vietnam in the fresh form but simply by directly adding in

the diet of animals without treatment (Nguyen, Luu et al. 2002). Some preliminary data showed the benefit of by-products to animals such as pig or chicken (Nguyen, Luu et al. 2002). However, there has been little work which elucidated the nutrient composition of such by-products, except some data on the whole distillate from the home-scale distillers (Nguyen, Luu et al. 2002).

The aim of this work was to identify and assess the nutrient values of distiller spent (DS) produced in 5 ethanol factories in the North, Centre and South of Vietnam, and to show their potential application for animal feeding, which may help to reduce the imported DDGS.

2. Materials and methods

2.1 Samples collection

Seven wet distiller spent (WDS) samples were collected from different ethanol factories located over the country. The raw materials used in these factories were either rice or cassava. There was one sample collected from a factory in Southern of Vietnam based on imported maize. The WDS samples were transported immediately after production directly to Hanoi University of Science and Technology and were dried following a protocol as follows: 90^{o}C for 30 min, 80^{o}C for 2.5-3 h and finally 70^{o}C for 1 h in a circulating dryer. The dried distiller spent (DDS) samples then were packed in plastic bags and stored at -10^{o}C for further analysis.

In this study, seven DDS samples were investigated. Six samples of DDS were collected from 5 different ethanol factories located in the North, Center and South of Vietnam (Table 23 and figure 14). Another sample (named as SLSF) was obtained from a pilot-scale simultaneous liquefaction saccharification and fermentation process (SLSF) carried out at Victory Viet Phap JSC in Hoa Binh province.

2.2. Analytical methods

Different compounds in DDS samples were analyzed by using the standard methods including phosphorus (ISO 6491:1998), calcium (ISO 06490:1985), lipid (ISO 6492:1999), ash (ISO 05984:2002), crude fiber (with Ankom filter bag technique), total crude protein (ISO 05983-1:2005). Starch was determined by the acid hydrolysis method, in which starch was hydrolyzed to the reducing sugar by HCl 2% for 2 hours in boiling water bath. The reduced sugar content was determined by using Dinitrosalicylic Acid (Miller 1959).

Figure 14: Ethanol production outlines applied in 5 factories

. A. Victory Viet Phap JSC (Hoa Binh province) and Saigon - Dongxuan JSC (Phu Tho province); B. HALICO (Hanoi Liquor JSC (Bac Ninh province); C. Tung Lam JSC (Dong Nai province); D. SLSF (Hoa Binh province)

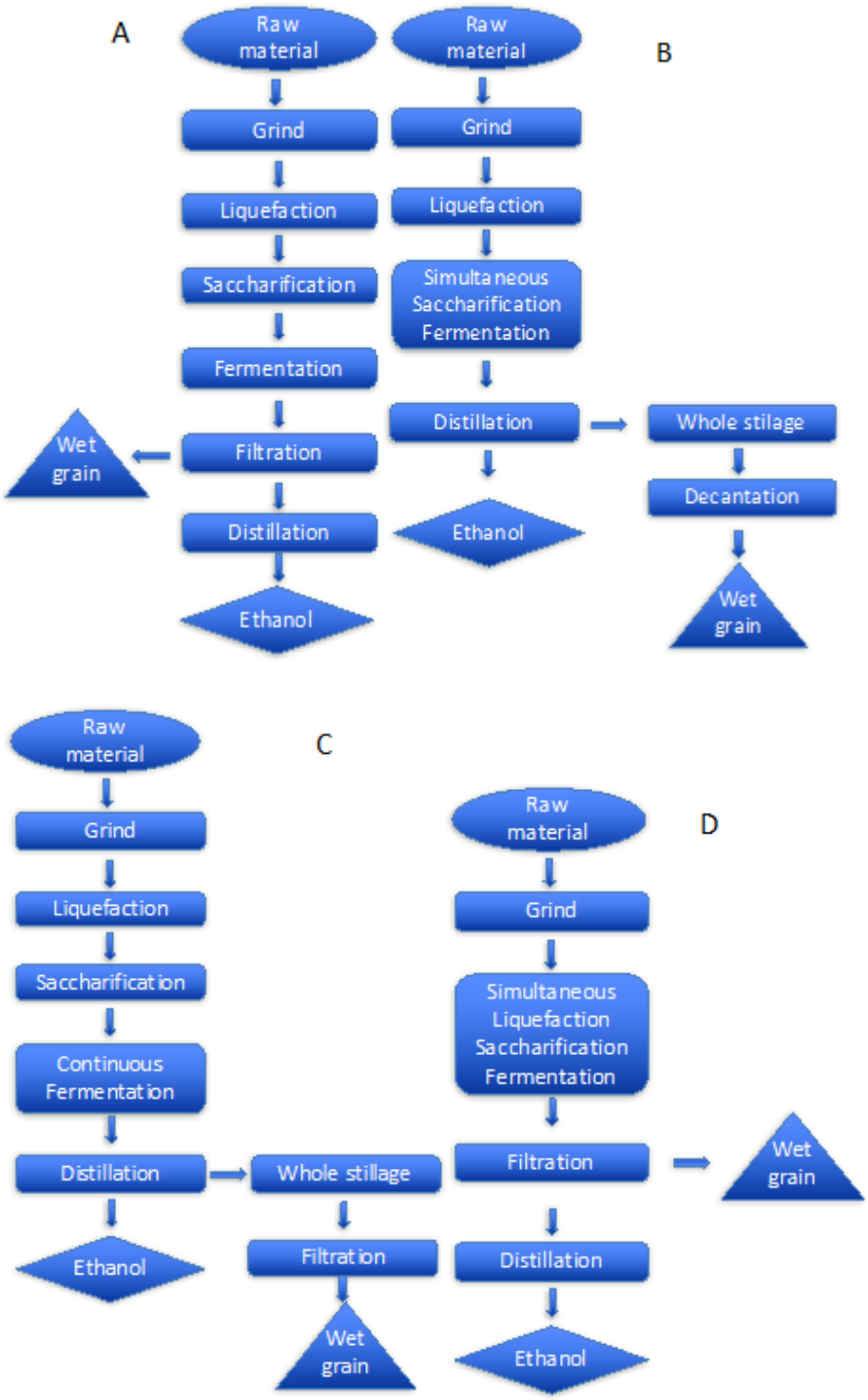


Table 23: Overview of ethanol production and Wet Distillers Spent (WDS) usage in different factories in Vietnam

Samples	**SLSF**	**SG-DX**	**VP**	**HALICO**	**BSR-BF**	**TL-CS**	**TL-CO**
Ethanol plants	Pilot	Saigon-Dongxuan JSC	Victory Viet Phap JSC	Hanoi Liquor JSC	Dung Quat bioethanol plant	Tung Lam limited liability company	Tung Lam limited liability company
Capacity (l/year)	150,000	1,500,000	1,200,000	10,000,000	100,000,000	76,000,000	76,000,000
End user	Beverage ethanol	Beverage ethanol	Beverage ethanol	Beverage ethanol	Fuel ethanol	Fuel ethanol	Beverage ethanol
Location	HoaBinh province North Vietnam	Phu Tho province North Vietnam	HoaBinh province North Vietnam	BacNinh province North Vietnam	Quang Ngai province Vietnam Center	Dong Nai province South Vietnam	Dong Nai province South Vietnam
Raw materials	Rice	Rice	Rice	Rice	Cassava	Cassava	Corn
Processing	Simultaneous liquefaction, saccharification and fermentation at pilot scale (500 l/batch)	Separate liquefaction, saccharification and fermentation	Separate liquefaction, saccharification and fermentation	Separated Liquefaction Simultaneous Saccharification and fermentation	Separated Liquefaction Simultaneous Saccharification and fermentation	Liquefaction, saccharification and continuous fermentation	Liquefaction, saccharification and continuous fermentation
Separation	Wet distillers grains (WDG) were filtered before distillation by a frame and plate filtration	WDG were filtered before distillation by a frame and plate filtration	WDG were filtered before distillation by a frame and plate filtration	WDG were separated after distillation by decanter	WDG were separated after distillation by decanter	WDG were filtered after distillation by a frame and plate filtration	WDG were filtered after distillation by a frame and plate filtration
Drying	No	No	No	No	Flash drying	Flash drying	Flash drying

2.3 Amino acid determination

DDS samples (around 20-40 mg of sample) were hydrolyzed in vapor phase of 1 ml HCl 6 M, 0.5% phenol for 24h at 120^{o}C. Afterward, the hydrolyzed DDS samples were re-suspended in deionized water for neutralization to pH 7 with NaOH and brought up to 10 ml of total volume. These solutions were filtered through 0.2µm membrane before applying for HPLC analysis.

The amino acid profiles were determined by using Agilent 1200 series (Germany) with DAD detector (at 338 nm of wave length). Amino acids in samples were derivatized with OPA reagent (Sigma, USA) in auto-sampler before injection for separation in C18 ElipseZorbax 5 µm, 4.6 x 150 mm (Agilent, US). The gradient elution was performed with buffer A Sodium phosphate 40 mM, pH 7.8 and buffer B consisting of methanol, acetonitrile and deionized water with respective ratio 45:45:10. The buffer A was changed during elution as following 100% (0 -1.9 min); to 50% (1.9 - 15.5 min), to 43% (15.5 - 21 min), to 0 (21 - 22 min), 0% (22-26 min); to 100% (26 -27 min); 100% (27 – 31 min). The analysis time was 31 min at flow rate of 1 ml/min. The temperature of separation was maintained at 30^{o}C.

2.4. Gross energy

Gross energy (GE) was measured by Bomb Calorimeter (Parr/USA). Digestible energy (DE) and metabolizable energy (ME) values were calculated using the following formulas (Spiehs, Whitney et al. 2002). DE (kcal/kg) = 4151- (122 x% Ash) + (23 x crude protein in %) + (38 x % EE) – (64 x crude fiber in %) and ME (kcal/kg) = DE x (1.003 – (0.0021 x crude protein in %)).

2.5. Data analysis

Descriptive statistical parameters such as mean values and standard errors were calculated using Microsoft Excel from at least duplicate measurements with the standard deviation was less than 5%.

3. Results

Seven WDS samples were collected from 5 factories are different in raw materials, capacity, ethanol production process and WDS treatment (Table 23, Fig. 14). Among, 4 WDS samples (named SLSF, SG-DX, VP and HALICO) were obtained from rice-based by-products; 2 WDS samples (BSR-BF and TL-CS) were obtained from cassava-based by-products; and the rest (TL-CO) was based on imported corn. Seven respective DDS samples were obtained and analyzed for the proximate components and amino acid profile. Obviously, DDS samples obtained from 5 factories were different in composition as shown in Table 23.

3.1. Proximate composition of rice-based and cassava DDS samples

The protein the in rice-based DDSs amounted from 52 – 80% of dry matter, while in the cassava-based DDSs, this value was only 12.00 – 16.40 % of dry matter. The protein composition in the corn-based DDS sample was estimated of 35.6% of dry matter.

The starch content was measured in range of 8.55% to 15.01% of dry matter for the rice-based samples, and of 10.90-25.4% for the cassava-based samples. Meanwhile, the starch contributed up to 26.63% of dry matter in the corn-based sample.

The fiber content was found inconsistent among these DDSs. Two cassava–based DDSs, BSR-BF and TL-CS contained the significantly high fiber, which was of 32.8% for the former and of 16.5% for the latter. The fiber content of the rice-based DDSs varied in a range of 9.00 – 11.58%, except the SLSF that was of 18.42%. The corn-based TL-CO contained a slightly lower of fiber compared to the rice-based DDS, amounting of 7.59% of dry matter.

Fat content was found as rather varied from sample to sample. Fat was at higher level in the rice-based samples such as SLSF (7.47%), SG-DX (9.70%) and the corn-based TL-CO (4.56%). Surprisingly, the rice-based HALICO contained rather low of fat (0.69%). The fat in cassava–based DDSs varied in a narrow range of 2.30 – 2.94% of dry matter.

The ash of rice-based DDS samples amounted from 1.44 – 2.61% of dry matter. This component was slightly higher in the corn – based DDS (3%). In contrast, ash contributed a high level in the cassava-based DDSs ranging from 9.05 to 2.08%.

Calcium and phosphorus were also determined for the rice-based DDSs and one cassava-based DDS sample. Phosphorus was at low level in almost of test samples (between 0.01 – 0.03% of dry matter). Calcium was very low in the rice-based SG-DX (0.02%), but much higher in the cassava-based DDS of BSR-BF.

Due to the higher level of protein and lower level of crude fiber, the digestible energy (DE) and the metabolizable energy (ME) of the rice-based DDSs were approximately four times higher to that of cassava-based DDSs (Table 24).

3.2. Amino acid profiles

The amino acid profiles of these DDSs samples were determined. It can be seen that the consistent result was observed (Table 3). The amino acid profile was generally similar among the DDSs which derived from same type of raw materials. In agreement to the protein composition, the amino acid was lower in DDS based on casava, corn than other rice-based DDSs. Most of essential amino acids with significant amount were determined in the DDSs, especially the rice-derived samples. Each of amino acid such as leucine, phenyl alanine, arginine, lysine contributed higher than 3% of dry matter in the DDSs from rice material. These amino acids amounted much lower in cassava- and corn-based DDSs, except leucin in the TL-CO sample was comparable to that of rice-based DDS (Table 25).

DDS samples contained also most of non-essential amino acids. Among aspartic acid and glutamic acid were found as the highest amino acids in most of DDSs regardingless the raw material. These amino acids contributed up to 7.7 – 9 % of dry matter in the rice-based DDSs (except the SLSL sample, due to the lower protein content of this sample) and 3.7 - 6.1% of dry matter in the corn-based TL-CO. Alanine was found also in these DDSs at remarkable level, especially in the DDS from rice and corn, in which more than 3% of dry matter was alanine. Amino acids of cassava-based DDS samples were lower in accordance to the lower protein ratio. These amino acids including arginine, leucine, asparatic acid, glutamic acid and alanine presented in these DDSs at level above 1%. In contrast, histidine, glycine, tyrosine and cysteine amounted below level of 0.34% (Table 25).

Table 24: Composition of Dried Distillers Spent (DDS) samples

Compositions		SLSF	SG-DX	VP	HALICO	BSR-BF	TL-CS	TL-CO	Ref DDGS
Ethanol plants/Province		Pilot at Victory Viet Phap JSC / Hoa Binh	Saigon-Dongxuan JSC / Phu Tho	Victory Viet Phap JSC / Hoa Binh	Hanoi Liquor JSC / Bac Ninh	Dung Quat bioethanol JSC / Quang Ngai	Tung Lam JSC / Dong Nai	Tung Lam JSC / Dong Nai	(Belyea, Rausch et al. 2004)
Raw materials		Rice	Rice	Rice	Rice	Cassava	Cassava	Corn	Corn
Crude Protein (% DM)		51.49	70.44	74.96	79.60	12.00	16.40	35.6	31.4
Non-protein (% DM)	Starch	15.01	14.29	11.77	8.55	25.40	10.90	26.63	5.3
	Crude Fiber	18.42	2.90	11.58	8.98	32.80	16.5	7.59	10.2
	Fats	7.47	9.70	2.11	0.69	2.3	2.94	4.56	12.0
	Ash	1.74	2.27	1.44	2.61	8.31	12.08	3.00	4.6
Calcium (% DM)		0.11	0.02	0.04	0.14	0.41	-	-	-
Phosphorus (% DM)		0.02	0.02	0.03	0.03	0.01	-	-	-
GE (kcal/kg DM)		5,092	6,154	5,476	5,408	4,132	2,863	4,006	-
DE (kcal/kg DM)		4,228	5,354	5,038	5,115	452	2,110	4,291	-
ME(kcal/kg DM)		3,783	4,556	4,260	4,275	441	2,044	3,983	-

Table 25: Profile of amino acids of DDG samples

Amino acids (%)	SLSF	SG-DX	VP	HALICO	BSR-BF	TL-CS	TL-CO
Essentials amino acids							
HIS	1.05	1.58	1.35	1.64	0.25	0.27	0.93
ARG	2.60	3.37	4.44	3.44	1.19	1.21	1.91
THR	1.87	2.09	2.23	2.28	0.45	0.70	0.99
VAL+MET	2.17	2.45	2.79	3.26	0.42	0.68	0.95
PHE	2.57	3.29	3.41	3.48	0.56	1.12	1.70
ISOLEU	2.22	2.19	2.39	2.79	0.51	0.89	1.07
LEU	3.86	4.46	4.85	4.68	0.90	1.45	4.61
LYS	2.53	3.60	3.59	4.16	0.88	0.99	0.99
Non-essential amino acids							
GLY	0.54	1.44	1.49	1.83	0.09	0.14	0.37
ASP	4.17	8.16	7.88	7.70	2.42	2.44	3.70
GLU	7.37	8.53	9.05	9.05	1.41	2.26	6.10
SER	1.95	2.38	2.38	2.48	0.55	0.73	1.38
ALA	3.14	3.28	3.67	3.21	1.04	1.54	3.24
TYR	1.50	2.55	2.51	3.09	0.14	0.25	1.20
CYS	1.11	2.00	2.23	3.25	0.20	0.34	0.57
Total protein (%DM)	51.49	72.44	74.96	79.60	11.01	16.40	35.60

The values were the averages of duplicate experiments

4. Discussion

The wet by-product from ethanol industry has been used for long time for purpose of animal feeding in Vietnam. However, the nutrient composition of this potential by-product has not been elucidated, except little data on the whole distillate from the small home–distillers using rice (Nguyen, Luu et al. 2002). In contrast, there are a huge bank of data for wheat- and corn-based DDGS which have been published several years (Belyea, Rausch et al. 2004, Belyea, Rausch et al. 2010, Liu 2011, Rosenfelder, Eklund et al. 2013, Alagón, Arce et al. 2016, Böttger and Südekum 2017, Böttger and Südekum 2017). In fact, rice and cassava have been known as more popular crops in Asian and African countries than maize and wheat. This study was the first systematic work focusing on the nutrient compositions of by-products from different ethanol factories in Vietnam using rice, cassava as raw materials.

4.1.Effect of technology and raw materials on composition of DDS

In this study, the variation of nutrient composition including crude protein, starch, fat, crude fiber and ash of 7 DDS samples collected from 5 ethanol factories was observed. The variation in composition was also proven for DDGS from corn and wheat (Belyea, Rausch et al. 2010, Liu 2011). The authors showed that the types of raw materials, the ethanol processing and WDG treatment definitely influenced on the composition of DDGS, and this was an explanation for the variation we observed with DDSs in this study. These DDSs were different not only in the raw material, but also in ethanol processing and treatment of WDS. Regardingless to the raw material as rice, cassava or corn, in general, the bioethanol manufacture starts with a liquefaction step of the hammer milling-ground raw material. The liquefaction was performed at high temperature of above 100^{o}C with supported by a heat stable α-amylase. A high pressure steam treatment (temperature of 105 -110^{o}C) also was applied in HALICO and Tung Lam limited liability company in order to archive the high extensive liquefaction of starch in raw material. A saccharification step was performed either separately (as in Saigon - Dongxuan JSC, Tung Lam limited liability company and Victory Viet-Phap JSC) or simultaneously with fermentation (as in HALICO and Dung Quat bioethanol factory). Tung Lam limited liability company carried out a continuous fermentation meanwhile others in batch mode. The SLSF sample was different from others because the liquefaction, saccharification and fermentation were simultaneously performed at 30^{o}C with yeast in the supporting of liquefying, saccharifying enzymes. Moreover, the WDS were differently harvested and treated from factory to others. The wet-spent grain was

separated by plate-frame filter before the alcohol distillation to avoid the blocking for distilling tower as at Victory Viet Phap JSC and Saigon – Dongxuan JSC. Meanwhile, in other factories, the WDS were separated by a high-speed decanter (HALICO, Dung Quat bioethanol factory) or by a frame-plate filter (Tung Lam) from whole stillage after the alcohol distillation. Moreover, in HALICO factory, the thin stillage after the decantation was partly reused to reduce the pH of rice mash. Interestingly, in Dung Quat bioethanol factory, spent from cassava starch process, which contained up to 50% of fiber, was combined with WDS from bioethanol process.

The composition of raw materials such as white rice, cassava chips and corn has been extensively elucidated. Based on dry matter, rice (milled rice or white rice) composes of less crude fiber (0.1 -0.8%) than corn (3%) or cassava (3.7- 4.0%) (Zhou, Robards et al. 2002, Montagnac, Davis et al. 2009, Nuss and Tanumihardjo 2010, Morgan and Choct 2016). On the other hand, white rice contains 7-8% of crude protein, which is less than that in corn (9.42%) but more than in cassava (1-3%) (Zhou, Robards et al. 2002, Montagnac, Davis et al. 2009, Nuss and Tanumihardjo 2010, Morgan and Choct 2016). Three types of raw material compose of comparable level of carbohydrate with mainly starch, i.e. 80% for rice and cassava and 72% for corn (Zhou, Robards et al. 2002, Montagnac, Davis et al. 2009, Nuss and Tanumihardjo 2010, Morgan and Choct 2016). The indigestible such as crude fiber in the raw material, which is mostly left in DDS, could result in the difference in compositions of DDSs. This can explain for the high level of protein in DDS from rice materials, which was determined as 2-3 times higher than in the corn-based DDS and 6 times higher than in the cassava-based DDSs (Table 24). Even though the thin stillage was not taken into account, the rice based DDSs in this study contained more protein than the corn DDGS which ranged from 26-32% (Cromwell, Herkelman et al. 1993, Spiehs, Whitney et al. 2002, Belyea, Rausch et al. 2004, Kim, Mosier et al. 2008, Liu 2011). Also, in this work the protein content in the corn based TL-CO was also slight higher than the range (35.6%) (Table 24). Yeast biomass have been known as a rich source of protein which was reported in range of 38.8 – 70.7% dry matter (Martini, Miller et al. 1979). The amount of protein in DDGS is affected by yeast as shown on review of Liu et al (Liu 2011). In an uncertain estimation, the authors indicated that yeast contribute approximately 5.3% of protein in DDGS, however, this number is much lower than number from rice- DDS in this study.

The higher fiber in cassava resulted in the higher of this parameter in cassava DDSs (up to 16.5% in TL-CS) in comparison to the rice DDSs (Table 24). The high content of fiber

in the cassava-based BSR-BF was explained by the addition of the cassava spent, a by-product of starch processing which contains up to 50% of fiber. The fiber content in the corn-based TL-CO in this study was in agreement to the reported range for DDGS (7.22 – 10.2 %) (Spiehs, Whitney et al. 2002, Belyea, Rausch et al. 2004, Monceaux and Kuehner 2009).

The content of residual starch in DDS indicated the effectiveness of heat treatment and the fermentation as well on the raw material (Belyea, Rausch et al. 2004). Starch content in corn-based DDGS ranges from 3 - 6% (Belyea, Rausch et al. 2004, Liu 2011). The starch content in DDS in this study might be overestimated because of the analysis method using hydrolysis of hydrochloride acid and DNS for reducing sugar determination.

Despite of the lower ash content in cassava (Montagnac, Davis et al. 2009, Morgan and Choct 2016), the ash content of cassava-DDS were higher than that in rice-based DDS (1.44 – 2.61%) and corn-based DDS (3%) (Table 24). We also found in this work that the fat content in the corn based TL-CO was 4.56%, which is a half to one-third of reported range from 10.2 – 14.5% of DDGS (Spiehs, Whitney et al. 2002, Belyea, Rausch et al. 2004, Kim, Mosier et al. 2008, Liu 2011).

There was no correlation between fat content in raw material and in DDS samples. For example, the fat content in rice (1-2%) and cassava (0.5-1.0%) is lower than in maize (4.72%) (Montagnac, Davis et al. 2009, Nuss and Tanumihardjo 2010, Morgan and Choct 2016), and the fat in the rice-DDG varied from 0.69% - 10.41% (Table 24).

The difference in composition data between the rice–based VP and the SLSF suggests that the ethanol processing technology leads the significant effect. The VP and the SLSF were from the same plant but different process show varied data in protein, starch, fiber and other components. Interestingly, the DDS from SLSF differs remarkably from that of VP on protein (51.49%), fiber (18.42%) and fat (7.47%). It is likely because of the uncooked and simultaneously saccharification and fermentation in SLSF process. The fiber in this process may not influence by heating, thus presenting in higher ratio in the DDS (Table 24).

4.2.The potential of rice- and cassava- based DDS for animal feeding

It is well-known that protein is one of most important parameters in animal feeding, thus alternative protein sources have been exploited and supplemented in feed formulation, such as soy, canola and cotton meal (Cai 2014). The protein content in oilseed meal is about 40 -55% (Broderick, Faciola et al. 2015) which is a little higher than protein content in DDGS from corn, but still much lower than protein in some rice-based DDS shown in this study.

Moreover, the profile of amino acids in the rice -based DDS is really suitable for animal feeding with the balance of essential and non-essential amino acid (Table 25). Similarly in rice as reported previously (Khoi, Dien et al. 1987), in the rice-based DDS the essential amino such as isoleucin, leucine, phenylalanine, valine, threonine also present at higher level than others. This also means the ethanol process did not change the amino acid profile of raw material to DDS as mentioned previously (Liu 2011). Lysine, an important amino acid for growth of swine and poutry (Liao, Wang et al. 2015), is considered as the first limiting in cereals such as maize, cassava and even in some varieties of rice (Torbatinejad, Rutherfurd et al. 2005). In some varieties of Vietnamese rice, lysine was in range of 3-5 % of total protein (Khoi, Dien et al. 1987) which is in agreement to the lysine content in rice DDSs found in this study (approximately 5% of total protein) (Table 25). Lysine presents in rice-based DDS at level of 2.5-4 times higher than in cassava -or corn-based DDS (Table 25). All together indicate the potential of rice-based DDS for animal feeding.

Cassava-based DDS contained the low level of protein but high of fiber. Fiber, even though does not generate energy, has to be included in the diet to maintain normal physiological functions in the digestive tract (Lindberg 2014).

5. Conclusions

In this study, the main proximate such as protein, lipid, ash, starch, amino acids, ash, calcium and phosphorous as well as gross energy of dried distiller spent based on rice, cassava and corn from different ethanol factories were firstly determined. Among, the rice-based DDSs were shown as the most potential for animal feeding with high crude protein and appropriate amino acid profile including essential amino acids in balance to non-essential ones. Similarly, cassava-based DDS also exhibited the valuable for animal feeding, especially the high level of fiber. Even though, the feeding experiments in which these DDS are supplemented should be performed to evaluate their digestibility and effectiveness to animal, these data suggest that the DDGs from bioethanol industry are potential and promising for animal feed in Vietnam.

CHAPTER IV:

PROTEIN ENRICHMENT OF CASSAVA-BASED DRIED DISTILLER'S GRAIN BY SOLID STATE FERMENTATION USING TRICHODERMA HARZIANUM AND YARROWIA LIPOLYTICA FOR FEED INDREDIENTS

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Protein enrichment of cassava-based dried distiller's grain by Solid State Fermentation using *Trichoderma harzianum* and *Yarrowia lipolytica* for feed ingredients

Mai-Dinh Vuong[1, 2], Nguyen-Tien Thanh[1], Chu-Ky Son[1, 2] * and Waché Yves[1, 2]

[1] School of Biotechnology and Food Technology (SBFT), Hanoi University of Science and Technology (HUST), 1 Dai Co Viet, Hai Ba Trung, Hanoi 100000, Vietnam

[2] International Joint Laboratory Tropical Bioresources & Biotechnology between School of Biotechnology and Food Technology, Hanoi University of Science and Technology and UMR PAM, Univ. Bourgogne Franche-Comté, AgroSup Dijon, PAM UMR A 02.102, F-21000 Dijon, France

* Corresponding author:

Assoc Prof. Son Chu-Ky; Email: **son.chuky@hust.edu.vn**

Protein enrichment of cassava-based dried distiller's grain by Solid State Fermentation using *Trichoderma harzianum* and *Yarrowia lipolytica* for feed ingredients

ABSTRACT

Cassava-based dried distiller's grain (Cassava-based DDG) is known as a by-product of the bio-ethanol industry with low nutritional value due to the presence of cyanide and to the low content of protein. More value can be added to cassava-based DDG through solid-state fermentation (SSF) using mold and yeast. SSF were conducted with cassava-based DDG in 8 and 5 days of fermentation, respectively. Under optimal conditions, the crude protein fraction of cassava-based DDG fermented by *Trichoderma harzianum* BiomaTH1 or *Yarrowia lipolytica* W29 was increased from 11.84% DM for unfermented sample to 15.29 and 14.06% DM, respectively. In addition, the total amino acids of fermented samples using *T. harzianum* and *Y. lipolytica* was increased from 11.01 % DM to 13.86 % DM and 12.39 % DM along with an increase in the essential amino acids content which enhanced by 55% and 22%, respectively, including limiting amino acids in pig feeds. The *in vitro* protein digestibility was improved significantly from 82.5% to 89.2 and 86.9% for *T. harzianum* and *Y. lipolytica* fermentation, respectively. Beside increasing the nutritional value, the SSF showed a clear effect in reducing cyanide content of raw cassava DDG from 62.3 mg/kg DM to 24.3 and 53.6 mg/kg. The obtained results indicated that the protein enrichment of this bio-ethanol by-product using mold and yeast fermentation could be very promising to be used efficiently as a cheap and abundant source of essential amino acids for animal feed ingredients in Vietnam. The nutritional projection of adding this cheap ingredient was discussed.

Keywords: *Cassava-based dried distiller's grain, solid state fermentation, Trichoderma harzianum, Yarrowia lipolytica, protein enrichment, animal feed ingredients*

1. Introduction

Despite being a country in Southeast Asia with a big tradition of agriculture, Vietnam imports annually 65-70% of raw materials for feed production, mostly from Europe and America, with a total import value of around 4 billion USD. These materials include dried-grain distillers with solubles (DDGS) from corn and wheat that are by-products from bio-ethanol plants. According to the Ministry of Industry and Trade, in comparison with other countries in the Asian region, the price of feed production in Vietnam is always 15-20% higher because of this importation. Therefore, the use of local available by-products and materials has been suggested as the most important alternative for livestock. Cassava is one of the most popular crops, playing a key role in Vietnam's agricultural structure. Besides being used as a rich source of starch in foods, cassava is considered an attractive raw materials for bioethanol production which would achieve 1.8 million liters in 2025, accounting for 5% of country's demand (Ministry-of-Industry 2007). In our previous research, the content of cassava based DDG was characterized as high in crude fiber (32.8%); low in protein (12%) and amino acids (5.16%) with limited interest for animal feeding (Taranu et al. 2019). Therefore, the question is how to increase value-added in this potential source, improve farm profitability, provide jobs, produce protein material for feed processing industries and reduce dependence on imported raw materials for feed production.

One of the most popular ways to enhance protein content in lignocellulosic substrate is solid-state fermentation (SSF). The advantage of this method is its low investment requirement. It has been used in many fields, including protein enrichment in animal feed production (Gelinas et al. 2007, Ugwuanyi et al. 2008). SSF is a process whereby an insoluble substrate containing sufficient moisture but without free water allows the microorganism to grow and metabolize. Besides some bacterial species, yeast genera such as *Saccharomyces, Yarrowia, Candida,* and filamentous fungi of *Aspergillus, Chaetomium, Paecilomyces, Penicillium,* and *Trichoderma* genera have been used for protein enrichment. *Trichoderma* species are considered useful or at least not harmful to humans and animals (Şişman et al. 2013). They have been known for producing many extracellular enzymes and are mostly used in food and textile industries to degrade complex polysaccharides (Ezekiel et al. 2013). For instance, *Trichoderma harzianum* actively takes part in the decomposition of plant residues in the soil (Harper et al. 1985). Its efficiency to enrich the protein content in various cellulosic agricultural by-products was reported in previous studies such as peels of mango, orange, apple, banana and tomato wastes (Osama A. Abo Siada et al. 2018); rice

polishing (Ahmed et al. 2017); sunflower lignocellulosic fraction (Parrado et al. 1993); and cassava root meal (Muindi et al. 1981).

On the other hand, the non-conventional yeast *Yarrowia lipolytica* was certified as GRAS (Generally Recognized As Safe) from FDA (American Food and Drug Administration) for use in food and pharmaceutical industries. Recently, it has received great attention as a potential source of single cell protein (SCP) (Ritala et al. 2017). This obligate aerobic yeast is well known for its ability to produce proteases, peptidases and lipases, increasing the nutritional value of the agricultural substrate. However, *Y. lipolytica* does not produce cellulases and hemicellulases so it cannot reduce the content of fiber in agricultural residues (Wang et al. 2014).

Many previous studies have shown that SSF processes using molds and yeasts not only increase the crude protein content but also enhance significantly the profile of essential amino acids in agricultural substrates at very low cost. The essential amino acids, especially lysine, leucine, methionine, valine, isoleucine, threonine have been commonly used in animal feeds, since they tend to be deficient in natural feedstuffs and cannot be synthesized by animals (Ahmed et al. 2017, Vong et al. 2018). This is why the addition of protein obtained from bio-processing of agro-byproducts to animal feed has played an increasingly important role in reduction in animal feed costs which account up to 2/3 or more of total animal production cost, especially in developing countries (Lemke et al. 2008, Ajila et al. 2012). In addition, with many environmental concerns about the use of wild fish for production of fish meal protein, leading to overfishing, ecological imbalance as well as unsustainability of fisheries, the partial replacement of this commercial protein ingredient feed by fermented agro-byproducts has been studied to meet the development needs of livestock sector (Nguyen et al. 2009, Hong et al. 2017).

Hence, using *Trichoderma harzianum* and *Yarrowia lipolytica* for solid state fermentation on cassava based DDG brings the following benefits: (i) being common microorganisms, well adapted to climates in Vietnam; (ii) not harmful to humans and animals; (iii) produce proteolytic enzymes that help increasing protein-value and reduce of anti-nutrients in byproduct; (iv) increase the value added of agro by-products. For these above reasons, our research focused on enrichment of protein and essential amino acids of cassava-based DDG; decrease of hydrogen cyanide – anti-nutrient by the SSF using *Trichoderma harzianum* and *Yarrowia lipolytica*. The nutritional projection of adding this cheap ingredient in pig feed to replace partially, about 10-40% protein from fish meal was

investigated. To realize this aim, the optimal conditions of fermentation process and nutritional compositions of fermented cassava-based DDG were carried out.

2. Materials and methods

2.1. Samples collection

The wet distiller grain (WDG) from a cassava-based bioethanol plant (BSR-BF) located in Quang Ngai province (Center of Vietnam) was separated after distillation by decanter and dried immediately in order to obtain cassava-based dried distiller's grain. Those samples were collected, transported immediately after production to Hanoi University of Science and Technology and dried using a circulating dryer to obtain cassava-based dried distiller grain (CDDG). CDDG samples were packed in plastic bags and stored at room temperature in a dry place for further usage.

2.2. Microorganisms and inoculum preparation

Trichoderma harzianum BiomaTH1 was from the collection of the microbiology pedagogic unit BioMA from AgroSup Dijon (France) and *Yarrowia lipolytica* W29 (ATCC 20460) was obtained from the UMR PAM laboratory collection of AgroSup Dijon–University of Burgundy (Dijon, France). *Trichoderma harzianum* BiomaTH1 was maintained on potato dextrose agar (PDA) slants at 4°C. The inoculum was grown in PDA for 5 days at 28°C. Spores were harvested and suspended into sterile distilled water. The spore concentration in suspension was determined by using a counting chamber (Marienfeld-Superior, Germany) under a 40X objective - Nikon EFD-3 microscope.

Yarrowia lipolytica W29 was cultured at 28 °C for 48 h on YPDA (Yeast Peptone Dextrose Agar: 20 g l^{-1} of glucose, 20 g l^{-1} of tryptone pancreatic digest of casein, 10 g l^{-1} of yeast extract and 15 g l^{-1} of agar). Cells were inoculated into 500 ml baffled Erlenmeyer containing 200 ml of YPD medium. Flasks were shaken for 24 h at 100 rpm at 28 °C until the cultures reached late logarithmic growth phase. Cells from the culture media were collected by centrifugation for 15 min at 4000 x g at 4 °C and washed twice with sterile saline solution (0.9% of NaCl (w/v)). The cell number was also determined by the counting chamber as mentioned above.

2.3. Solid state fermentation (SSF)

10 g of CDDG were weighed into 250 ml Erlenmeyer flask using cotton plugs to facilitate air transfer. Distilled water and NaOH 0.1 N or HCl 0.1 N were added into substrate to obtain desired moisture and pH. These flasks were autoclaved at 121°C for 15 min and allowed to cool to ambient temperature. The samples were inoculated separately

with spores of *T. harzianum* BiomaTH1 (at 5 ×10^6 spores g^{-1}) or with *Y. lipolytica* W29 cells (at 10^6 cells g^{-1} of CDDG). The SSF process was carried out at 28°C for 5 and 8 days for yeast and mold fermentation, respectively (fig. 15). For both microorganisms, a sample was taken every day and dried at 50°C for 24 h for further analysis.

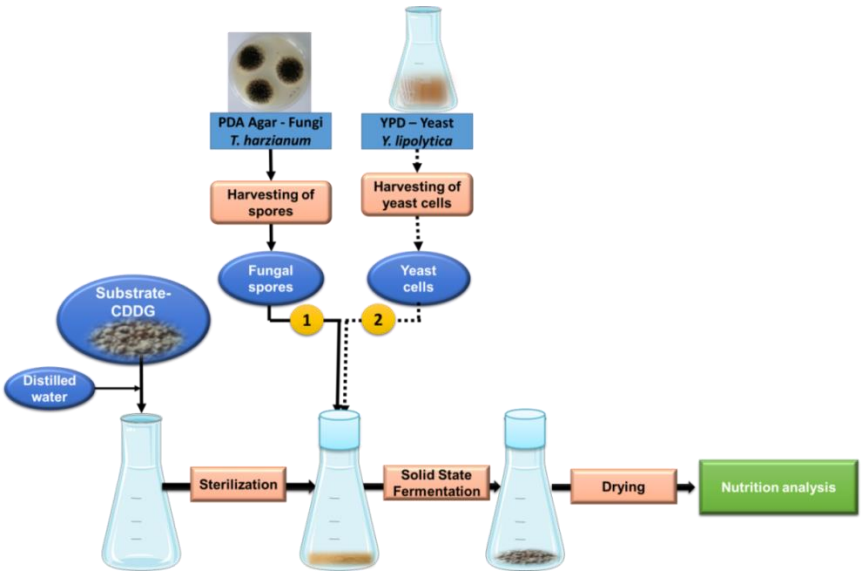

Figure 15: Solid State Fermentation process using 1) *T. harzianum* BiomaTH1 and 2) *Y. lipolytica* W29

2.4. Optimization of protein production from *T. harzianum* BiomaTH1 and *Y. lipolytica* W29

Three factors: initial moisture content, initial pH, nitrogen supplementation, affecting the crude protein levels of fermented CDDG under SSF process, were optimized by using a search technique varying one factor at a time approach which has been used extensively in SSF process (Zhang et al. 2008, Ahmed, Mustafa et al. 2017).

First the initial moisture content was investigated: the fermentation was conducted as described above under various initial moisture content (60%, 70%, 80% w/w for *T. harzianum* BiomaTH1 and 70%, 75%, 80% w/w for *Y. lipolytica* W29)

The best moisture conditions were used to evaluate the best initial pH content from the three initial pH contents 4, 5 and 6.

Then, keeping the two previous factors at their optimized level, nitrogen supplementation was investigated: the effect of additional nitrogenous sources on protein enrichment of CDDG was tested by adding urea and ammonium sulfate ranging from 0.5 to 1% w/w.

2.5. Evaluation of using fermented cassava based DDG for partial replacement of fish meal in pig feed

To evaluate the effectiveness of the use of fermented by-products of cassava bioethanol as a fishmeal's alternative source in animal feed, fishmeal was partially replaced by fermented CDDG with a ratio ranging from 0-50% DM in formulation. Based on the nutrient composition of each ingredient (Table 26), the nutritional value of different formulations was assessed for their ability to meet the nutritional needs of animals, including crude protein, crude fiber, lysine, leucine and metabolizable energy (ME). The economy benefit of different formulas blended with fermented cassava based DDG was also investigated.

Table 26: Composition of animal feed ingredients (expressed in % dry matter)

Ingredients (express in % DM)	Crude protein	Crude fiber	Lysine	Leucine	ME (kcal Kg^{-1})	Price (USD Kg^{-1})
Fish meal [a, b]	57.89	1.50	5.25	3.34	3090.00	1.50
Soybean meal [a, b]	43.40	5.05	3.39	3.79	3362.00	0.30
Maize [a, b]	9.80	2.00	0.27	1.10	3298.00	0.25
Cassava root meal [a, b]	2.87	2.25	0.07	0.12	3152.00	0.24
Rice bran [a, b]	13.00	7.77	0.55	0.98	2671.00	0.22
CDDGTH [1]	15.30	30.00	0.95	1.62	1784.00	0.24
CDDGYL [2]	14.10	33.56	0.71	1.10	1571.00	0.24
Salt						0.25
Premix						2.00

[1] CDDGTH: Cassava-based dried distiller's grain fermented by *T. harzianum* BiomaTH1

[2] CDDGYL: Cassava-based dried distiller's grain fermented by *Y. lipolytica* W29

Ingredient composition (IC) (Crude protein; crude fiber, lysine and leucine) in animal feed formulation and formulation cost (FC) were calculated using the following formulas:

IC (g kg^{-1}) = IC in ingredient (%) * Quantity of ingredient (g) in 1 kg formulation

FC (USD kg^{-1}) = Price of an ingredient unit (USD kg^{-1}) * Quantity of ingredient (g) in 1 kg formulation

Digestible and metabolizable energy (DE and ME) values were calculated using the following formulas (Spiehs et al. 2002) based on nutrient compositions in feed:

1. DE kcal kg^{-1} = 4151 − (122 × % Ash) + (23 × % Crude Protein) + (38 × % Fat) − (64 × % Crude Fiber)

2. ME kcal kg^{-1} = DE × (1.2003 − (0.0021 × % Crude Protein))

2.6. Analytical methods

Cassava-based DDG compositions were analyzed for moisture (AOAC 927.05 (2005)); crude protein (ISO 059831:2005) which was afterwards calculated by multiplying the total nitrogen content of the sample with a factor of 6.25 using Kjeldahl method, crude fiber was determined by ANKOM bag method (ANKOM Technology Corp., Fairport, NY); fats (ISO 6492:1999), ash (AOAC 930.30 (1930)). Total sugar was determined by the acid hydrolysis method, in which starch was hydrolyzed to the reducing sugar by HCl 2% for 2 hours in boiling water bath (Le Thanh M et al. 2007). Cyanide content was measured by titration with $AgNO_3$ (Pohlandt 1983).

The amino acid profiles were determined by using Agilent 1200 series HPLC systems (Germany) with DAD detector (at 338 nm). To determine the amino acid profiles, the samples (around 20-40 mg) were hydrolyzed in vapor phase of 1 ml HCl 6 M, 0.5% phenol for 24 h at 120^{o}C. Afterward, the hydrolyzed samples were re-suspended in deionized water for neutralization to pH 7 with NaOH and brought up to 10 ml of total volume. Hydrolyzed samples were then filtered through 0.2 µm Sartorius membranes. Amino acids in samples were derivatized with OPA reagent (Sigma, USA) in the autosampler of system. After 2 min of reaction, derivatized amino acids were injected and separated in the C18 ElipseZorbax 5 µm, 4.6 x 150 mm column (Agilent, US). The gradient elution was performed at flow rate of 1 ml/min with two buffers including sodium phosphate 40 mM pH 7.8 (buffer A) and mixture of HPLC grade methanol/acetonitrile/deionized water with volume ratio of 45/45/10 (buffer B). During elution, the ratio of buffer A and B was changed, in which ratio of buffer A controlled as following: maintained at 100% for 1.9 min, decreased to 50% until 15.5 min, decreased to 43% until 21 min, decreased to 0 % until 22 min, maintained at 0% until 26 min, increased to 100% until 27 min and then maintained at 100% until 31 min before starting the next injection. The temperature of separation was maintained at 30^{o}C (Taranu, Nguyen et al. 2019).

The *in vitro* protein digestibility was evaluated based on method using AOAC (1999) method (AOAC 1999). Briefly, initial ground samples were de-fatted by Soxhlet extraction with petroleum ether. Thereafter, 0.5 g of defatted sample was suspended in HCl 0.075 mol/L with pepsin solution 0.002% (pepsin, activity 1:10,000, Sigma-Aldrich, St. Louis, MO, USA) and agitated for 16 h at 45°C, and the resulting digested solution was filtered. Crude protein content of the indigestible residue remaining on the filter was determined. The amount of protein digested by pepsin was subtraction of the initial protein to the indigestible protein.

2.7. Experimental design used for solid state fermentation using *T. harzianum* BiomaTH1 and *Y. lipolytica* W29

Table 27: Experimental design used for solid state fermentation using *T. harzianum* BiomaTH1 and *Y. lipolytica* W29

Experiment steps	Factors		Experimental domain		Response
			T. harzianum BiomaTH1	*Y. lipolytica* W29	
Optimization of SSF process	Moisture content (% w/w)		60; 70; 80	70; 75; 80	Crude protein; Cell growth
	pH		4; 5;6		
	Nitrogen supplementation (% w/w)	Urea	0; 5; 1		Crude protein
		Ammonium sulfate	0; 5; 1		
Comparison of sample with and without SSF process	Without fermentation		No fermentation		Nutritional properties; Digestible and metabolizable energy; *In-vitro* protein digestibility
	With fermentation		Optimized conditions		
Evaluation of use of fermented CDDG for partial replacement of fish meal	Nutritional composition (% of fish meal protein replaced by crude protein from fermented CDDG)		0; 12.5; 25; 37.5; 50		Nutritional properties; Economy benefit

The experimental design for SSF process of cassava based DDG using two strains of microorganisms was summarized and shown in Table 27. Firstly, the CDDG was analyzed for proximate compositions. Then, in the goal of improving its nutritional properties, this by-product was incubated with *T. harzianum* BiomaTH1 (for 8 days) or with *Y. lipolytica* W29 for (5 days) by using solid-state-fermentation method (SSF). The fermentation conditions were optimized for both strains of *T. harzianum* BiomaTH1 and *Y. lipolytica* W29 concerning initial moisture content, pH and supplemented nitrogen sources. Using

optimized conditions, the SSFs of CDDG with mold and yeast were carried out to evaluate the efficiency of nutritional enhancement of strains. The nutrients and protein digestibility of fermented CDDG by both strains were evaluated and compared to that of non-fermented CDDG. Finally, the potential applications of these treated CDDG were estimated, especially for partial replacement of fish meal in feed formulation in terms of nutrition and cost.

3. Results and Discussion

3.1. Proximate composition of cassava-based DDG

The moisture and pH of CDDG used as fermentation medium were 5.7 % DM and 4.02, respectively. The proximate composition of CDDG was analyzed in this study in which protein, total sugar and crude fiber content corresponded to 11.9; 28.6 and 34.3% DM, respectively. In addition to the low protein content and high fiber content, CDDG was characterized by a low content of total essential amino acids (5.16%) and the presence of hydrogen cyanide (62.3 mg kg^{-1}) which may cause depressed thyroid function, decreased utilization of oxygen and decreased weight gain in animal. The cyanide content in CDDG is lower than other cassava by-products in Vietnam. According to a study by Ho (Ho 2014) , cyanide content of cassava residues from starch extraction process obtained by factory and household varies from 270 to 331 mg kg^{-1} dry weight. That could be explained by the variety of cassava and the pre-treatment process. This latter involves soaking in water, followed by flash drying as well as boiling and fermentation which can reduce the cyanide content due to the dissolution of glucosides in water (Cooke et al. 1978). The cyanide content in CDDG was higher than the threshold recommended by the EFSA (European Food Safety Authority), which is less than 50 mg kg^{-1} feed, to prevent acute toxicity in animals (EFSA 2007).

3.2. Cassava-based DDG particle sizes

CDDG particle size was measured using a sieve analysis. Three fractions of CDDG based on particle size (21.6% < 0.5mm; 0.5mm < 19.5% < 1 mm and 1mm < 58.9% < 2.5mm) were found. It is noted that the small particle sizes decrease interparticle space leading to reduction in substrate porosity (Camacho-Ruiz et al. 2003) which pose problem in aeration. In contrast, larger particles provide better aeration but lesser surface area which limits the growth of the filamentous organism (Pandey 1992). According to Membrillo, dry sugar cane bagasse using blend of particle size with average diameter size of 1.68 mm was most suitable for protein enrichment with *Pleurotus ostreatus* strains (Membrillo et al. 2008). Mixed-culture (*Bacillus sublitis, Saccharomyces sp.* and *Lactococcus lactis*), the results indicated that the optimal sizes in SSF of soybean meal ranging from 1.0 to 1.4 mm gave the highest peptide production. SSF of soybean meal using mixed-culture (*Bacillus sublitis, Saccharomyces sp.* and *Lactococcus lactis*) indicated that the optimal sizes ranging from 1.0 to 1.4 mm gave the highest peptide production (Guan et al. 2014). Therefore, this

blend of particle sizes is promising to address the aeration and surface area problems and improve the permeability conditions of the media, the contact surface between the substrate and the microorganisms.

3.3. Effect of initial moisture content on protein enrichment

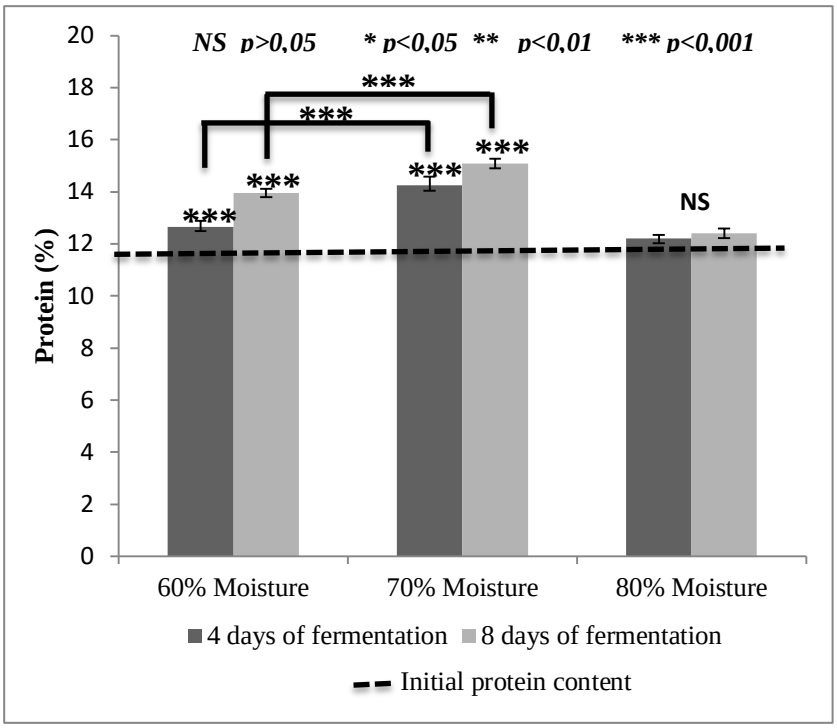

Figure 16: Effect of moisture on crude protein of cassava-based DDG during solid state fermentation using *T. harzianum* BiomaTH1

Moisture level is one of the most important factors that influence directly fungal growth, depending on nature of substrate and micro-organism in SSF process. Proper moisture content varies between 35 and 80% w/w, and especially, more important for cellulosic substrates which are known for their high water absorption capacity (Raimbault 1998). The increase in protein content is closely related to the growth of microorganisms. Under appropriate moisture conditions, thriving microorganisms promote the use of available nutrients in the substrate, especially carbon sources, resulting in an increase in microbial biomass and a decrease in dry matter mass (Ugalde et al. 2002). As can be seen on fig. 16, the moisture of 70% w/w had a clear impact on protein content in substrate which was increased significantly to 15.09±0.19% DM compared to 13.96±0.16 and 12.63±0.19% DM for the samples managed the moisture at 60 and 80% w/w, respectively, after 8 days of fermentation.

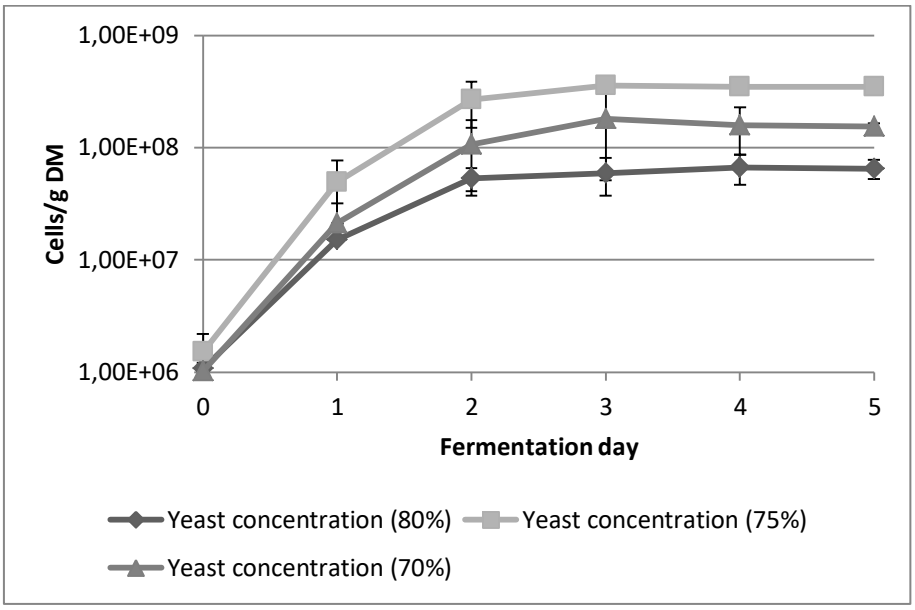

Figure 17: Growth of *Y. lipolytica* W29 at different initial moisture content during solid state fermentation

For protein enrichment using *Y. lipolytica*, the growth of *Y. lipolytica* is shown in fig. 17. The initial moisture content of 75% w/v led to the best performance of yeast cells. After 5 days of fermentation, the yeast number was increased from 1×10^6 cells/g DM to 3.7×10^8 cells/g DM. On the one hand, for both used microbes, the high initial moisture of substrate (80% w/w) had no positive effect on biomass production. This could be caused by compaction of the substrate, reduction in porosity of the interparticle space and consequent interference with oxygen transfer, leading the reduction of microorganism growth. On the other hand, insufficient quantity of water does not allow a good diffusion of solutes and gases, leading to a cellular inhibition because of a lack of substrates or through too high concentration of inhibitive metabolites in or near the cells (Gervais et al. 2003). In other research on optimization of SSF using *T. harzianum*, when the initial moisture content was set to 75%, the sporulation decreased significantly (Zhang et al. 2015).

3.4. Effect of initial pH

Along with initial moisture content, an appropriate pH is also a key factor facilitating microorganism growth through the synthesis of biomass and the degradation of available nutrient sources. The influences of pH on microbial growth were shown in fig. 18 and fig. 19. Maximal protein production obtained after 8 days of fermentation for *T. harzianum* at pH 4.0 was 15.27±0.19% DM. The best growth performance of *Y. lipolytica* was at pH 5.0,

reaching 5.58×10^{8} cell g^{-1} or protein production of 14.06±0.09% DM after 5 days of fermentation. This result was consistent with previous investigation which confirmed that acidic pH favored the growth of *Trichoderma* species and *Y. lipolytica* (INRA-CIRAD-AFZ 2004, Singh et al. 2014). The optimal pH for fungal biomass protein production from *T. harzianum* using rice polishing, cassava root meal, cellulosic agricultural wastes were around 4.0-4.5 (Muindi et al. 1981, Osama et al. 2013, Ahmed, Mustafa et al. 2017). According to Osama et al, after 10 days of fermentation using *T. harzianum*, crude protein of tomato leaves, sugar beet leaves and sugar beet pulp was increased to 18.12; 13.23 and 16.85% DM from 15.12; 10.62 and 14.31% DM respectively (Osama, Khaled et al. 2013). For protein enrichment of biofuel waste using *Y. lipolytica A-101* at optimized pH (5.0), the protein concentration was increased to 8.% DM - a 44% increase as compared to the original (3.65% DM) (Jach et al. 2020). The conditions can thus be considered as optimal for the CDDG source of substrate which has an initial pH of 4.02.

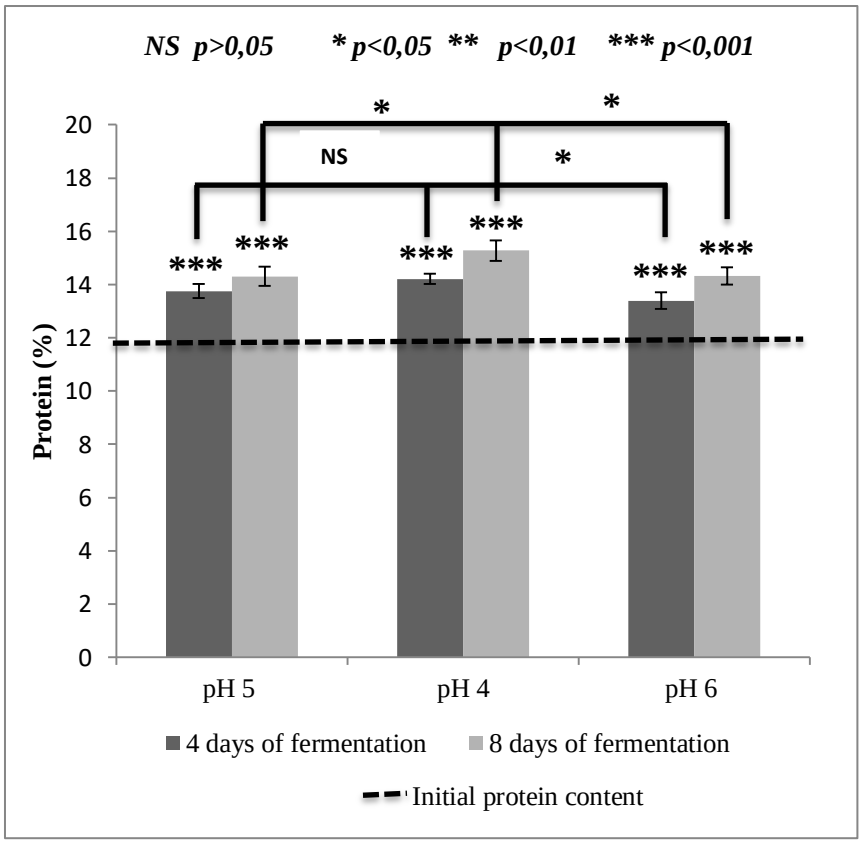

Figure 18: Effect of pH on crude protein of cassava-based DDG during solid state fermentation using *T. harzianum* BiomaTH1

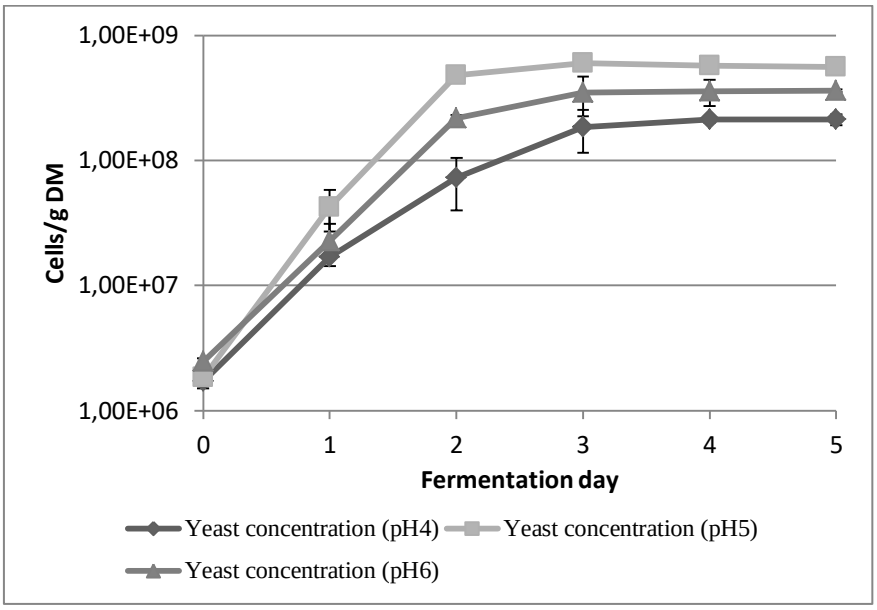

Figure 19: Growth of *Y. lipolytica* W29 at different initial pH during solid state fermentation

3.5. Effect of nitrogenous sources

An addition of nitrogen sources for protein enrichment during fermentation of cassava residues was reported in many previous studies (Yang 1988, Roussos et al. 1993, Correia et al. 2007, Bayitse et al. 2015). Fungi and yeast require an inorganic or organic nitrogen source as nutrient to synthesize the biomass and the chitin/chitosan which is a nitrogen containing biopolymer for their cell wall (Moore 1996). To evaluate whether the nitrogen source was a limiting factor for the production of proteins by the two strains, urea and ammonium sulfate were used. Results were shown in table 28. Without addition of N, the initial protein content was 11.96±0.17% DM. In the presence of urea, the initial crude protein content of fermentation medium for both microorganisms was 13.45±0.12 and 14.72±0.14% DM with supplement of urea of 0.5 and 1% w/w respectively. Meanwhile, the supplement of ammonium sulfate with a ratio of 0.5 and 1% w/w resulted in an increase of initial crude protein of 12.65±0.18 and 13.36±0.12% DM, respectively.

For *T. harzianum*, after 8 days fermentation, the supplement of urea of 0.5% and 1 % has led the increase of the crude protein of fermented samples to 17.10±0.16; 18.82±0.14%, respectively. Similarity, the increase of crude protein was also observed with ammonium sulfate, which was 16.18±0.16 and 17.05±0.1 % DM with supplemented ammonium sulfate of 0.5 and 1% w/w respectively. The improvement of crude protein was then estimated of around 27-28%.

For *Y. lipolytica*, after 5 days of fermentation, by using the same concentration of urea and ammonium sulfate, the crude protein achieved 15.83±0.10 (for 0.5% urea); 17.29±0.24 (for 1% urea) and 14.92±0.18 (0.5% ammonium sulfate) and 15.79±0.18% DM (1% ammonium sulfate). The crude protein improvement ranged from 17.62-18.21%. However, the results were not significatively different for both nitrogenous sources ($P \leq 0.05$) compared to without supplementation. This happens for both strains. In other words, nitrogen supplementation had no effect on protein enrichment for both tested strains. Maybe, a suitable ratio of N:C in this study (approximately 1:5) facilitates the fermentation process. Previous study recommended this ratio between 1:4 and 1:7 for SCP production from *Trichoderma album* by using sugar beet residue as the substrate (Yang et al. 1986).

Table 28: Effect of nitrogenous sources on protein enrichment (crude protein-expressed by % dry matter) during SSF using *T. harzianum* BiomaTH1 and *Y. lipolytica* W29

Fermentation day	Microorganism for SSF	Without supplementation	Urea (% w/w)		Ammonium sulfate (% w/w)	
			0.5	1.0	0.5	1.0
0	For both strains	11.96±0.17	13.45±0.12	14.72±0.14	12.65±0.18	13.36±0.12
5	***Y. lipolytica***	14.06±0.09	15.83±0.10	17.29±0.24	14.92±0.18	15.79±0.18
Change of crude protein (%)		2.11±0.10	2.38±0.09	2.58±0.16	2.27±0.10	2.43±0.07
Protein improvement (%)*		17.62±0.99[a]	17.71±0.75[a]	17.50±1.09[a]	17.94±0.88[a]	18.21±0.37[a]
8	***T. harzianum***	15.27±0.19	17.10±0.16	18.82±0.14	16.18±0.16	17.05±0.13
Change of crude protein (%)		3.32±0.36	3.64±0.23	4.10±0.09	3.53±0.13	3.69±0.17
Protein improvement (%)*		27.73±0.92[b]	27.1±1.89[b]	27.89±0.72[b]	27.91±1.26[b]	27.65±1.44[b]

* *Protein improvement* = Change of crude protein after 5days (for *Y. lipolytica*) or 8 days (*T. harzianum*) of fermentation/Crude protein of initial samples (0 days of fermentation).

Values are means of three replicates per treatment. Means in a row with no common letters differ significantly ($P < 0.05$)

3.6. Changes in nutritional value of cassava-based DDG substrate after solid state fermentation

Under optimized initial pH and moisture of CDDG substrate, nutritional changes and cyanide content during the course of fermentation were investigated. The total cyanide content of CDDG fermented by *T. harzianum* and *Y. lipolytica* reduced from 62.3 mg kg^{-1} DM to 24.3 mg kg^{-1} DM and 53.6 mg kg^{-1} respectively. This result was in accordance with a FAO (1981) study reporting that a longer fermentation period reduces the content of free

hydrocyanic acid (FAO 1981). A previous study showed that the HCN content was reduced significantly by yeast and mold due to their ability of utilizing cyanogenic glycoside (Oboh et al. 2007).

Tables 29 and 30 showed that the SSF using filamentous fungi and yeast enhanced the nutrient compositions of cassava DDG, helping to increase crude proteins and amino acid content. The crude protein content was enriched significantly during fermentation, especially for *T.harzianum*, a 27.8% increase in crude protein was recorded at the 7th day. Meanwhile, the *Y. lipolytica* resulted in a crude protein increase of 16.6% at the 4th. The loss of dry matter during fermentation could be a possible reason for an enrichment in the nitrogen ratio (Shi et al. 2015). Crude fiber was gradually decreased during fermentation with *T. harzianum* from the initial value of 34.28% to 30%. The increase of crude fiber observed during the initial phase of fermentation can be explained by the utilization of the available nutrients by the fungi and the later reduction was due to the degradation of non-starch polysaccharide to fungal protein. However, the crude fiber was almost unchanged during fermentation by yeast. A diet with reasonable fiber content helps also to increase satiety for pregnant sows, improve reproduction efficiency, reduce environmental cost of pig production via a reduction of the nitrogen loss in manure (Jarrett et al. 2018). The ash content increased and reached a maximum on the 8th day (9.37±0.1%) using mold fermentation and 5th day (8.88±0.16%) using yeast fermentation with an increase of 18.5% and 12%, respectively, from initial content. This can be explained by the increase in mold and yeast biomass during the course of fermentation as well as the degradation in organic matter caused by the SSF process. It can be noted that the percentage of the decrease in the total sugar content from the initial value was 28% using mold and 25.7% using yeast fermentation. A slight reduction in crude fat which was 7.6 and 4.0% respectively during fermentation process using *T. harzianum* and *Y. lipolytica* could be explained as assimilation of lipids from cassava DDG possibility for biomass production. Loss of lipid in agricultural wastes during SSF due to its conversion into fungal biomass or maybe as a result of the lipolytic activity of these microorganisms using *Trichoderma* and *Y. lipolytica* strains were previously reported (Toscano et al. 2013, Yan et al. 2018). These changes resulted in a significant improvement in metabolizable energy (ME) which was 1571±63 kcal kg^{-1} and 1784±79 kcal $kg^{-1}$for mold and yeast fermentation, respectively, compared to that of non-fermented sample (1377±61 kcal kg^{-1}).

Table 29: Proximate composition (expressed by % dry matter) of unfermented mix and fermented products using *T. harzianum* BiomaTH1

Time (days)	Crude protein	Crude fat	Crude fiber	Crude ash	Total sugar
0	11.96±0.17^{a}	2.49±0.03^{a}	34.28±1.02^{a}	7.91±0.17^{a}	28.41±2.11^{a}
1	12.40±0.01^{a}	2.53±0.04^{a}	34.39±0.73^{a}	8.14±0.31^{a}	26.11±0.55^{a}
2	13.78±0.33^{b}	2.59±0.04^{a}	35.13±1.35^{a}	8.66±0.09^{b}	23.47±0.40^{b}
3	13.91±0.08^{b}	2.57±0.16^{a}	34.42±0.85^{a}	8.86±0.03^{b}	23.53±0.19^{b}
4	14.16±0.01^{c}	2.63±0.04^{a}	32.17±0.44^{b}	9.04±0.04^{c}	23.07±1.16^{b}
5	14.46±0.07^{d}	2.55±0.07^{a}	30.23±0.11^{c}	9.16±0.03^{c}	22.76±0.18^{b}
6	14.91±0.11^{e}	2.49±0.03^{a}	29.76±0.19^{c}	9.15±0.06^{c}	22.46±0.52^{b}
7	15.28±0.02^{e}	2.34±0.04^{b}	29.95±1.13^{c}	9.19±0.01^{c}	20.78±0.33^{c}
8	15.27±0.19^{e}	2.30±0.03^{b}	30.01±0.87^{c}	9.37±0.02^{d}	20.48±1.18^{c}

Values are means of three replicates per treatment. Means in a column with no common letters differ significantly (P <0.05)

Table 30: Proximate composition (expressed by % dry matter) of unfermented mix and fermented products using *Y. lipolytica* W29

Time (days)	Crude protein	Crude fat	Crude fiber	Crude ash	Total sugar
0	12.11±0.19^{a}	2.53±0.01^{a}	32.70±1.70^{a}	7.93±0.17^{a}	28.78±1.90^{a}
1	12.46±0.09^{a}	2.52±0.02^{a}	32.00±0.61^{a}	8.40±0.09^{b}	26.40±0.47^{a}
2	13.42±0.04^{b}	2.49±0.04^{a}	32.36±0.59^{a}	8.54±0.14^{b}	23.71±1.01^{b}
3	13.84±0.05^{c}	2.46±0.02^{b}	32.28±1.28^{a}	8.61±0.03^{b}	22.31±0.55^{b}
4	14.12±0.07^{d}	2.44±0.05^{b}	32.16±0.16^{a}	8.85±0.06^{c}	21.73±1.39^{b}
5	14.06±0.09^{d}	2.43±0.05^{b}	33.56±0.46^{a}	8.88±0.16^{c}	21.37±1.29^{b}

Values are means of three replicates per treatment. Means in a column with no common letters differ significantly (P <0.05)

The changes in the amino acids composition in the fermented products using *T. harzianum* and *Y. lipolytica* are given in Table 31. The total amino acid of fermented samples increased to 13.86 and 12.39 (% DM), respectively from unfermented sample 11.01 (% DM) along with an improvement of essential amino acids profile. The samples

fermented by *T. harzianum* and *Y. lipolytica* showed a significant increase in the essential amino acids content which rose to 55% DM and 22% DM compared to initial sample, respectively. For mold fermentation, essential amino acids whose concentration increased were histidine (40%), arginine (18%), valine and methionine (271%), phenylalanine (52%), isoleucine (98%), leucine (80%), and lysine (8%). Only essential amino acid threonine showed a reduction in concentration of 40%. Some non-essential amino acids also increased their concentration after 8 days including alanine (37%) and tyrosine (86%). After 5 days of fermentation by yeast, except arginine and lysine, other essential amino acids which showed increased during solid state fermentation include histidine (24%), threonine (257%), valine and methionine (169%), phenylalanine (20%), isoleucine (41%), leucine (22%). Some non-essential amino acids got reduced except glutamic (42%), serine (56%), tyrosine (71%) which showed increase on fermentation. Higher percentage of essential amino acids was present in cassava based DDG after fermentation, especially lysine, valine, methionine, threonine and isoleucine that are essential amino acids in those of pig feeds due to not being synthesized by animals (D'Mello 2003). Essential amino acids content of fermented cassava DDG by fungi and yeast were 8.01 and 6.30% DM respectively, being relatively competitive compared to other commercial cereal byproducts used widely for animal feed such as barley distillers grains (10.38%), brewers grains (9.12%), maize DDGS from ethanol production (11.94%), wheat distillers grains with starch > 7% (9.72%) (INRA-CIRAD-AFZ 2004). Those fermented products were considered as cofeeding with commercially available protein which decreases the cost of protein ingredient for feeds.

Table 31: Amino acid profile of unfermented and fermented products using *T. harzianum* BiomaTH1 and by *Y. lipolytica* W29

No	Amino acid	Unfermented cassava DDG	Cassava DDG fermented by	
			T. harzianum	***Y. lipolytica***
1	APS	2.42±0.50	1.85±0.03	1.54±0.03
2	GLU	1.41±0.05	1.22±0.02	2.01±0.04
3	SER	0.55±0.02	1.06±0.02	0.86±0.02
4	HIS*	0.25±0.02	0.35±0.01	0.31±0.01
5	THR*	0.45±0.03	0.27±0.02	1.61±0.05
6	GLY	0.09±0.01	0.03±0.01	0.63±0.01
7	ARG*	1.19±0.04	1.40±0.05	0.05±0.01
8	ALA	1.04±0.06	1.43±0.04	1.04±0.03
9	TYR	0.14±0.03	0.26±0.02	0.24±0.02
10	CYS	0.2±0.03	Trace	Trace
11	VAL+MET*	0.42±0.02	1.56±0.06	1.13±0.04
12	PHE*	0.56±0.03	0.85±0.02	0.67±0.02

13	ISO*	0.51±0.03	1.01±0.03	0.72±0.02
14	LEU*	0.90±0.04	1.62±0.04	1.10±0.04
15	LYS*	0.88±0.03	0.95±0.03	0.71±0.02
	Essential amino acids	5.16±0.04	8.01±0.04	6.30±0.03
	Total amino acids	11.01±0.06	13.86±0.03	12.62±0.02

* Essential amino acids ($p \leq 0.05$)

Those results are consistent with the previous study on protein enrichment by *T. harzianum* using rice polishing which showed that all essential amino acids in substrate were increased significantly after 3 days of fermentation (Ahmed, Mustafa et al. 2017). In another study, using fungi and yeast improved nutritional of okara in which the amino acids in *Rhizopus oligosporus* mono-culture and *Rhizopus oligosporus* and *Yarrowia lipolytica* co-culture were increased by 2.3 and 2.5-fold respectively (Vong, Hua et al. 2018). Depending on the substrate and the fermentation condition used in SSF, the composition of the single cell protein (SCP) biomass of the microorganism varies (Ugalde and Castrillo 2002). The SCP biomass obtained by these two strains via fermentation using agro-industrial wastes as substrate has been showed to be rich in essential amino acids (Ghanem 1992, Ahmed, Mustafa et al. 2017, Yan, Han et al. 2018). Therefore, the increase in amino acids in the substrate can be explained by the loss of dry matter, the SCP biomass production and the extracellular proteases secreted by *T. harzianum* and *Y. lipolytica*. Proteolysis of CDDG proteins releases peptides and free amino acids; the latter can be further deaminated and catabolized by microorganisms. This explained the reduction in some essential amino acids which were utilized for the production of enzymes and other organic compounds by the filamentous fungi and yeast strain.

3.7. *In vitro* protein digestibility

Regarding to nitrogen source, the nutritional quality of animal feed depends on amino acid composition, availability of essential amino acids, protein digestibility, and physiological capacity of utilization of specific amino acids after digestion and absorption (Bergner 1994). Protein digestibility is an important index to estimate the protein availability for intestinal absorption after digestion which reflects on the efficiency of protein utilization on diet. The result showed that the crude protein digestibility of initial CDDG is 82.5±0.6% which is near wheat DDGS (79.6-92.2%) and corn DDGS (71.8-79.6%) (Ahmed, Mustafa et al. 2017). The protein digestibility of CDDG fermented by *T. harzianum* and *Y. lipolytica* were increased significantly at 89.2±1.4 and 86.9±0.6% compared to that in unfermented

CDDG (82.5±0.6%). Other researchers (Vong, Hua et al. 2018) also reported that SSF increased the protein content and quality of several substrates. Increases in *in vitro* protein digestibility could be explained by elimination of undesirable factors and protein hydrolysis during solid state fermentation, which result in proteins that are more vulnerable to enzyme action.

3.8. Potential use of fermented cassava –based DDG for partial replacement of fish meal in pig animal feed in Vietnam

CDDG fermented by fungi and yeast contained a crude protein content of 15.3% and 14.1% DM; ME of 1784.0 and 1571.7 (kcal kg^{-1}) along with an important amount of essential amino acids. It could be thus a suitable candidate as a source of crude protein and amino acids with low cost to partly replace imported animal ingredient feed, especially fishmeal which was showed in fig. 20.

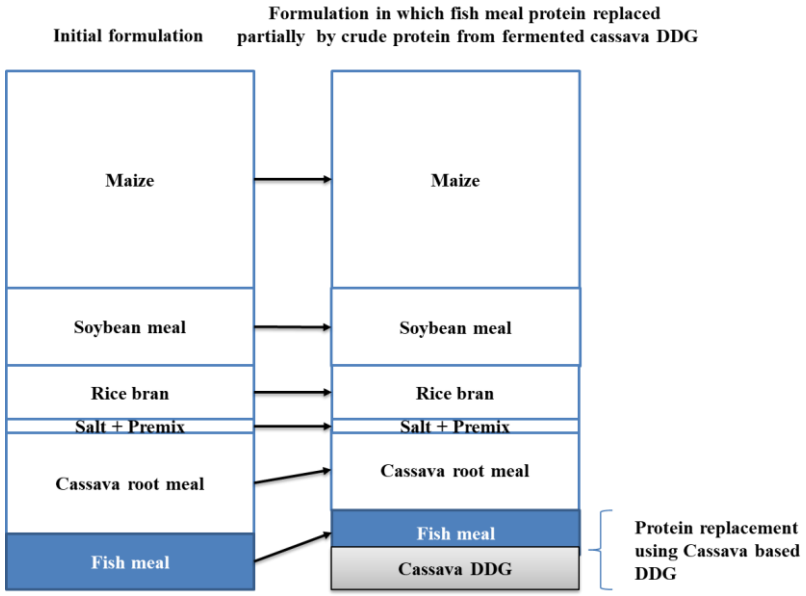

Figure 20: Formulation with partial replacement of fish meal by fermented cassava-based DDG for farrowing sows pig (Left column without and right column with cassava-based DDG)

The recommendation for mixing feed varies depending on the purpose and stage of development of the pig. For example, for farrowing sows (exotic), recommendation of nutrient requirements including crude protein, fiber, lysine and ME is 180; 70; 9.5-11 (g kg^{-1} formulation) and 3000 kcal kg^{-1} DM respectively (Viet et al. 2014). Table 32 shows the pig feed formulation which consists in a mixture of ingredients, including starch, protein and amino acids based on locally available ingredients with cheap cost. In Vietnam, although

used as a common source of protein and amino acids for animal feed, fishmeal which is characterized by high cost, limited supply, variable quality and unsustainable production that leads to depletion of ecosystems, environmental damage, and the collapse of local fisheries due to over-exploitation of marine sources, could be replaced by fermented CDDG. As our calculation, the replacement ration of fish meal by fermented CDDG was of 12.5; 25; 37.5 and 50% DM in the feed diet for farrowing pig with the purpose of reducing feed costs, maintaining nutritional needs, including CP; CF, lysine and ME as shown (Table 32).

Table 32: Impact of replacement of 0 to 50% of the fishmeal by fermented cassava-based DDG on the formulation, nutritional composition and price of the pig feeding

Formulation	**FM0***	**FM12,5***		**FM25***		**FM37,5***		**FM50***	
Ingredient (express in % DM)	m (g)								
Fish meal	80.00	70.00	70.00	60.00	60.00	50.00	50.00	40.00	40.00
Soybean meal	160.00	160.00	160.00	160.00	160.00	160.00	160.00	160.00	160.00
Maize	402.00	402.00	402.00	402.00	402.00	402.00	402.00	402.00	402.00
Rice bran	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Cassava root meal	200.00	169.32	164.96	134.97	125.86	100.62	86.75	66.02	47.38
CDDGTH	0.00	40.68	0.00	85.03	0.00	129.38	0.00	173.98	0.00
CDDGYL	0.00	0.00	45.04	0.00	94.14	0.00	143.25	0.00	192.62
Salt	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Premix	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
SUM (g)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
CP (g kg^{-1})	180.39	179.94	180.48	179.95	179.96	179.96	179.97	180.01	180.01
ME (kcal kg^{-1})	3141.97	3086.94	3071.38	3026.88	3014.42	2966.83	2947.86	2906.44	2880.94
CF (g kg^{-1})	33.48	44.84	46.05	57.22	59.75	69.60	73.45	82.05	87.23
Lysine (g kg^{-1})	11.67	11.51	11.55	11.39	11.47	11.26	11.38	11.13	11.30
Leu (g kg^{-1})	14.85	15.14	15.20	15.48	15.62	15.82	16.03	16.17	16.45
CDDG TH, % of formulation	0.00	4.07	4.50	8.50	9.41	12.94	14.33	17.40	19.26
CDDG TH, % of fish meal	0.00	13.44	14.88	28.09	31.10	42.74	47.32	57.47	63.62
Formulation price (USD kg^{-1})	0.36	0.34	0.34	0.32	0.31	0.29	0.29	0.27	0.27

FM0: All supplementary protein from fish meal

FM12.5: 12.5% of fish meal protein replaced by crude protein from CDDG fermented by *T. harzianum* BiomaTH1 (CDDGTH) or by *Y. lipolytica* W29 (CDDGYL)

FM25: 25% of fish meal protein replaced by crude protein from CDDGTH or CDDGYL

FM37.5: 37.5% of fish meal protein replaced by crude protein from CDDGTH or CDDGYL

FM50: 50% of fish meal protein replaced by crude protein from CDDGTH or CDDGYL

It can be seen that all replacement rates helped significantly increase fiber, leucine content and maintain nutrient requirements (CF, lysine and ME) for pig. However, the replacement rate of 37.5% in pig feed formulation is the most effective, reaching 2967-2968 kcal kg^{-1} ME; 69.6-73.4 g kg^{-1} CF; 11.26-11.38 g kg^{-1} lysine; 15.82-16.3 g kg^{-1} leucine. In addition, this ratio reduces up to 19.5% of final formulation cost compared to without replacement. There is no doubt that using fermented CDDG – a promising source of industrial by-products as a substitute for fishmeal in animal diet reduces the dependence on imported protein materials as well as negative impacts on environment due to overexploitation of marine resources for making industrial fishmeal, promoting sustainable development in Vietnam.

4. Conclusions

In this work, the nutritional composition of CDDG in Vietnam bioprocessed by solid state fermentation was determined. The fermentation of this by-product with *T. harzianum* BiomaTH1 for 8 days and with *Y. lipolytica* W29 for 5 days could enrich the crude protein content without supplementing source of nutrient when the substrate was moistened at 70% and 75% w/w respectively. The nutritional value of the fermented products as potential livestock feeds was evaluated by taking into account their composition in protein, especially amino acids content. Using *T. harzianum* BiomaTH1 and *Y. lipolytica* W29 for SSF process, the crude protein was increased significantly from 12.0% to 15.29% and 14.06% DM respectively. In addition, the essential amino acids-key-protein source for animal feed was enhanced 55% and 22% DM. Moreover, this process using fungi and yeast helped improve *in vitro* protein digestibility of fermented CDDG from 82.5% to 89.2% and 86.9% respectively. The use of yeast or mold in SSF as pig ingredient feed depends on efficiency and fermentation time. In this study, although the use of *T. harzianum* increased the protein content compared to *Y. lipolytica*, but the fermentation time (days) was much longer (1.6 times higher) as well as reduced more the fiber and starch content of the substrate. An important factor of SSF which using agro-industrial residues resources as substrate for pigs and poultry feeds production being rich in amino acids content with value-added at low production costs allows SSF to be economically viable. Furthermore, along with economic efficiency, reducing environmental treatment cost and environmental problems make SSF process attractive, and have great potential for application in the current conditions of Vietnam, where the investment in agriculture and livestock development still remains limited.

These findings could encourage animal feed mills to utilize these protein-enriched sources more efficiently at low cost.

GENERAL CONCLUSION ET PERSPECTIVES

GENERAL CONCLUSION

The VHG technology has been widely applied for ethanol production to increase ethanol content and plant productivity. Thanks to advances in biotechnology, the SLSF using native starch-hydrolyzing enzymes has been introduced to increase production efficiency, to reduce energy consumption and production cost. This study focused on development a SLSF-VHG of cassava flour at lab and pilot scale using commercial enzyme which is able to hydrolyze starch at room temperature. The results showed no significant effect of auxiliary enzymes (cellulase and β-glucanase) and yeast nutrient on ethanol performance. In contrast, the combination of a high concentration of hydrolyzing native enzyme (Stargen 002 at 2565 GAU/kg CF) and glucoamylase (Amigase Mega L at 0.105% w/w) had a positive impact on ethanol performance. At lab scale, the SLSF process finished after 144 h fermentation with an ethanol concentration of 14.4% v/v corresponding to the theoretical ethanol yield of 86.0%. At pilot scale (1000 L), the ethanol concentration reached 13.9% v/v corresponding to a yield of 83.1% of the theoretical ethanol along with greenhouse gas emissions of 186.281 kg CO_2eq. The quantity of enzymes and inoculation rate of yeast were optimized to maintain a high ethanol yield and decrease production cost.

A large amount of by-products in the form of wet distillers spent is generated each year from ethanol plants in Vietnam. After distillation, the solid matter from whole stillage is filtered, and then dried to obtain the dried distillers grains (DDG). Cassava-based DDG was characterized by a high fiber content (16-33%) and a low protein content (12-16%) with limited interest for animal feeding whereas the rice-based DDG had a high protein content (55-80% of DM). To increase the nutritional value of this ethanol byproduct, solid-state fermentation (SSF) using mold *Trichoderma harzianum* BiomaTH1 and yeast *Yarrowia lipolytica* W29 was used. Cassava DDG was fermented by SSF process for 8 (using the mold) and 5 days (using the yeast). Under optimal conditions, from 11.84% DM for unfermented sample, the crude protein of cassava-based DDG fermented by *T. harzianum* BiomaTH1 or *Y. lipolytica* W29 was increased to 15.29 and 14.06% DM, respectively.

To sum up, a closed process including a no-cooking process at very high gravity for ethanol production and valorization of distillery by-products has been successfully established. These results are an important driving force for the development of the ethanol and feed industry in Vietnam.

PERSPECTIVE

This work has raised several points which merit further study:

(i) The starch granules with a lower diameter are more susceptible to enzymes compared to those of higher diameter due to their higher contact area. Therefore, a smaller granule size has a positive impact on enzymatic hydrolysis of starch (Franco 1992, Tester, Qi et al. 2006). The average granule size of normal cassava starch is 15.6 µm, which is higher than that of rice starch (3-10 µm). This also contributes to explain the low enzymatic hydrolysis efficiency of cassava starch using SLSF technology. Therefore, hybrid or genetically modified cassava varieties with a small starch granule size (< 10 µm) in Vietnam may be an interesting solution for no-cooking process (Oral discussion with Dr. Chu-Ky Son).

(ii) Yeast cells are subjected to various stresses during the fermentation process. One of the main factors that lead to a decrease in fermentation efficiency is the high ethanol concentration at the end of fermentation (Panchal and Stewart 1980, Stewart, D'Amore et al. 1988, Jakobsen and Piper 1989, Pátková, Šmogrovičová et al. 2000). Therefore, ethanol should be removed from the fermentation broth as it is formed. The vacuum fermentation has been widely applied for the ethanol fermentation to remove ethanol by decreasing the boiling point temperature of ethanol-water mixture in broth (Nguyen et al. 2011). Integrating this method into SLSF-VHG of cassava flour has the potential to increase fermentation efficiency and shorten fermentation duration.

(iii) Other applications of by-products from cassava-based ethanol plants.

- Production of nanocellulose:

  Nanocellulose can be applied in food industry as the texturing agent, the biodegradable package. It has been studied and extracted from various fibrous residues. Firstly, the pretreatment of biomass by acid-chlorite or alkaline treatment is an important step for removing other non-cellulosic components. Then, nanocellulose is extracted from cellulose fibrils by acid hydrolysis or enzymatic hydrolysis (Phanthong et al. 2018). Promoting research and application of biodegradable package is an inevitable trend in the world.

Therefore, these by-products are potential candidates for nanocellulose production.

- Production of fiber-rich supplements:

  By using enzymatic method to remove other non-fiber components (protein, starch…), the fiber content in cassava-based DDG is increased. The fiber obtained can be compressed in tablet form and used as functional food (Oral discussion with Dr. Chu-Ky Son)

REFERENCE

Aastrup, S. (1979). "The relationship between the viscosity of an acid flour extract of barley and its β-glucan content." Carlsberg Research Communications **44**(5): 289-304.

Agu, R. C., T. Bringhurst and J. Brosnan (2006). "Production of Grain Whisky and Ethanol from Wheat, Maize and Other Cereals." Journal of The Institute of Brewing **112**: 314-323.

Ahmed, S., G. Mustafa, M. Arshad and M. I. Rajoka (2017). "Fungal biomass protein production from Trichoderma harzianum using rice polishing." BioMed Research International **2017**.

Ajila, C., S. Brar, M. Verma, R. Tyagi, S. Godbout and J. Valéro (2012). "Bio-processing of agro-byproducts to animal feed." Critical reviews in biotechnology **32**(4): 382-400.

Åkerberg, C., G. Zacchi, N. Torto and L. Gorton (2000). "A kinetic model for enzymatic wheat starch saccharification." Journal of Chemical Technology & Biotechnology **75**(4): 306-314.

Alagón, G., O. N. Arce, E. Martínez-Paredes, L. Ródenas, V. J. Moya, E. Blas, C. Cervera and J. J. Pascual (2016). "Nutritive value of distillers dried grains with solubles from barley, corn and wheat for growing rabbits." Animal Feed Science and Technology **222**: 217-226.

Alfenore, S., X. Cameleyre, L. Benbadis, C. Bideaux, J.-L. Uribelarrea, G. Goma, C. Molina-Jouve and S. Guillouet (2004). "Aeration strategy: a need for very high ethanol performance in Saccharomyces cerevisiae fed-batch process." Applied Microbiology and Biotechnology **63**(5): 537-542.

AOAC (1999). "Official method 971.09. Official Methods of Analysis of AOAC International, 16th edition 5th revision." AOAC International: Gaithersburg, MD 20877-2417, USA. .

Association, W. B. (2020). "WBA Global Bioenergy Statistics; 2020. Available from: www.worldbioenergy.org/global-bioenergy-statistics."

Assamoi, A. A., J. Destain, F. Delvigne, G. Lognay and P. Thonart (2007). "Solid-state Fermentation of Xylanase from Penicillium canescens 10-10c in a Multi-layer-packed Bed Reactor". Biotechnology for Fuels and Chemicals, Springer**:** 87-98.

Assamoi, A. A., J. Destain and P. Thonart (2009). "Microbial aspects of endo-β-1, 4-xylanase production in solid-state fermentation by Penicillia: the case of Penicillium canescens." Biotechnologie, Agronomie, Société et Environnement **13**(2): 281-294.

Bajaj, R., N. Singh, A. Kaur and N. Inouchi (2018). "Structural, morphological, functional and digestibility properties of starches from cereals, tubers and legumes: a comparative study." Journal of Food Science and Technology **55**: 3799-3808.

Baker, R. (1997). "Reassessment of Some Fruit and Vegetable Pectin Levels." Journal of Food Science **62**: 225-229.

Balat, M. and H. Balat (2009). "Recent trends in global production and utilization of bio-ethanol fuel." Applied energy **86**(11): 2273-2282.

Balcerek, M. and K. Pielech-Przybylska (2013). "Effect of simultaneous saccharification and fermentation conditions of native triticale starch on the dynamics and efficiency of process and composition of the distillates obtained." Journal of Chemical Technology & Biotechnology **88**(4): 615-622.

Bals, B., V. Balan and B. Dale (2009). "Integrating alkaline extraction of proteins with enzymatic hydrolysis of cellulose from wet distiller's grains and solubles." Bioresource technology **100**(23): 5876-5883.

Bandara, N., L. Chen and J. Wu (2011). "Protein extraction from triticale distillers grains." Cereal chemistry **88**(6): 553-559.
Bayitse, R., X. Hou, G. Laryea and A.-B. Bjerre (2015). "Protein enrichment of cassava residue using Trichoderma pseudokoningii (ATCC 26801)." AMB Express **5**(1): 80.
Bellissimi, E. and W. Ingledew (2005). "Analysis of commercially available active dry yeast used for industrial fuel ethanol production." Journal of the American Society of Brewing Chemists **63**(3): 107-112.
Belyea, R. L., K. D. Rausch, T. E. Clevenger, V. Singh, D. B. Johnston and M. E. Tumbleson (2010). "Sources of variation in composition of DDGS." Animal Feed Science and Technology **159**(3): 122-130.
BenitoInfante, R., O. OmarEGarcía and C. J. Rivera (2013). Characterization of dietary fiber and pectin of cassava bread obtained from different regions of Venezuela.
Bera, M. and R. Mukherjee (1989). "Solubility, emulsifying, and foaming properties of rice bran protein concentrates." Journal of food science **54**(1): 142-145.
Bergner, H. (1994). "Determination of the protein quality of food and animal feed." Archiv fur Tierernahrung **45**(4): 293-332.
Bhattacharyya, B., S. Banerjee and T. Ghosh (2008). "Bioreactors: functions in fermentation processes." Advances in Fermentation Technology: 172-201.
Blandino, A., M. Al-Aseeri, S. Pandiella, D. Cantero and C. Webb (2003). "Cereal-based fermented foods and beverages." Food research international **36**(6): 527-543.
Blazek, J. G., E. (2010). "Effect of enzymatic hydrolysis on native starch granule structure." Biomacromolecules **12**: 3275-3289.
Bootsma, J. A., M. Entorf, J. Eder and B. H. Shanks (2008). "Hydrolysis of oligosaccharides from distillers grains using organic–inorganic hybrid mesoporous silica catalysts." Bioresource technology **99**(12): 5226-5231.
Boros, D., R. R. Marquardt, B. Slominski and W. Guenter (1993). "Extract viscosity as an indirect assay for water-soluble pentosan content in rye." Cereal Chemistry **70**: 575-580.
Böttger, C. and K. H. Südekum (2017). "Within plant variation of distillers dried grains with solubles (DDGS) produced from multiple raw materials in varying proportions: Chemical composition and in vitro evaluation of feeding value for ruminants." Animal Feed Science and Technology **229**: 79-90.
Bourdichon, F., S. Casaregola, C. Farrokh, J. C. Frisvad, M. L. Gerds, W. P. Hammes, J. Harnett, G. Huys, S. Laulund and A. Ouwehand (2012). "Food fermentations: microorganisms with technological beneficial use." International journal of food microbiology **154**(3): 87-97.
Box, G. E. and N. R. Draper (1959). "A basis for the selection of a response surface design." Journal of the American Statistical Association **54**(287): 622-654.
Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical biochemistry **72**(1-2): 248-254.
Brander, M., Sood, A., Wylie, C., Haughton, A., & Lovell, J. (2011). "Technical paper| Electricity-specific emission factors for grid electricity." Ecometrica, Emissionfactors. com. Available from: https://ecometrica.com/assets/Electricity-specific-emission-factors-for-grid-electricity.pdf
Breisha, G. Z. (2010). "Production of 16% ethanol from 35% sucrose." Biomass and bioenergy **34**(8): 1243-1249.
Broderick, G. A., A. P. Faciola and L. E. Armentano (2015). "Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows." Journal of Dairy Science **98**(8): 5672-5687.

Cai, C. (2014). "Assessment of the feasibility of including high levels of oilseed meals in the diets of juvenile Chinese mitten crabs (Eriocheir sinensis): Effects on growth, non-specific immunity, hepatopancreatic function, and intestinal morphology." Animal feed science and technology **v. 196**: pp. 117-127-2014 v.2196.

Camacho-Ruiz, L., N. Perez-Guerra and R. P. Roses (2003). "Factors affecting the growth of Saccharomyces cerevisiae in batch culture and in solid sate fermentation." Electronic Journal of Environmental, Agricultural and Food Chemistry **2**(5): 531-542.

Cao, X., H. Wen, C. Li and Z. Gu (2009). "Differences in functional properties and biochemical characteristics of congenetic rice proteins." Journal of cereal science **50**(2): 184-189.

Carboué, Q., I. Perraud-Gaime, M. Tranier and S. Roussos (2017). "Production of microbial enzymes by solid state fermentation for food applications." Microbial Enzyme Technology for Food Applications: 437-451.

Celus, I., K. Brijs and J. A. Delcour (2007). "Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates." Journal of agricultural and food chemistry **55**(21): 8703-8710.

Chandi, G. K. and D. S. Sogi (2007). "Biochemical characterisation of rice protein fractions." International journal of food science & technology **42**(11): 1357-1362.

Chandorkar, K. and N. Badenhuizen (1966). "How Meaningful are Determinations of Glucosyltransferase Activities in Starch-Enzyme Complexes?" Starch-Stärke **18**(4): 91-95.

Chao, B., R. Liu, X. Zhang, X. Zhang and T. Tan (2017). "Tannin extraction pretreatment and very high gravity fermentation of acorn starch for bioethanol production." Bioresource Technology **241**: 900-907.

Chatzifragkou, A., P. C. Prabhakumari, O. Kosik, A. Lovegrove, P. R. Shewry and D. Charalampopoulos (2016). "Extractability and characteristics of proteins deriving from wheat DDGS." Food chemistry **198**: 12-19.

Chen, H. (2013). "Modern solid state fermentation." Netherlands: Springer.

Chu-Ky, S., T.-H. Pham, K.-L. T. Bui, T.-T. Nguyen, K.-D. Pham, H.-D. T. Nguyen, H.-N. Luong, V.-P. Tu, T.-H. Nguyen and P.-H. Ho (2016). "Simultaneous liquefaction, saccharification and fermentation at very high gravity of rice at pilot scale for potable ethanol production and distillers dried grains composition." Food and Bioproducts Processing **98**: 79-85.

Compton, S. J. and C. G. Jones (1985). "Mechanism of dye response and interference in the Bradford protein assay." Analytical biochemistry **151**(2): 369-374.

Cooke, R. and E. Maduagwu (1978). "The effects of simple processing on the cyanide content of cassava chips." International Journal of Food Science & Technology **13**(4): 299-306.

Cookman, D. J. and C. E. Glatz (2009). "Extraction of protein from distiller's grain." Bioresource technology **100**(6): 2012-2017.

Corredor, D., S. Bean, T. Schober and D. Wang (2006). "Effect of Decorticating Sorghum on Ethanol Production and Composition of DDGS." Cereal Chemistry **83**: 17-21.

Correia, R., M. Magalhaes and G. Macêdo (2007). "Protein enrichment of pineapple waste with Saccharomyces cerevisiae by solid state bioprocessing." Journal of Scientific and Industrial Research **66**: 259-262.

Council, U. S. G. (2017). DDGS Weekly Market Report. **2017**.

Coursey, D. G. (1973). Cassava as food: toxicity and technology. Chronic cassava toxicity, IDRC, Ottawa, ON, CA.

Cromwell, G. L., K. L. Herkelman and T. S. Stahly (1993). "Physical, chemical, and nutritional characteristics of distillers dried grains with solubles for chicks and pigs." J Anim Sci **71**(3): 679-686.

D'Mello, J. F. (2003). Amino acids in animal nutrition, CABI publishing.

Dalmia, B. K. and Z. L. Nikolov (1991). "Characterization of glucoamylase adsorption to raw starch." Enzyme and microbial technology **13**(12): 982-990.

Das, R. and A. M. Kayastha (2019). "Enzymatic hydrolysis of native granular starches by a new β-amylase from peanut (Arachis hypogaea)." Food Chemistry **276**: 583-590.

Demirbas, A. (2009). "Biofuels securing the planet's future energy needs." Energy conversion and management **50**(9): 2239-2249.

Devantier, R., S. Pedersen and L. Olsson (2005). "Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain." Applied microbiology and biotechnology **68**(5): 622-629.

Diptee, R., J. Smith, I. Alli and S. Khanizadeh (1989). "Application of response surface methodology in protein extraction studies from brewer's spent grain." Journal of food processing and preservation **13**(6): 457-474.

Djelal, H., S. Chniti, M. Jemni, A. Weill, W. Sayed and A. Amrane (2017). "Identification of strain isolated from dates (Phœnix dactylifera L.) for enhancing very high gravity ethanol production." Environmental Science and Pollution Research **24**(11): 9886-9894.

Duan, G., N. Dunn-Coleman, O. Lantero, C. E. Pilgrim and J. K. Shetty (2009). "Acid fungal protease in fermentation of insoluble starch substrates." U.S. Patent No. 7,563,607. 21 Jul.

Duodu, K., J. R. Taylor, P. Belton and B. Hamaker (2003). "Factors affecting sorghum protein digestibility." Journal of Cereal Science **38**: 117-131.

Durand, A. (1998). "La fermentation en milieu solide." Biofutur **1998**(181): 41-43.

Durand, A. (2003). "Bioreactor designs for solid state fermentation." Biochemical Engineering Journal **13**(2-3): 113-125.

Durand, A., R. Renaud, J. Maratray and S. Almanza (1997). The INRA-Dijon reactors: designs and applications. Advances in solid state fermentation, Springer**:** 71-92.

Ebrahimi, F., M. Khanahmadi, S. Roodpeyma and M. J. Taherzadeh (2008). "Ethanol production from bread residues." Biomass and bioenergy **32**(4): 333-337.

EFSA (2007). "Opinion of the Scientific Panel on contaminations in the food chain on a request from the Commision related to cyanogenic compounds as undesirable sustances in animal feed." The EFSA Journal **437**: 1-67.

Eggum, B. O. (1979). "The nutritional value of rice in comparison with other cereals." In Proceedings of workshop on chemical aspects of rice grain quality 91-111.

Ellepola, S. W., S. M. Choi and C. Y. Ma (2005). "Conformational study of globulin from rice (Oryza sativa) seeds by Fourier-transform infrared spectroscopy." International Journal of Biological Macromolecules **37**(1-2): 12-20.

Espinosa-Pardo, F. A., R. Savoire, P. Subra-Paternault and C. Harscoat-Schiavo (2020). "Oil and protein recovery from corn germ: Extraction yield, composition and protein functionality." Food and Bioproducts Processing **120**: 131-142.

European Biofuels Technology Platform (2010). "Strategic research agenda 2010 update: Innovation driving sustainable biofuels." Available from: https://www.etipbioenergy.eu/images/SRA_2010_update_web.pdf

Ezekiel, O. O. and O. C. Aworh (2013). "Solid state fermentation of cassava peel with Trichoderma viride (ATCC 36316) for protein enrichment." International Journal of Agricultural and Biological Engineering **7**: 667-674.

Fannon, J. E., R. Hauber and J. N. Bemiller (1992). "Surface pores of starch granules." Cereal Chemistry **69**: 284-288.
FAO (1981). "Food loss prevention in perishable crops." Food and Agricultural Organization of the United Nations Agricultural Services Bulletin No.43.
Festel, G., M. Würmseher, C. Rammer, E. Boles and M. Bellof (2014). "Modelling production cost scenarios for biofuels and fossil fuels in Europe." Journal of Cleaner Production **66**: 242-253.
Franco, C. P., São José do Rio; Ciacco, C. (1992). "Factors that Affect the Enzymatic Degradation of Natural Starch Granules -Effect of the Size of the Granules." Starch-starke **44**: 422-426.
Fujii, M., T. Homma and M. Taniguchi (1988). "Synergism of α-amylase and glucoamylase on hydrolysis of native starch granules." Biotechnology and bioengineering **32**(7): 910-915.
Fujii, M. and Y. Kawamura (1985). "Synergistic action of α-amylase and glucoamylase on hydrolysis of starch." Biotechnology and bioengineering **27**(3): 260-265.
Gantelet, H. and F. Duchiron (1999). "A new pullulanase from a hyperthermophilic archaeon for starch hydrolysis." Biotechnology letters **21**(1): 71-75.
Gawande, P. and M. Kamat (1999). "Production of Aspergillus xylanase by lignocellulosic waste fermentation and its application." Journal of Applied Microbiology **87**(4): 511-519.
Gelinas, P. and J. Barrette (2007). "Protein enrichment of potato processing waste through yeast fermentation." Bioresource technology **98**(5): 1138-1143.
Gervais, P. and P. Molin (2003). "The role of water in solid-state fermentation." Biochemical Engineering Journal **13**(2-3): 85-101.
Ghanem (1992). "Single cell protein production from beet pulp by mixed culture." Qatar University Science Journal
Gibreel, A., J. R. Sandercock, J. Lan, L. A. Goonewardene, A. C. Scott, R. T. Zijlstra, J. M. Curtis and D. C. Bressler (2011). "Evaluation of value-added components of dried distiller's grain with solubles from triticale and wheat." Bioresource technology **102**(13): 6920-6927.
Gibreel, A., J. R. Sandercock, J. Lan, L. A. Goonewardene, R. T. Zijlstra, J. M. Curtis and D. C. Bressler (2009). "Fermentation of barley by using Saccharomyces cerevisiae: examination of barley as a feedstock for bioethanol production and value-added products." Applied and environmental microbiology **75**(5): 1363-1372.
Gibson, B. R., S. J. Lawrence, J. P. Leclaire, C. D. Powell and K. A. Smart (2007). "Yeast responses to stresses associated with industrial brewery handling." FEMS microbiology reviews **31**(5): 535-569.
Godoy, M. G., G. M. Amorim, M. S. Barreto and D. M. Freire (2018). "Agricultural residues as animal feed: protein enrichment and detoxification using solid-state fermentation." Current developments in biotechnology and bioengineering, Elsevier**:** 235-256.
Gohel, V. and G. Duan (2012). "Conventional process for ethanol production from Indian broken rice and pearl millet." Bioprocess and biosystems engineering **35**(8): 1297-1308.
Gohel, V. and G. Duan (2012). "No-cook process for ethanol production using Indian broken rice and pearl millet." International journal of microbiology **2012**.
González, L. C., M. A. Loubes and M. P. Tolaba (2018). "Incidence of milling energy on dry-milling attributes of rice starch modified by planetary ball milling." Food Hydrocolloids **82**: 155-163.

Gorinstein, S., M. Zemser, M. Friedman, W. Rodrigues, P. Martins, N. Vello, G. Tosello and O. Paredes-López (1996). "Physicochemical characterization of the structural stability of some plant globulins." Food chemistry **56**(2): 131-138.

Guan, J., G. Yang, H. Yin, F. Jia and J. Wang (2014). "Particle size for improvement of peptide production in mixed-culture solid-state fermentation of soybean meal and the corresponding kinetics." Am. J. Agr. For **2**(1): 1-6.

Gunawan, S., T. Widjaja, S. Zullaikah and L. Ernawati (2014). "Effect of fermenting cassava with Lactobacillus plantarum, Saccharomyces cereviseae, and Rhizopus oryzae on the chemical composition of their flour." International Food Research Journal **22**(3): 1280-1287.

Gunst, R. F. (1996). "Response Surface Methodology: Process and Product Optimization Using Designed Experiments." Technometrics **38**(3): 284-286

Guo, X., J. Zhang, Y. Ma and S. Tian (2013). "Optimization of limited hydrolysis of proteins in rice residue and characterization of the functional properties of the products." Journal of Food Processing and Preservation **37**(3): 245-253.

Hamada, J. (1997). "Characterization of protein fractions of rice bran to devise effective methods of protein solubilization." Cereal Chemistry **74**(5): 662-668.

Hamaker, B. R. and B. A. Bugusu (2003). "Overview: sorghum proteins and food quality." Workshop on the proteins of sorghum and millets: enhancing nutritional and functional properties for Africa [CD](Pretoria: South Africa).

Hargono, A. C. Kumoro and B. Jos (2015). "Comparative study on the conventional and non thermal simultaneous saccharification and fermentation of Manihot glaziovii root starch." AIP Conference Proceedings **1699**(1): 030013

Hargono, H., B. Jos and A. C. Kumoro (2018). "Kinetics of the hydrolysis of Cassava starch by Glucoamylase and a granular starch hydrolyzing enzyme." Scientific Study & Research. Chemistry & Chemical Engineering, Biotechnology, Food Industry **19**(4): 443.

Hawashi, M., A. Altway, T. Widjaja and S. Gunawan (2019). "Optimization of process conditions for tannin content reduction in cassava leaves during solid state fermentation using Saccharomyces cerevisiae." Heliyon **5**(8): e02298.

Harper, S. and J. Lynch (1985). "Colonization and decomposition of straw by fungi." Transactions of the British Mycological Society **85**(4): 655-661.

Henry, R. J. (1987). "Pentosan and (1A' 3),(1A' 4)-²-Glucan concentrations in endosperm and wholegrain of wheat, barley, oats and rye." Journal of Cereal Science **6**: 253-258.

Higuchi, Y., A. Ohashi, H. Imachi and H. Harada (2005). "Hydrolytic activity of alpha-amylase in anaerobic digested sludge." Water Science and Technology **52**(1-2): 259-266.

Ho, D.T, Pham, T.M., Nguyen, M. T (2014). "Determination of total cyanide content in cassava and cassava residue." Journal of Fisheries Science and Technology (Vietnam) **1**, 195-200.

Hölker, U. and J. Lenz (2005). "Solid-state fermentation-are there any biotechnological advantages?" Current opinion in microbiology **8**(3): 301-306.

Hong, T. T. T., P. T. B. Lien, D. T. Hai, P. T. Hang and N. H. Quan (2017). "Protein-enriched cassava root pulp as partial replacement for fish meal in diets for growing pigs." Chemical analysis **30**(3): 97.

Hoover, R. and Y. Zhou (2003). "In vitro and in vivo hydrolysis of legume starches by#±-amylase and resistant starch formation in legume" a review." Carbohydrate Polymers **54**: 401-417.

Hosny, W. M., Davis, R. T., and Werle, M. J. (1979). "Improvements to the solution of the viscous shock layer equations (No. AFL-78-11-45)." Cincinnati Univ Oh Dept of Aerospace Engineering and Engineering Mechanics.

Hou, L., Y. Zhu and Q. Li (2010). "Characterization and preparation of broken rice proteins modified by proteases." Food Technology and Biotechnology **48**(1): 50.

Hounsa, C.-G., E. V. Brandt, J. Thevelein, S. Hohmann and B. A. Prior (1998). "Role of trehalose in survival of Saccharomyces cerevisiae under osmotic stress." Microbiology **144**(3): 671-680.

İçöz, E., K. M. Tuğrul, A. Saral and E. İçöz (2009). "Research on ethanol production and use from sugar beet in Turkey." Biomass and bioenergy **33**(1): 1-7.

Ingledew, W., K. Thomas, S. Hynes and J. McLeod (1999). "Viscosity concerns with rye mashes used for ethanol production." Cereal chemistry **76**(3): 459-464.

INRA-CIRAD-AFZ. (2004). "Feed tables: Composition and nutritive values of feeds for cattle, sheep, goats, pigs, poultry, rabbits, horses and salmonids." Retrieved 24/06, 2019. Available from: https://feedtables.com/

Izmirlioglu, G. and A. Demirci (2015). "Enhanced bio-ethanol production from industrial potato waste by statistical medium optimization." International journal of molecular sciences **16**(10): 24490-24505.

Jach, M. E., T. Baj, M. Juda, R. Świder, B. Mickowska and A. Malm (2020). "Statistical evaluation of growth parameters in biofuel waste as a culture medium for improved production of single cell protein and amino acids by Yarrowia lipolytica." AMB Express **10**(1): 1-12.

Jakobsen, M. and J. Piper (1989). "Performance and osmotolerance of different strains of lager yeast in high gravity fermentations." Technical quarterly-Master Brewers Association of the Americas (USA).

Jambrak, A. R., Z. Herceg, D. Šubarić, J. Babić, M. Brnčić, S. R. Brnčić, T. Bosiljkov, D. Čvek, B. Tripalo and J. Gelo (2010). "Ultrasound effect on physical properties of corn starch." Carbohydrate Polymers **79**(1): 91-100.

Jane, J.-l. (2006). "Current understanding on starch granule structures." Journal of Applied Glycoscience **53**(3): 205-213.

Jane, J., T. Kasemsuwan, S. Leas, H. Zobel and J. Robyt (1994). "Anthology of Starch Granule Morphology by Scanning Electron Microscopy." Starch-starke **46**: 121-129.

Jao, C.-L., W.-C. Ko and K.-C. Hsu (2011). "Studies on the cooking conditions and mechanical koji-making of black beans." Procedia Food Science **1**: 1388-1395.

Jarrett, S. and C. J. Ashworth (2018). "The role of dietary fibre in pig production, with a particular emphasis on reproduction." Journal of animal science and biotechnology **9**(1): 1-11.

Joannis-Cassan, C., J. Riess, F. Jolibert and P. Taillandier (2014). "Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup." Biomass and Bioenergy **70**: 165-173.

Jobling, S. (2004). "Improving starch for food and industrial applications." Current opinion in plant biology **7**(2): 210-218.

Jones, A. M. and W. Ingledew (1994). "Fuel alcohol production: Assessment of selected commercial proteases for very high gravity wheat mash fermentation." Enzyme and microbial technology **16**(8): 683-687.

Jones, A. M. and W. M. Ingledew (1994). "Fuel alcohol production: optimization of temperature for efficient very-high-gravity fermentation." Appl Environ Microbiol **60**(3): 1048-1051.

Jones, A. M., K. C. Thomas and W. M. Ingledew (1994). "Ethanolic fermentation of blackstrap molasses and sugarcane juice using very high gravity technology." Journal of agricultural and food chemistry **42**(5): 1242-1246.

Kamerling, J. P. (2007). "Comprehensive glycoscience. Introduction to Glycoscience; Synthesis of Carbohydrates." Elsevier.

Kamm, B., P. R. Gruber and M. Kamm (2006). "Biorefineries-industrial processes and products." Wiley-VCH Weinheim.

Karimi, K., G. Emtiazi and M. J. Taherzadeh (2006). "Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae." Enzyme and microbial technology **40**(1): 138-144.

Kawa-Rygielska, J., W. Pietrzak and A. Czubaszek (2012). "Characterization of fermentation of waste wheat-rye bread mashes with the addition of complex enzymatic preparations." Biomass and Bioenergy **44**: 17-22.

Kazuo, I., I. Masaru and M. Noshi (1998). "Stabilization of amylolytic enzymes by modification with periodate-oxidized soluble starch." The Korean Journal of Food And Nutrition **11**(5): 561-564.

Kelsall, D. and R. Piggot (2009). Grain milling and cooking for alcohol production: Design for the options in dry milling. The alcohol textbook**:** 161-176.

Khanahmadi, M., R. Roostaazad, D. A. Mitchell, M. Miranzadeh, R. Bozorgmehri and A. Safekordi (2006). "Bed moisture estimation by monitoring of air stream temperature rise in packed-bed solid-state fermentation." Chemical engineering science **61**(17): 5654-5663.

Khanahmadi, M., R. Roostaazad, A. Safekordi, R. Bozorgmehri and D. A. Mitchell (2004). "Investigating the use of cooling surfaces in solid-state fermentation tray bioreactors: modelling and experimentation." Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology **79**(11): 1228-1242.

Khanal, S. K. (2011). "Anaerobic biotechnology for bioenergy production: principles and applications." John Wiley & Sons.

Khanal, S. K., M. Montalbo, J. Van Leeuwen, G. Srinivasan and D. Grewell (2007). "Ultrasound enhanced glucose release from corn in ethanol plants." Biotechnology and bioengineering **98**(5): 978-985.

Khoi, B. H., L. D. Dien, R. Lásztity and A. Salgó (1987). "The protein and the amino acid composition of some rice and maize varieties grown in North Vietnam." Journal of the Science of Food and Agriculture **39**(2): 137-143.

Khongsay, N., L. Laopaiboon, P. Jaisil and P. Laopaiboon (2012). "Optimization of agitation and aeration for very high gravity ethanol fermentation from sweet sorghum juice by Saccharomyces cerevisiae using an orthogonal array design." Energies **5**(3): 561-576.

Kim, Y., N. S. Mosier, R. Hendrickson, T. Ezeji, H. Blaschek, B. Dien, M. Cotta, B. Dale and M. R. Ladisch (2008). "Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage." Bioresour Technol **99**(12): 5165-5176.

Kingsly, A., K. E. Ileleji, C. L. Clementson, A. Garcia, D. E. Maier, R. L. Stroshine and S. Radcliff (2010). "The effect of process variables during drying on the physical and chemical characteristics of corn dried distillers grains with solubles (DDGS)–Plant scale experiments." Bioresource Technology **101**(1): 193-199.

Kollaras, A., J. M. Kavanagh, G. L. Bell, D. Purkovic, S. Mandarakas, P. Arcenal, W. S. Ng, K. S. Routledge, D. H. Selwood and P. Koutouridis (2011). "Techno-economic implications of improved high gravity corn mash fermentation." Bioresource technology **102**(16): 7521-7525.

Kosugi, A., A. Kondo, M. Ueda, Y. Murata, P. Vaithanomsat, W. Thanapase, T. Arai and Y. Mori (2009). "Production of ethanol from cassava pulp via fermentation with a surface-engineered yeast strain displaying glucoamylase." Renewable Energy **34**(5): 1354-1358.

Krishna, C. (2005). "Solid-state fermentation systems—an overview." Critical reviews in biotechnology **25**(1-2): 1-30.

Kubota, M., Y. Saito, T. Masumura, T. Kumagai, R. Watanabe, S. Fujimura and M. Kadowaki (2010). "Improvement in the in vivo digestibility of rice protein by alkali extraction is due to structural changes in prolamin/protein body-I particle." Bioscience, biotechnology, and biochemistry **74**(3): 614-619.

Kumagai, T., H. Kawamura, T. Fuse, T. Watanabe, Y. Saito, T. Masumura, R. Watanabe and M. Kadowaki (2006). "Production of rice protein by alkaline extraction improves its digestibility." Journal of nutritional science and vitaminology **52**(6): 467-472.

Lacerda, L. D., D. C. Leite and N. P. Silveira (2019). "Relationships between enzymatic hydrolysis conditions and properties of rice porous starches." Journal of Cereal Science **89**: 102819.

Landolfo, S., G. Zara, S. Zara, M. Budroni, M. Ciani and I. Mannazzu (2010). "Oleic acid and ergosterol supplementation mitigates oxidative stress in wine strains of Saccharomyces cerevisiae." International journal of food microbiology **141**(3): 229-235.

Laopaiboon, L., S. Nuanpeng, P. Srinophakun, P. Klanrit and P. Laopaiboon (2009). "Ethanol production from sweet sorghum juice using very high gravity technology: effects of carbon and nitrogen supplementations." Bioresource technology **100**(18): 4176-4182.

Laopaiboon, L., P. Thanonkeo, P. Jaisil and P. Laopaiboon (2007). "Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by Saccharomyces cerevisiae." World Journal of Microbiology and Biotechnology **23**(10): 1497-1501.

Laroche, M., V. Perreault, A. Marciniak, A. Gravel, J. Chamberland and A. Doyen (2019). "Comparison of Conventional and Sustainable Lipid Extraction Methods for the Production of Oil and Protein Isolate from Edible Insect Meal." Foods **8**(11): 572.

Le Thanh, M., Nguyen Thi, H., Pham Thu, T., Nguyen Thanh, H., Le Lan, C. (2007). "Analytical Methods in Fermentation Technology." Science and Technology Publishing House, Hanoi, Vietnam,.

Lee, R. A. and J.-M. Lavoie (2013). "From first-to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity." Animal Frontiers **3**(2): 6-11.

Lee, Y. and K. Kim (1990). "Gelatinization and Liquefaction of Starch with a Heat Stable α-Amylase." Journal of food science **55**(5): 1365-1366.

Lehmann, U. and F. Robin (2007). "Slowly digestible starch–its structure and health implications: a review." Trends in Food Science & Technology **18**(7): 346-355.

Lehrfeld, J. (1989). "High-performance liquid chromatography analysis of phytic acid on a pH-stable, macroporous polymer column." Cereal Chemistry **66**: 510-515.

Lemke, U., M. Mergenthaler, R. Roßler, L. Huyen, P. Herold, B. Kaufmann and A. V. Zarate (2008). "Pig production in Vietnam-A review." Pig News and Information **29**(2): 1R.

Lesaffre, L. b. "Industrial ethanol." https://lesaffreadvancedfermentations.com/ethanol_yeast/.

Li, J., T. Vasanthan and D. C. Bressler (2012). "Improved cold starch hydrolysis with urea addition and heat treatment at subgelatinization temperature." Carbohydrate Polymers **87**(2): 1649-1656.

Li, K., S. Liu and X. Liu (2014). "An overview of algae bioethanol production." International Journal of Energy Research **38**(8): 965-977.

Li, Q., Y. Ma, C. Mao and C. Wu (2009). "Grafting modification and structural degradation of multi-walled carbon nanotubes under the effect of ultrasonics sonochemistry." Ultrasonics sonochemistry **16**(6): 752-757.
Li, X., H. Xiong, K. Yang, D. Peng, H. Peng and Q. Zhao (2012). "Optimization of the biological processing of rice dregs into nutritional peptides with the aid of trypsin." Journal of food science and technology **49**(5): 537-546.
Li, Z., L. Cai, Z. Gu and Y.-C. Shi (2014). "Effects of granule swelling on starch saccharification by granular starch hydrolyzing enzyme." Journal of agricultural and food chemistry **62**(32): 8114-8119.
Li, Z., D. Wang and Y.-C. Shi (2017). "Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch." Journal of the Taiwan Institute of Chemical Engineers **70**: 229-235.
Liao, S. F., T. Wang and N. Regmi (2015). "Lysine nutrition in swine and the related monogastric animals: muscle protein biosynthesis and beyond." SpringerPlus **4**: 147.
Lin, Y., W. Zhang, C. Li, K. Sakakibara, S. Tanaka and H. Kong (2012). "Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742." Biomass and bioenergy **47**: 395-401.
Lindberg, J. E. (2014). "Fiber effects in nutrition and gut health in pigs." Journal of Animal Science and Biotechnology **5**(1): 15.
Linoj, K., D. Prabha, G. Anandajit and M. Sameer (2006). "Liquid biofuels in South Asia: Resources and technologies." Asian Biotechnology and Development Review **8**(2): 31-49.
Liu, K. S. (2011). "Chemical composition of distillers grains, a review." J Agri Food Chem **59**(5): 1508-1526.
Liu, R. and F. Shen (2008). "Impacts of main factors on bioethanol fermentation from stalk juice of sweet sorghum by immobilized Saccharomyces cerevisiae (CICC 1308)." Bioresource technology **99**(4): 847-854.
Longo, M. A., F. J. Deive, A. Domínguez and M. Sanromán (2008). "Solid-state fermentation for food and feed application." Current developments in solid-state fermentation, Springer**:** 379-411.
Lund, D. and K. J. Lorenz (1984). "Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization." Critical Reviews in Food Science & Nutrition **20**(4): 249-273.
Lund, M. N. and C. A. Ray (2017). "Control of Maillard reactions in foods: Strategies and chemical mechanisms." Journal of Agricultural and Food Chemistry **65**(23): 4537-4552.
Manchun, S., J. Nunthanid, S. Limmatvapirat and P. Sriamornsak (2012). Effect of ultrasonic treatment on physical properties of tapioca starch. Advanced materials research, Trans Tech Publ.
Mahanta, N., A. Gupta and S. Khare (2008). "Production of protease and lipase by solvent tolerant Pseudomonas aeruginosa PseA in solid-state fermentation using Jatropha curcas seed cake as substrate." Bioresource technology **99**(6): 1729-1735.
Mai, D. V., Nguyen, T. T., Chu-Ky, S., and Waché, Y. (2020). "Protein Enrichment of Cassava-Based Dried Distiller's Grain by Solid State Fermentation Using Trichoderma Harzianum and Yarrowia Lipolytica for Feed Ingredients." Waste and Biomass Valorization: 1-14.
Marchal, L. and J. Tramper (1999). "Hydrolytic gain during hydrolysis reactions; implications and correction procedures." Biotechnology techniques **13**(5): 325-328.
Marques, S., L. Alves, J. Roseiro and F. Gírio (2008). "Conversion of recycled paper sludge to ethanol by SHF and SSF using Pichia stipitis." Biomass and bioenergy **32**(5): 400-406.

Marquez, T., C. Millan and J.-M. Salmon (2009). "Plasma membrane sterols are involved in yeast's ability to adsorb polyphenolic compounds resulting from wine model solution browning." Journal of agricultural and food chemistry **57**(17): 8026-8032.
Martini, A. E. V., M. W. Miller and A. Martini (1979). "Amino acid composition of whole cells of different yeasts." Journal of Agricultural and Food Chemistry **27**(5): 982-984.
Masłyk, E., W. Leszczyński and A. Gryszkin (2003). "Modification-induced changes in potato starch susceptibility to amylolytic enzyme action." Polish journal of food and nutrition sciences **12**(Suppl. 1): 54-56.
Matser, A. M. and P. A. M. Steeneken (1998). "Origins of the Poor Filtration Characteristics of Wheat Starch Hydrolysates." Cereal Chemistry **75**(3): 289-293.
Mawal, Y., M. Mawal, M. Sainani and P. Ranjekar (1990). "Rice seed storage proteins: a structural insight." Plant Science **70**(1): 73-80.
Membrillo, I., C. Sánchez, M. Meneses, E. Favela and O. Loera (2008). "Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by Pleurotus ostreatus strains." Bioresource Technology **99**(16): 7842-7847.
Meyer, R. D., D. M. Steinberg and G. Box (1996). "Follow-up designs to resolve confounding in multifactor experiments." Technometrics **38**(4): 303-313.
Mikulski, D., G. Kłosowski and A. Rolbiecka (2014). "Effect of phytase application during high gravity (HG) maize mashes preparation on the availability of starch and yield of the ethanol fermentation process." Applied biochemistry and biotechnology **174**(4): 1455-1470.
Mikulski, D., A. J. Rolbiecka and G. R. Kłosowski (2017). "Potential influence of compounds released in degradation of phytates on the course of alcoholic fermentation of high gravity mashes–simulation with analogs of these compounds." Polish Journal of Chemical Technology **19**(3): 27-34.
Miller, G. L. (1959). "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar." Analytical Chemistry **31**(3): 426-428.
Ministry-of-Industry (2007). Development strategy for beverage ethanol production in Vietnam from 2007-2025.
Mitchell, D. A., M. Berovič and N. Krieger (2006). "Solid-state fermentation bioreactor fundamentals: Introduction and overview." Solid-State Fermentation Bioreactors, Springer**:** 1-12.
Mitsuda, H., K. Yasumoto, K. Murakami, T. Kusano and H. Kishida (1967). "Studies on the proteinaceous subcellular particles in rice endosperm: electron-microscopy and isolation." Agricultural and Biological Chemistry **31**(3): 293-300.
Mittal, G. S. (1992). "Food biotechnology: techniques and applications." Technomic Pub. Co.
Mogg, R. (2004). "Biofuels in Asia: Thailand relaunches 'Gasohol'for automotive use." Refocus **5**(3): 44-47.
Mohnen, D. (2008). "Pectin structure and biosynthesis." Current opinion in plant biology **113**: 266-277.
Mojović, L., S. Nikolić, M. Rakin and M. Vukasinović (2006). "Production of bioethanol from corn meal hydrolyzates." Fuel **85**(12-13): 1750-1755.
Molaverdi, M., K. Karimi, M. Khanahmadi and A. Goshadrou (2013). "Enhanced sweet sorghum stalk to ethanol by fungus Mucor indicus using solid state fermentation followed by simultaneous saccharification and fermentation." Industrial Crops and Products **49**: 580-585.
Monceaux, D. A. and D. Kuehner (2009). "Dryhouse technologies and DDGS production." The Alcohol Textbook - 5th edition. Ingledew W.M., Kelsall D.R., Austin G.D. and Kluhspies C.**:** 303-322.

Montagnac, J. A., C. R. Davis and S. A. Tanumihardjo (2009). "Nutritional Value of Cassava for Use as a Staple Food and Recent Advances for Improvement." Comprehensive Reviews in Food Science and Food Safety **8**(3): 181-194.
Monteiro, B., P. Ferraz, M. Barroca, S. H. da Cruz, T. Collins and C. Lucas (2018). "Conditions promoting effective very high gravity sugarcane juice fermentation." Biotechnology for biofuels **11**(1): 251.
Montesinos, T. and J.-M. Navarro (2000). "Production of alcohol from raw wheat flour by amyloglucosidase and Saccharomyces cerevisiae." Enzyme and Microbial Technology **27**(6): 362-370.
Montet, D. and R. C. Ray (2016). "Fermented Foods, Part I: Biochemistry and Biotechnology." CRC Press.
Moore, E. (1996). "Fundamentals of the fungi." Prentice Hall, Upper Saddle River, New Jersey **4th ed. Englewood Cliffs, NJ: Prentice-Hall**: 251-258.
Moorthy, S. N. (2002). "Physicochemical and Functional Properties of Tropical Tuber Starches: A Review." Starch - Stärke **54**(12): 559-592.
Morales, E., R. Domingos and D. Angelis (2018). "Improvement of protein bioavailability by solid-state fermentation of babassu mesocarp flour and cassava leaves." Waste and Biomass Valorization **9**(4): 581-590.
Morgan, N. K. and M. Choct (2016). "Cassava: Nutrient composition and nutritive value in poultry diets." Animal Nutrition **2**(4): 253-261.
Morita, T. and S. KIRIYAMA (1993). "Mass production method for rice protein isolate and nutritional evaluation." Journal of food science **58**(6): 1393-1396.
Moser, B. R. (2011). "Biodiesel production, properties, and feedstocks." Biofuels: 285-347.
Mousdale, D. M. (2008). Biofuels: biotechnology, chemistry, and sustainable development, CRC press.
Muindi, P. J. and J. F. Hanssen (1981). "Nutritive value of cassava root meal enriched by Trichoderma harzianum for chickens." Journal of the Science of Food and Agriculture **32**(7): 647-654.
Mukhtar, K., M. Asgher, S. Afghan, K. Hussain and S. Zia-ul-Hussnain (2010). "Comparative study on two commercial strains of Saccharomyces cerevisiae for optimum ethanol production on industrial scale." BioMed Research International **2010**.
Mussatto, S. I., G. Dragone, P. M. Guimarães, J. P. A. Silva, L. M. Carneiro, I. C. Roberto, A. Vicente, L. Domingues and J. A. Teixeira (2010). "Technological trends, global market, and challenges of bio-ethanol production." Biotechnology advances **28**(6): 817-830.
Mustafa, S. R., A. Husaini, C. N. Hipolito, H. Hussain, N. Suhaili and H. A. Roslan (2016). "Application of response surface methodology for optimizing process parameters in the production of amylase by Aspergillus flavusNSH9 under solid state fermentation." Brazilian Archives of Biology and Technology **59**.
Myers, R. H., D. C. Montgomery, G. G. Vining, C. M. Borror and S. M. Kowalski (2004). "Response surface methodology: a retrospective and literature survey." Journal of quality technology **36**(1): 53-77.
Naidu, K., V. Singh, D. B. Johnston, K. D. Rausch and M. E. Tumbleson (2007). "Effects of ground corn particle size on ethanol yield and thin stillage soluble solids." Cereal chemistry **84**(1): 6-9.
OECD-FAO (2015). Agricultural Outlook 2015. Paris, France: OECD Publishing; 2015. https://doi.org/1787/agr_outlook-2015-en."
Neves, M. A. d., T. Kimura, N. Shimizu and K. Shiiba (2006). "Production of alcohol by simultaneous saccharification and fermentation of low-grade wheat flour." Brazilian Archives of Biology and Technology **49**(3): 481-490.

Nghiem, N. P., F. Taylor, D. B. Johnston, J. K. Shetty and K. B. Hicks (2011). "Scale-up of ethanol production from winter barley by the EDGE (enhanced dry grind enzymatic) process in fermentors up to 300 l." Applied biochemistry and biotechnology **165**(3-4): 870-882.

Nguyen, C.-N., T.-M. Le and S. Chu-Ky (2014). "Pilot scale simultaneous saccharification and fermentation at very high gravity of cassava flour for ethanol production." Industrial crops and products **56**: 160-165.

Nguyen, N. X. D., H. M. Luu and P. Udén (2002). "Tropical fibre sources for pigs—digestibility, digesta retention and estimation of fibre digestibility in vitro." Anim Feed Sci Tech **102**(1–4): 109-124.

Nguyen, T. C., S. Chu-ky, H. N. Luong and H. Nguyen (2020). "Effect of Pretreatment Methods on Enzymatic Kinetics of Ungelatinized Cassava Flour Hydrolysis." Catalysts **10**: 760.

Nguyen, T. N., D. A. Davis and I. P. Saoud (2009). "Evaluation of alternative protein sources to replace fish meal in practical diets for juvenile tilapia, Oreochromis spp." Journal of the World Aquaculture Society **40**(1): 113-121.

Nguyen, V. D., J. Auresenia, H. Kosuge, R. R. Tan and Y. Brondial (2011). "Vacuum fermentation integrated with separation process for ethanol production." Biochemical engineering journal **55**(3): 208-214.

Nigam, P. S. and A. Pandey (2009). Solid-state fermentation technology for bioconversion of biomass and agricultural residues. Biotechnology for agro-industrial residues utilisation, Springer**:** 197-221.

Nikolić, S., L. Mojović, M. Rakin and D. Pejin (2009). "Bioethanol production from corn meal by simultaneous enzymatic saccharification and fermentation with immobilized cells of Saccharomyces cerevisiae var. ellipsoideus." Fuel **88**(9): 1602-1607.

Nikolić, S., L. Mojović, M. Rakin, D. Pejin and J. Pejin (2010). "Ultrasound-assisted production of bioethanol by simultaneous saccharification and fermentation of corn meal." Food Chemistry **122**(1): 216-222.

Nuss, E. T. and S. A. Tanumihardjo (2010). "Maize: A Paramount Staple Crop in the Context of Global Nutrition." Comprehensive Reviews in Food Science and Food Safety **9**(4): 417-436.

Oates, C. G. (1997). "Towards an understanding of starch granule structure and hydrolysis." Trends in Food Science & Technology **8**(11): 375-382.

Oboh, G. and A. Akindahunsi (2003). "Biochemical changes in cassava products (flour & gari) subjected to Saccharomyces cerevisae solid media fermentation." Food chemistry **82**(4): 599-602.

Oboh, G. and M. Oladunmoye (2007). "Biochemical changes in micro-fungi fermented cassava flour produced from low-and medium-cyanide variety of cassava tubers." Nutrition and health **18**(4): 355-367.

Ofuya, C. and S. Obilor (1994). "The effects of solid-state fermentation on the toxic components of cassava peel." Process Biochemistry **29**(1): 25-28.

Öhgren, K., R. Bura, G. Lesnicki, J. Saddler and G. Zacchi (2007). "A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover." Process Biochemistry **42**(5): 834-839.

Ortiz, S. M. and M. Añón (2001). "Enzymatic hydrolysis of soy protein isolates. DSC study." Journal of thermal analysis and calorimetry **66**(2): 489-499.

Osama A. Abo Siada, M. S. Negm, M. E. Basiouny, M. A. Fouad and S. Elagroudy (2018). "Protein Enrichment of Agro–Industrial Waste by Trichoderma harzianum EMCC 540

through Solid State Fermentation for Use as Animal Feed." Journal of Geography, Environment and Earth Science International **13**(4): 1-12.
Osama, A. S., M. A. Khaled and M. H. Abir (2013). "Bioconversion of Some Agricultural Wastes into Animal Feed by Trichoderma spp." Journal of American Science **9**(6): 203-212.
Owuama, C. I. and N. Okafor (1990). "Use of unmalted sorghum as a brewing adjunct." World Journal of Microbiology and Biotechnology **6**: 318-322.
Panchal, C. and G. Stewart (1980). "The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain." Journal of the Institute of Brewing **86**(5): 207-210.
Pandey, A. (1991). "Aspects of fermenter design for solid-state fermentations." Process Biochemistry **26**(6): 355-361.
Pandey, A. (1992). "Recent process developments in solid-state fermentation." Process biochemistry **27**(2): 109-117.
Pandey, A. (2011). "Biofuels: alternative feedstocks and conversion processes." Academic Press.
Pandey, A., C. R. Soccol and C. Larroche (2008). "Current developments in solid-state fermentation." Springer Science & Business Media.
Pandey, A., C. R. Soccol, P. Nigam, D. Brand, R. Mohan and S. Roussos (2000). "Biotechnological potential of coffee pulp and coffee husk for bioprocesses." Biochemical Engineering Journal **6**(2): 153-162.
Pandey, A., C. R. Soccol, P. Nigam and V. T. Soccol (2000). "Biotechnological potential of agro-industrial residues. I: sugarcane bagasse." Bioresource technology **74**(1): 69-80.
Pandey, A., C. R. Soccol, J. A. Rodriguez-Leon and P. S.-N. Nigam (2001). "Solid State Fermentation in Biotechnology: Fundamentals and Applications" Reference Book.
Pandya, J. J. and A. Gupte (2012). "Production of xylanase under solid-state fermentation by Aspergillus tubingensis JP-1 and its application." Bioprocess and biosystems engineering **35**(5): 769-779.
Paraman, I., N. Hettiarachchy and C. Schaefer (2008). "Preparation of rice endosperm protein isolate by alkali extraction." Cereal Chemistry **85**(1): 76-81.
Paraman, I., N. Hettiarachchy, C. Schaefer and M. I. Beck (2006). "Physicochemical properties of rice endosperm proteins extracted by chemical and enzymatic methods." Cereal chemistry **83**(6): 663-667.
Parrado, J. and J. Bautista (1993). "Protein enrichment of sunflower lignocellulosic fraction by Trichoderma harzianum S/G 2431 in low moisture content media." Bioscience, biotechnology, and biochemistry **57**(2): 317-318.
Pátková, J., D. Šmogrovičová, P. Bafrncova and Z. Dömény (2000). "Changes in the yeast metabolism at very high-gravity wort fermentation." Folia microbiologica **45**(4): 335-338.
Pátková, J., D. Šmogrovičová, Z. Dömény and P. Bafrncová (2000). "Very high gravity wort fermentation by immobilised yeast." Biotechnology Letters **22**(14): 1173-1177.
Pejin, D., L. Mojovi , V. Vv urovj , J. Pejin, S. Den j and M. Rakin (2009). "Fermentation of wheat and triticale hydrolysates: A comparative study." Fuel **88**: 1625-1628.
Pejin, D. J., L. V. Mojović, J. D. Pejin, O. S. Grujić, S. L. Markov, S. B. Nikolić and M. N. Marković (2012). "Increase in bioethanol production yield from triticale by simultaneous saccharification and fermentation with application of ultrasound." Journal of Chemical Technology & Biotechnology **87**(2): 170-176.
Phanthong, P., P. Reubroycharoen, X. Hao, G. Xu, A. Abudula and G. Guan (2018). "Nanocellulose: Extraction and application." Carbon Resources Conversion **1**(1): 32-43.

Pielech-Przybylska, K., M. Balcerek, P. Patelski and U. Dziekonska-Kubczak (2019). "Solutions for improvement of saccharification and fermentation of high gravity rye mashes." International Agrophysics **33**(1).

Pietrzak, W. and J. Kawa-Rygielska (2014). "Ethanol fermentation of waste bread using granular starch hydrolyzing enzyme: Effect of raw material pretreatment." Fuel **134**: 250-256.

Pietrzak, W. and J. Kawa-Rygielska (2015). "Simultaneous saccharification and ethanol fermentation of waste wheat–rye bread at very high solids loading: Effect of enzymatic liquefaction conditions." Fuel **147**: 236-242.

Pinu, F. R., P. J. Edwards, R. C. Gardner and S. G. Villas-Boas (2014). "Nitrogen and carbon assimilation by Saccharomyces cerevisiae during Sauvignon blanc juice fermentation." FEMS yeast research **14**(8): 1206-1222.

Piotrowicz, I. B. B., & Salas-Mellado, M. M. (2017). "Protein concentrates from defatted rice bran: preparation and characterization." Food Science and Technology 37: 165-172.

Piyachomkwan, K., R. Wunsuksri, S. Wanlapatit, P. Chatakanonda and K. Sriroth (2007). "Application of granular starch hydrolyzing enzymes for ethanol production." Starch. Progress in Basic and Applied Sciences, ed. Tomasik P., Yuryev V., Bertoft E., Polish Society of Food Technologists, Małopolska Branch, Kraków: 183-190.

Pohlandt, A. (1983). "A critical evaluation of methods applicable to the determination of cyanides." Journal of the Southern African Institute of Mining and Metallurgy **83**(1): 11-19.

Pomeranz, Y. (1976). "Advances in cereal science and technology." American Association of Cereal Chemists.

Poonsrisawat, A., S. Wanlapatit, A. Paemanee, L. Eurwilaichitr, K. Piyachomkwan and V. Champreda (2014). "Viscosity reduction of cassava for very high gravity ethanol fermentation using cell wall degrading enzymes from Aspergillus aculeatus." Process Biochemistry **49**(11): 1950-1957.

Potter, A. L. and E. A. McComb (2008). "Carbohydrate composition of potatoes. Pectin content." American Potato Journal **34**: 342-346.

Praspaliauskas, M., N. Pedišius, D. Čepauskienė and M. Valantinavičius (2020). "Study of chemical composition of agricultural residues from various agro-mass types." Biomass Conversion and Biorefinery **10**(4): 937-948.

Puligundla, P., V. S. R. Obulam and C. Mok (2014). "Preparation of very high gravity cassava mashes and subsequent fermentation to ethanol using Saccharomyces bayanus." Rom. Biotech. Lett **19**(6): 9925-9931.

Puligundla, P., D. Smogrovicova, V. S. R. Obulam and S. Ko (2011). "Very high gravity (VHG) ethanolic brewing and fermentation: a research update." Journal of industrial microbiology & biotechnology **38**(9): 1133-1144.

Raimbault, M. (1998). "General and microbiological aspects of solid substrate fermentation." Electronic Journal of Biotechnology **1**(3): 26-27.

Reddy, L. V., O. V. S. Reddy and Y.-J. Wee (2011). "Production of ethanol from mango (Mangifera indica L.) peel by Saccharomyces cerevisiae CFTRI101." African Journal of Biotechnology **10**: 4183-4189.

Rees, E. M. and G. G. Stewart (1999). "Effects of magnesium, calcium and wort oxygenation on the fermentative performance of ale and lager strains fermenting normal and high gravity worts." Journal of the Institute of Brewing **105**(4): 211-218.

Richardson, C. and E. Hatfield (1978). "The limiting amino acids in growing cattle." Journal of Animal Science **46**(3): 740-745.

Ritala, A., S. T. Häkkinen, M. Toivari and M. G. Wiebe (2017). "Single cell protein—state-of-the-art, industrial landscape and patents 2001–2016." Frontiers in microbiology **8**: 2009.

Robertson, G. H., D. W. Wong, C. C. Lee, K. Wagschal, M. R. Smith and W. J. Orts (2006). "Native or raw starch digestion: a key step in energy efficient biorefining of grain." Journal of Agricultural and Food Chemistry **54**(2): 353-365.

Rocha, T. d. S., A. P. d. A. Carneiro and C. M. L. Franco (2010). "Effect of enzymatic hydrolysis on some physicochemical properties of root and tuber granular starches." Food Science and Technology **30**(2): 544-551.

Rolland-Sabaté, A., T. Sánchez, A. Buléon, P. Colonna, B. Jaillais, H. Ceballos and D. Dufour (2012). "Structural characterization of novel cassava starches with low and high-amylose contents in comparison with other commercial sources." Food Hydrocolloids **27**: 161-174.

Rooney, L. and R. Pflugfelder (1986). "Factors affecting starch digestibility with special emphasis on sorghum and corn." Journal of animal science **63 5**: 1607-1623.

Rosenfelder, P., M. Eklund and R. Mosenthin (2013). "Nutritive value of wheat and wheat by-products in pig nutrition: A review." Animal Feed Science and Technology **185**(3): 107-125.

Rosset, M., V. R. Acquaro and A. D. P. Beléia (2014). "Protein Extraction from Defatted Soybean Flour with V iscozyme L Pretreatment." Journal of Food Processing and Preservation **38**(3): 784-790.

Roussos, S., M. Raimbault, J.-P. Prebois and B. Lonsane (1993). "Zymotis, a large scale solid state fermenter design and evaluation." Applied Biochemistry and Biotechnology **42**(1): 37-52.

Sangsurasak, P., M. Nopharatana and D. Mitchell (1996). "Mathematical modeling of the growth of filamentous fungi in solid state fermentation." Journal of Scientific and Industrial Research **55**(5-6): 333-342.

Sakdaronnarong, C., S. Sraphet, N. Srisawad, R. Chantasod, W. Jonglertjunya and K. Triwitayakorn (2020). "Rheological characteristics and genotype correlation of cassava root for very high gravity ethanol production: The influence of cassava varieties and harvest times." Biotechnology and Applied Biochemistry **67**(1): 105-116.

Sakdaronnarong, C., N. Srimarut, R. Chantasod, S. Sraphet, K. Triwitayakorn and W. Jonglertjunya (2018). "Enzyme Matching Design Approach on Very High Gravity Liquefaction and Saccharification of Cassava Root and Cassava Starch for Ethanol Fermentation." BioResources **13**(2): 2304-2327.

Sapińska, E., M. Balcerek and K. Pielech-Przybylska (2013). "Alcoholic fermentation of high-gravity corn mashes with the addition of supportive enzymes." Journal of Chemical Technology & Biotechnology **88**(12): 2152-2158.

Sathe, S., S. Deshpande and D. K. Salunkhe (1982). "Functional Properties of Lupin Seed (Lupinus mutabilis) Proteins and Protein Concentrates." Journal of Food Science **47**: 491-497.

Sawai, H. and Y. Morita (1968). "Studies on rice glutelin." Agricultural and Biological Chemistry **32**(1): 76-80.

Schofield, P., D. Mbugua and A. Pell (2001). "Analysis of condensed tannins: a review." Animal Feed Science and Technology **91**: 21-40.

Schubert, C. (2006). "Can biofuels finally take center stage?" Nature biotechnology **24**(7): 777-784.

Schulze, K. (1956). "The effect of phosphate supply on the rate of growth and fat formation in yeasts." Applied microbiology **4**(4): 207.

Sella, S. R. B. R., B. P. Guizelini, L. P. d. S. Vandenberghe, A. B. P. Medeiros and C. R. Soccol (2009). "Lab-Scale production of Bacillus atrophaeus' spores by solid state fermentation in fifferent types of bioreactors." Brazilian Archives of Biology and Technology **52**(SPE): 159-170.

Shadbahr, J., F. Khan and Y. Zhang (2017). "Kinetic modeling and dynamic analysis of simultaneous saccharification and fermentation of cellulose to bioethanol." Energy Conversion and Management **141**: 236-243.

Shanavas, S., G. Padmaja, S. Moorthy, M. Sajeev and J. Sheriff (2011). "Process optimization for bioethanol production from cassava starch using novel eco-friendly enzymes." Biomass and Bioenergy **35**(2): 901-909.

Shariffa, Y., A. Karim, A. Fazilah and I. Zaidul (2009). "Enzymatic hydrolysis of granular native and mildly heat-treated tapioca and sweet potato starches at sub-gelatinization temperature." Food Hydrocolloids **23**(2): 434-440.

Sharma, V., K. D. Rausch, J. V. Graeber, S. J. Schmidt, P. Buriak, M. Tumbleson and V. Singh (2010). "Effect of resistant starch on hydrolysis and fermentation of corn starch for ethanol." Applied biochemistry and biotechnology **160**(3): 800-811.

Shen, L., X. Wang, Z. Wang, Y. Wu and J. Chen (2008). "Studies on tea protein extraction using alkaline and enzyme methods." Food Chemistry **107**(2): 929-938.

Shen, Y., J.-S. Guo, Y.-P. Chen, H.-D. Zhang, X.-X. Zheng, X.-M. Zhang and F.-W. Bai (2012). "Application of low-cost algal nitrogen source feeding in fuel ethanol production using high gravity sweet potato medium." Journal of biotechnology **160**(3-4): 229-235.

Shen, Y., H. D. Zhang, X. X. Zheng, X. M. Zhang, J. S. Guo and Y. P. Chen (2011). "Very high gravity fermentation using sweet potato for fuel ethanol production." Advanced Materials Research, Trans Tech Publ.

Shetty, J. K., O. Lantero and N. Dunn-Coleman (2006). "State–of-the-art Production of Fuel Ethanol using Granular Starch Hydrolyzing Enzymes (GSHE)." https://dc.engconfintl.org/cgi/viewcontent.cgi?article=1001&context=bioenergy_i

Shewry, P. and B. Miflin (1985). "Seed storage proteins of economically important cereals." Advances in Cereal Science and Technology **7**: 1-83

Shi, C., J. He, J. Yu, B. Yu, Z. Huang, X. Mao, P. Zheng and D. Chen (2015). "Solid state fermentation of rapeseed cake with Aspergillus niger for degrading glucosinolates and upgrading nutritional value." Journal of animal science and biotechnology **6**(1): 13.

Shoja, A., E. Hamid, P. Hejazi, F. Vasheghani and A. Rinzema (2007). "Evaluation of strategies for temperature and moisture control in solid state packed bed bioreactors." Iranian Journal of Biotechnology: 219-225.

Siada, O. A. A., M. Negm, M. Basiouny, M. Fouad and S. Elagroudy (2018). "Protein enrichment of agro–industrial waste by trichoderma harzianum EMCC 540 through solid state fermentation for use as animal feed." J. Geogr. Environ. Earth Sci. Int. **13**(4): 1-12.

Sievert, D. and Y. Pomeranz (1990). "Enzyme-resistant starch. II, Differential scanning calorimetry studies on heat-treated starches and enzyme-resistant starch residues." Cereal Chemistry **67**: 217-221.

Silva, E. M. S., A. Peres, A. C. Silva, M. C. B. D. M. Leal, L. M. Lião and V. O. Almeida (2019). "Sorghum starch as depressant in mineral flotation: part 1 - extraction and characterization." Journal of materials research and technology **8**: 396-402.

Singh, A., M. Shahid, M. Srivastava, S. Pandey, A. Sharma and V. Kumar (2014). "Optimal physical parameters for growth of Trichoderma species at varying pH, temperature and agitation." Virology and Mycology **3**(1): 127-134.

Singhania, R. R., A. K. Patel, C. R. Soccol and A. Pandey (2009). "Recent advances in solid-state fermentation." Biochemical Engineering Journal **44**(1): 13-18.

Singleton, G. (2003). Impacts of rodents on rice production in Asia (No. 2169-2019-1613).

Şişman, T., Ö. Gür, N. Doğan, M. Özdal, Ö. F. Algur and T. Ergon (2013). "Single-cell protein as an alternative food for zebrafish, Danio rerio: a toxicological assessment." Toxicology and industrial health **29**(9): 792-799.

Sivakumar, G., D. R. Vail, J. Xu, D. M. Burner, J. O. Lay, X. Ge and P. J. Weathers (2010). "Bioethanol and biodiesel: Alternative liquid fuels for future generations." Engineering in Life Sciences **10**(1): 8-18.

Slaughter, S. L., P. R. Ellis and P. J. Butterworth (2001). "An investigation of the action of porcine pancreatic α-amylase on native and gelatinised starches." Biochimica et Biophysica Acta (BBA)-General Subjects **1525**(1-2): 29-36.

Snow, P. and K. O'dea (1981). "Factors affecting the rate of hydrolysis of starch in food." The American journal of clinical nutrition **34 12**: 2721-2727.

Solomon, B. D., J. R. Barnes and K. E. Halvorsen (2007). "Grain and cellulosic ethanol: History, economics, and energy policy." Biomass and Bioenergy **31**(6): 416-425.

Sørensen, S. O., M. Pauly, M. Bush, M. Skjøt, M. McCann, B. Borkhardt and P. Ulvskov (2000). "Pectin engineering: modification of potato pectin by in vivo expression of an endo-1,4-beta-D-galactanase." Proceedings of the National Academy of Sciences of the United States of America **97 13**: 7639-7644.

Spiehs, M., M. Whitney and G. C. Shurson (2002). "Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota." Journal of animal science **80**(10): 2639-2645.

Spier, M. R., L. Vandenberghe, A. B. P. Medeiros and C. R. Soccol (2011). "Application of different types of bioreactors in bioprocesses." Bioreactors: design, properties and applications. Nova Science Publishers Inc: New York: 55-90.

Srichuwong, S., M. Fujiwara, X. Wang, T. Seyama, R. Shiroma, M. Arakane, N. Mukojima and K. Tokuyasu (2009). "Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol." Biomass and bioenergy **33**(5): 890-898.

Srichuwong, S., T. Orikasa, J. Matsuki, T. Shiina, T. Kobayashi and K. Tokuyasu (2012). "Sweet potato having a low temperature-gelatinizing starch as a promising feedstock for bioethanol production." Biomass and Bioenergy **39**: 120-127.

Srikanta, S., S. Jaleel, N. Ghildyal and B. Lonsane (1992). "Techno-economic feasibility of ethanol production from fresh cassava tubers in comparison to dry cassava chips." Food/Nahrung **36**(3): 253-258.

Sriroth, K., K. Piyachomkwan, S. Wanlapatit and S. Nivitchanyong (2010). "The promise of a technology revolution in cassava bioethanol: From Thai practice to the world practice." Fuel **89**(7): 1333-1338.

Sriroth, K., K. Piyachomkwan, S. Wanlapatit, K. Thitipraphunkul and M. Laddee (2006). "Study on utilization of by-products from ethanol process for value addition." The Department of Alternative Energy Development and Efficiency, Ministry of Energy. Cassava and Starch Technology Research Unit. Retrieve from http://cassava.org.

Sriroth, K., V. Vichukit, E. Sarobol, K. Piyachomkwan, S. Wanlapatit, K. Amornitikul, N. Termvejsayanon and B. Lamchaiyaphum (2007). "Development of raw material management plan for ethanol industry in Thailand." Kasetsart University-Thailand.

Staniszewski, M., W. Kujawski and M. Lewandowska (2007). "Ethanol production from whey in bioreactor with co-immobilized enzyme and yeast cells followed by pervaporative recovery of product–Kinetic model predictions." Journal of Food Engineering **82**(4): 618-625.

Stewart, G., T. D'Amore, C. Panchal and I. Russell (1988). "Factors that influence the ethanol tolerance of brewer's yeast strains during high gravity wort fermentations." Technical quarterly-Master Brewers Association of the Americas (USA).

Strąk-Graczyk, E. and M. Balcerek (2020). "Effect of Pre-hydrolysis on Simultaneous Saccharification and Fermentation of Native Rye Starch." Food and Bioprocess Technology: 1-14.

Strąk-Graczyk, E., M. Balcerek and K. Przybylsk (2019). "Simultaneous saccharification and fermentation of native rye, wheat and triticale starch." Journal of the science of food and agriculture.

Sugimoto, T., T. Makita, K. Watanabe and H. Shoji (2012). "Production of multiple extracellular enzyme activities by novel submerged culture of Aspergillus kawachii for ethanol production from raw cassava flour." Journal of industrial microbiology & biotechnology **39**(4): 605-612.

Sujka, M. and J. Jamróz (2010). "Characteristics of pores in native and hydrolyzed starch granules." Starch-starke **62**: 229-235.

Sun, H., P. Zhao, X. Ge, Y. Xia, Z. Hao, J. Liu and M. Peng (2010). "Recent advances in microbial raw starch degrading enzymes." Applied biochemistry and biotechnology **160**(4): 988-1003.

Suryaningsih, R. and Irhas (2014). "Bioenergy Plants in Indonesia: Sorghum for Producing Bioethanol as an Alternative Energy Substitute of Fossil Fuels." Energy Procedia **47**: 211-216.

Tan, K. T., K. T. Lee and A. R. Mohamed (2008). "Role of energy policy in renewable energy accomplishment: the case of second-generation bioethanol." Energy policy **36**(9): 3360-3365.

Tang, D.-S., G.-M. Yin, Y.-Z. He, S.-Q. Hu, B. Li, L. Li, H.-L. Liang and D. Borthakur (2009). "Recovery of protein from brewer's spent grain by ultrafiltration." Biochemical Engineering Journal **48**(1): 1-5.

Taranu, I., T.-T. Nguyen, K.-D. Pham, M. A. Gras, G. C. Pistol, D. E. Marin, C. Rotar, M. Habeanu, P.-H. Ho, T.-M. Le, T. T. H. Bui, D. V. Mai and S. Chu-ky (2019). "Rice and Cassava Distillers Dried Grains in Vietnam: Nutritional Values and Effects of Their Dietary Inclusion on Blood Chemical Parameters and Immune Responses of Growing Pigs." Waste and Biomass Valorization **10**(11): 3373-3382.

Taylor, N. and J. Cluskey (1962). "Wheat gluten and its glutenin component: Viscosity, diffusion and sedimentation studies." Archives of biochemistry and biophysics **97**(2): 399-405.

Tester, R., X. Qi and J. Karkalas (2006). "Hydrolysis of native starches with amylases." Animal Feed Science and Technology **130**: 39-54.

Thatipamala, R., S. Rohani and G. Hill (1992). "Effects of high product and substrate inhibitions on the kinetics and biomass and product yields during ethanol batch fermentation." Biotechnology and bioengineering **40**(2): 289-297.

Thomas, K., S. Hynes and W. Ingledew (1996). "Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation." Process Biochemistry **31**(4): 321-331.

Thomas, K. and W. Ingledew (1995). "Production of fuel alcohol from oats by fermentation." Journal of industrial microbiology **15**(2): 125-130.

Thomas, K. C. and W. Ingledew (1990). "Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes." Applied and environmental Microbiology **56**(7): 2046-2050.

Thomas, K. C. and W. Ingledew (1992). "Relationship of low lysine and high arginine concentrations to efficient ethanolic fermentation of wheat mash." Canadian journal of microbiology **38 7**: 626-634.

Thomas, K. C. and W. Ingledew (2005). "Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes." Journal of Industrial Microbiology **10**: 61-68.

Tomasik, P. and D. Horton (2012). Enzymatic conversions of starch. Advances in carbohydrate chemistry and biochemistry, Elsevier. **68:** 59-436.

Torbatinejad, N. M., S. M. Rutherfurd and P. J. Moughan (2005). "Total and Reactive Lysine Contents in Selected Cereal-Based Food Products." Journal of Agricultural and Food Chemistry **53**(11): 4454-4458.

Toscano, L., G. Montero, L. Cervantes, M. Stoytcheva, V. Gochev and M. Beltrán (2013). "Production and partial characterization of extracellular lipase from Trichoderma harzianum by solid-state fermentation." Biotechnology & Biotechnological Equipment **27**(3): 3776-3781.

Trakarnpaiboon, S., N. Srisuk, K. Piyachomkwan, S.-T. Yang and V. Kitpreechavanich (2017). "L-Lactic acid production from liquefied cassava starch by thermotolerant Rhizopus microsporus: Characterization and optimization." Process Biochemistry **63**: 26-34.

Treimo, J., S. I. Aspmo, V. G. Eijsink and S. J. Horn (2008). "Enzymatic solubilization of proteins in brewer's spent grain." Journal of agricultural and food chemistry **56**(13): 5359-5365.

Tu-Viet, P., D. Pham-Kim, O. Nguyen-Cong and S. Chu-Ky (2016). "Assessment of potential use of alcohol by-products for animal feed production in Vietnam." Journal of Science and Development (Vietnam) **14**(1): 36-45.

Tumuluru, J. S., C. C. Conner and A. N. Hoover (2016). "Method to produce durable pellets at lower energy consumption using high moisture corn stover and a corn starch binder in a flat die pellet mill." JoVE (Journal of Visualized Experiments)(112): e54092.

Udeh, H., T. Kgatla and A. Jideani (2014). "Effect of mineral ion addition on yeast performance during very high gravity wort fermentation." International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering **8**(11): 1208-1216.

Ueda, S., C. T. Zenin, D. A. Monteiro and Y. K. Park (1981). "Production of ethanol from raw cassava starch by a nonconventional fermentation method." Biotechnology and Bioengineering **23**(2): 291-299.

Ugalde, U. and J. Castrillo (2002). Single cell proteins from fungi and yeasts. Applied mycology and biotechnology, Elsevier. **2:** 123-149.

Ugwuanyi, J. O., L. M. Harvey and B. McNeil (2008). "Protein enrichment of corn cob heteroxylan waste slurry by thermophilic aerobic digestion using Bacillusstearothermophilus." Bioresource technology **99**(15): 6974-6985.

Umsza-Guez, M. A., A. B. Díaz, I. d. Ory, A. Blandino, E. Gomes and I. Caro (2011). "Xylanase production by Aspergillus awamori under solid state fermentation conditions on tomato pomace." Brazilian Journal of Microbiology **42**(4): 1585-1597.

Uribe, S. and J. G. Sampedro (2003). "Measuring solution viscosity and its effect on enzyme activity." Biological procedures online **5**(1): 108-115.

Uthumporn, U., Y. N. Shariffa and A. Karim (2011). "Hydrolysis of Native and Heat-Treated Starches at Sub-Gelatinization Temperature Using Granular Starch Hydrolyzing Enzyme." Applied Biochemistry and Biotechnology **166**: 1167-1182.

Van Beynum, G. M. A. and J. Roels (1985). "Starch conversion technology." Marcel Dekker Inc., New York

Vergara-Barberán, M., M. Lerma-García, J. Herrero-Martínez and E. Simó-Alfonso (2015). "Use of an enzyme-assisted method to improve protein extraction from olive leaves." Food chemistry **169**: 28-33.
Viet, T. Q., V. N. Trung, D. Van Cai and N. T. Van (2014). "Nutrition, feeds and feeding for pig production in Vietnam: Current status and future research—A review." Nairobi, Kenya: ILRI.
Villas-Bôas, S. G., E. Esposito and D. A. Mitchell (2002). "Microbial conversion of lignocellulosic residues for production of animal feeds." Animal Feed Science and Technology **98**(1-2): 1-12.
Villegas-Torres, M., J. Ward and G. Lye (2015). "The protein fraction from wheat-based dried distiller's grain with solubles (DDGS): extraction and valorization." New biotechnology **32**(6): 606-611.
Vohra, M., J. Manwar, R. Manmode, S. Padgilwar and S. Patil (2014). "Bioethanol production: Feedstock and current technologies." Journal of Environmental Chemical Engineering **2**(1): 573-584.
Vong, W. C., X. Y. Hua and S.-Q. Liu (2018). "Solid-state fermentation with Rhizopus oligosporus and Yarrowia lipolytica improved nutritional and flavour properties of okara." LWT **90**: 316-322.
Wada, M., J. Kato and I. Chibata (1979). "A new immobilization of microbial cells." European journal of applied microbiology and biotechnology **8**(4): 241-247.
Walker, G. J. and P. M. Hope (1963). "The action of some α-amylases on starch granules." Biochemical Journal **86**(3): 452-462.
Wang, P., V. Singh, H. Xue, D. B. Johnston, K. D. Rausch and M. Tumbleson (2007). "Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry-grind corn processing." Cereal Chemistry **84**(1): 10-14.
Wang, W., H. Wei, M. Alahuhta, X. Chen, D. Hyman, D. K. Johnson, M. Zhang and M. E. Himmel (2014). "Heterologous expression of xylanase enzymes in lipogenic yeast Yarrowia lipolytica." PLoS One **9**(12): e111443.
Wang, X., W. Gao, J. Zhang, H. Zhang, J. Li, X. He and H. Ma (2010). "Subunit, amino acid composition and in vitro digestibility of protein isolates from Chinese kabuli and desi chickpea (Cicer arietinum L.) cultivars." Food Research International **43**(2): 567-572.
Wang, Y., M. Tilley, S. Bean, X. S. Sun and D. Wang (2009). "Comparison of methods for extracting kafirin proteins from sorghum distillers dried grains with solubles." Journal of agricultural and food chemistry **57**(18): 8366-8372.
Watanabe, T., S. Srichuwong, M. Arakane, S. Tamiya, M. Yoshinaga, I. Watanabe, M. Yamamoto, A. Ando, K. Tokuyasu and T. Nakamura (2010). "Selection of stress-tolerant yeasts for simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash to ethanol." Bioresource technology **101**(24): 9710-9714.
Wee, L. L., M. Annuar, S. Ibrahim and Y. Chisti (2011). "Enzyme-mediated production of sugars from sago starch: statistical process optimization." Chemical Engineering Communications **198**(11): 1339-1353.
Wolf, W. J. and J. W. Lawton Jr (1997). "Isolation and characterization of zein from corn distillers' grains and related fractions." Cereal chemistry **74**(5): 530-536.
Wu, X., R. Zhao, S. R. Bean, P. A. Seib, J. S. McLaren, R. L. Madl, M. Tuinstra, M. C. Lenz and D. Wang (2007). "Factors Impacting Ethanol Production from Grain Sorghum in the Dry-Grind Process." Cereal Chemistry **84**(2): 130-136.
Wu, Y., X. Li, W. Xiang, C. Zhu, Z. Lin, Y. Wu, J. Li, S. Pandravada, D. D. Ridder, G. Bai, M. L. Wang, H. N. Trick, S. R. Bean, M. R. Tuinstra, T. T. Tesso and J. Yu (2012).

"Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin." Proceedings of the National Academy of Sciences **109**(26): 10281-10286.

Xia, N., J.-M. Wang, Q. Gong, X.-Q. Yang, S.-W. Yin and J.-R. Qi (2012). "Characterization and In Vitro digestibility of rice protein prepared by enzyme-assisted microfluidization: Comparison to alkaline extraction." Journal of Cereal Science **56**(2): 482-489.

Xu, H. and G. Duan (2010). "Effect of temperature on the no cook, very high gravity ethanol fermentation process." Sheng wu gong cheng xue bao= Chinese journal of biotechnology **26**(3): 330-334.

Xu, Q.-S., Y.-S. Yan and J.-X. Feng (2016). "Efficient hydrolysis of raw starch and ethanol fermentation: a novel raw starch-digesting glucoamylase from Penicillium oxalicum." Biotechnology for biofuels **9**(1): 216.

Yan, J., B. Han, X. Gui, G. Wang, L. Xu, Y. Yan, C. Madzak, D. Pan, Y. Wang and G. Zha (2018). "Engineering Yarrowia lipolytica to simultaneously produce lipase and single cell protein from agro-industrial wastes for feed." Scientific reports **8**(1): 1-10.

Yang, S., A. Durand and H. Blachere (1986). "Protein enrichment of sugar beet residue with conidia of Trichoderma album by solid state fermentation." Chinese Journal of Microbiology and Immunology **19**: 11-22.

Yan, S., X. Wu, S. R. Bean, J. F. Pedersen, T. Tesso, Y. R. Chen and D. Wang (2011). "Evaluation of waxy grain sorghum for ethanol production." Cereal chemistry **88**(6): 589-595.

Yang, L., J.-H. Chen, H. Zhang, W. Qiu, Q.-H. Liu, X. Peng, Y.-N. Li and H.-K. Yang (2012). "Alkali treatment affects in vitro digestibility and bile acid binding activity of rice protein due to varying its ratio of arginine to lysine." Food chemistry **132**(2): 925-930.

Yang, S.-S., A. Durand and H. Blachere (1986). "Protein Enrichment of Sugar Beet Residue with Conidia of Trichoderma Album by Solid State Fermentation." Chinese J. Microbiol. Immunol. **19**.

Yang, S. S. (1988). "Protein enrichment of sweet potato residue with amylolytic yeasts by solid-state fermentation." Biotechnology and bioengineering **32**(7): 886-890.

Yingling, B., Y. Zongcheng, W. Honglin and C. Li (2011). "Optimization of bioethanol production during simultaneous saccharification and fermentation in very high-gravity cassava mash." Antonie van Leeuwenhoek **99**(2): 329-339.

Yong, T., L. Sixin, X. Haibo, L. Kaimian and L. Congfa (2011). Process optimization for high gravity ethanol fermentation of cassava flour. 2011 International Conference on Materials for Renewable Energy & Environment, IEEE.

Yue, G., J. Yu, X. Zhang and T. Tan (2012). "The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice." Biomass and bioenergy **39**: 48-52.

Zabed, H., G. Faruq, J. N. Sahu, M. S. Azirun, R. Hashim and A. Nasrulhaq Boyce (2014). "Bioethanol production from fermentable sugar juice." The Scientific World Journal **2014**.

Zabed, H., J. Sahu, A. Suely, A. Boyce and G. Faruq (2017). "Bioethanol production from renewable sources: Current perspectives and technological progress." Renewable and Sustainable Energy Reviews **71**: 475-501.

Zaldivar, J., J. Nielsen and L. Olsson (2001). "Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration." Applied microbiology and biotechnology **56**(1-2): 17-34.

Zhai, C., C. M. Lu, X. Zhang, G. J. Sun and K. Lorenz (2001). "Comparative Study on Nutritional Value of Chinese and North American Wild Rice." Journal of Food Composition and Analysis **14**: 371-382.

Zhang, J. and Q. Yang (2015). "Optimization of solid-state fermentation conditions for Trichoderma harzianum using an orthogonal test." Genetics and Molecular Research **14**(1): 1771-1781.

Zhang, L., Q. Chen, Y. Jin, H. Xue, J. Guan, Z. Wang and H. Zhao (2010). "Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG)." Fuel Processing Technology **91**(12): 1845-1850.

Zhang, L., H. Zhao, M. Gan, Y. Jin, X. Gao, Q. Chen, J. Guan and Z. Wang (2011). "Application of simultaneous saccharification and fermentation (SSF) from viscosity reducing of raw sweet potato for bioethanol production at laboratory, pilot and industrial scales." Bioresource technology **102**(6): 4573-4579.

Zhang, X., H. Mo and Z. Li (2003). "A solid-state bioreactor coupled with forced aeration and pressure oscillation." Biotechnology letters **25**(5): 417-420.

Zhang, Z. Y., B. Jin, Z. H. Bai and X. Y. Wang (2008). "Production of fungal biomass protein using microfungi from winery wastewater treatment." Bioresource Technology **99**(9): 3871-3876.

Zhao, L., X. Zhang, J. Xu, X. Ou, S. Chang and M. Wu (2015). "Techno-economic analysis of bioethanol production from lignocellulosic biomass in China: Dilute-acid pretreatment and enzymatic hydrolysis of corn stover." Energies **8**(5): 4096-4117.

Zheng, D., K. Zhang, K. Gao, Z. Liu, X. Zhang, O. Li, J. Sun, X. Zhang, F. Du and P. Sun (2013). "Construction of novel Saccharomyces cerevisiae strains for bioethanol active dry yeast (ADY) production." PLoS One **8**(12).

Zhou, Z., K. Robards, S. Helliwell and C. Blanchard (2002). "Composition and functional properties of rice." International Journal of Food Science & Technology **37**(8): 849-868.

Zobel, H. F. (1984). "Gelatinization of starch and mechanical properties of starch pastes." Starch: Chemistry and technology. Elsevier**:** 285-309.

PUBLICATION

1. **Mai, D. V**., Nguyen, T. T., Chu-Ky, S., and Waché, Y. (2020). "Protein Enrichment of Cassava-Based Dried Distiller's Grain by Solid State Fermentation Using Trichoderma Harzianum and Yarrowia Lipolytica for Feed Ingredients." Waste and Biomass Valorization: 1-14.

2. Waché, Y., Do, T. L., Do, T. B. H., Do, T. Y., Haure, M., Ho, P. H., Anal, K. A., Le, V. V. M., Li, W. J., Licandro, H., Lorn, D., Ly-Chantain, M, H., Ly, S., Mahakarnchanakul, W., **Mai, D. V.**, Minh, H, Nguyen, H. D., Nguyen, T. K. C., Nguyen, T. M. T., Nguyen, T. T. T., Nguyen, V. A., Pham, H. V., Pham, T. A., Phan, T. T., Tan, R., Tien, T. N., Tran, Therry., Try, S., Phi, Q. T., Valentin, D., Vo, V. Q. B., Vongkamjan, K., Vu, D. C., Vu, N. T and Chu-Ky, S. (2018). "Prospects for food fermentation in South-East Asia, topics from the tropical fermentation and biotechnology network at the end of the AsiFood Erasmus+ Project." Frontiers in microbiology **9**(2278).

3. Taranu, I., Nguyen, T. T., Pham, K. D., Gras, M. A., Pistol, G. C., Marin, D. E., Rotar, C., Habeanu, M., Ho, P. H., Le, T. M., Bui, T. T. H., **Mai, D. V**., Chu-ky, S**.** (2019). "Rice and cassava distillers dried grains in Vietnam: nutritional values and effects of their dietary inclusion on blood chemical parameters and immune responses of growing pigs." Waste and Biomass Valorization **10**(11): 3373-3382.

POSTER AND PUBLIC PRESENTATTION

1. Journée des doctorants UMR-PAM 2017 - Oral presentation «Optimization of the Simultaneous Liquefaction, Saccharification and Fermentation process at Very High Gravity for ethanol production and Valorization of ethanol by-products for animal feeds»
2. Congrès SFM - Société Française de Microbiologie 2018 – Poster "Protein enrichment of Cassava-based dried distiller's grain by Solid State Fermentation using Trichoderma harzianum and Yarrowia lipolytica"
3. Forum des Jeunes Chercheurs 2018 – Oral presentation "Protein enrichment of Cassava dry distillers' grains by solid state fermentation using Trichoderma harzianum for animal feed"

4. Journée des doctorants UMR-PAM 2019 – Poster “Protein enrichment of cassava-based dried distiller’s grain by Solid State Fermentation using Trichoderma harzianum and Yarrowia lipolytica for feed ingredients”