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Production of Single Cell Proteins (SCP) by *Cupriavidus necator* : Impact of environmental parameters, carbon and nitrogen sources.

Siwar Ismail

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THÈSE

**En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**
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Toulouse

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Siwar ISMAIL**

Le 12 avril 2022

**Production des Protéines d'Organismes Unicellulaires (POU) par
Cupriavidus necator : Impact des paramètres environnementaux,
des sources de carbone et d'azote.**

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Thèse dirigée par

Nathalie GORRET et Stéphane GUILLOUET

Jury

M. Stéphane DELAUNAY, Rapporteur

M. Fadhel BEN CHAABANE, Rapporteur

M. Mokhtar HAMDY, Rapporteur

M. Pierre-Yves PONTALIER, Examineur

Mme Agnès HOCQUELLET, Examinatrice

Mme Nathalie GORRET, Directrice de thèse

M. Stéphane GUILLOUET, Co-directeur de thèse

M. Claude MARANGES, Président

Nom : ISMAIL

Prénom : Siwar

Titre : Production des Protéines d'Organismes Unicellulaires (POU) par *Cupriavidus necator* : Impact des paramètres environnementaux, des sources de carbone et d'azote.

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Résumé

En raison de l'augmentation rapide de la population mondiale, de nombreux pays en cours de développement sont concernés par des problèmes de malnutrition (famine, insécurité alimentaire, etc.). En particulier, l'insuffisance en sources de protéines devient un problème majeur pour la nutrition humaine et animale et leur demande augmente de plus en plus. Dans ce contexte, les Protéines d'Organismes Unicellulaires (POU) produites par des micro-organismes peuvent être exploitées comme une source de protéines alternative et non conventionnelle. L'objectif de ces travaux était d'étudier les dynamiques de croissance et de production de POU ainsi que leur composition par *Cupriavidus necator*, en réponse à des conditions environnementales différentes et en utilisant différents types de sources de carbone (glucose, acide formique et acide oléique) et d'azote (sulfate d'ammonium et urée). Une approche mono-factorielle en condition d'environnement contrôlé a été utilisée afin d'étudier l'impact de la température, du pH, des sources de carbone et d'azote dans des cultures en bioréacteurs. Les résultats ont été comparés en termes de croissance bactérienne, de composition de la biomasse (en protéines et en acides nucléiques) et via la composition élémentaire de la biomasse. Des analyses complémentaires ont également été effectuées par cytométrie en flux afin de suivre la morphologie cellulaire, la perméabilité membranaire, et la présence potentielle de PHB, bio polymère naturellement produit par *C. necator*. Les résultats concernant la température ont montré que ce paramètre avait un impact sur la croissance ainsi que sur la production et la composition des POU. A 40°C, la croissance était plus faible et de faibles quantités de protéines et d'acides nucléiques ont été obtenues. La même conclusion a été obtenue à pH5. Concernant la nature de la source d'azote, le sulfate d'ammonium a donné les meilleurs résultats, comparé à l'urée, comme source d'azote. De plus, l'acide formique s'est avéré être une source de carbone prometteuse pour la production de protéines comparé au glucose et à l'acide oléique avec un pourcentage en protéines totales élevé autour de 90%_{DW} (mesuré par la méthode de Dumas).

Mots clés : *Cupriavidus necator*, protéines, acides nucléiques, température, pH, source d'azote, source de carbone

Last-name: ISMAIL

First-name: Siwar

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Abstract

Due to the fast increase in world population, many developing countries are facing malnutrition problems (famine, food insecurity, etc.). Particularly, the deficiency of protein sources is becoming a serious problem for both human and animals' nutrition. Thus, the demand for protein sources has increased. In this context, Single Cell Proteins (SCP) produced by microorganisms could be exploited as an alternative and non-conventional protein source. The aim of this work was to study growth dynamics and SCP production and composition by *Cupriavidus necator*, under various environmental conditions stress and using different carbon (glucose, formic acid and oleic acid) and nitrogen (ammonium sulfate and urea) sources types. A mono-factorial approach under well-controlled conditions was used in order to investigate the impact of temperature, pH, carbon and nitrogen sources in bioreactor cultures. Results were compared in terms of bacterial growth, biomass composition (proteins and nucleic acids) and via the elemental biomass composition. Complementary analyses were also done using flow cytometry in order to study cell morphology, membrane permeability and presence of PHB production, a biopolymer naturally produced by *C. necator*. The temperature evaluation results showed that this parameter had an impact on growth and SCP production and composition. At 40°C, the growth was lower and low amounts of proteins, and nucleic acids were obtained. The same conclusion was obtained at pH5. Regarding the nitrogen source, the ammonium sulfate provided better results than urea as a nitrogen source. Added to that, the formic acid was found as a promising carbon source for protein production compared to glucose and oleic acid with a high total protein percentage of 90%_{DW} (obtained with the Dumas's method).

Keywords: *Cupriavidus necator*, microbial protein, nucleic acids, temperature, pH, nitrogen source, carbon source

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COMMUNICATIONS

➤ Poster presentation

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μ	Specific growth rate (h^{-1})
μ_{max}	Maximal specific growth rate (h^{-1})
AA	Amino Acid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
d	Unit for day
CDW	Cell Dry Weight
CHONS	Carbon, hydrogen, oxygen, nitrogen, sulfur
Cys	Cysteine
D	Dilution
DNA	Deoxyribonucleic acid
DW	Cell Dry Weight
FAO	Food and Agriculture Organization
FSC	Forward Scatter Signal
g	Unit for gram
h	Unit for hour
HPLC	high performance liquid chromatography
HPIC	High Performance Ionic Chromatography
Ile	Isoleucine
IPA	Isopropanol
l	Unit for liter
Leu	Leucine
Lys	Lysine
min	Unit for minute
Met	Methionine
NA	Not available
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NR	Nile Red
OD	Optical density
pH	Potential of hydrogen
PHA	PolyHydroxyAlkanoates
PHB	Poly- β -hydroxybutyrate
Phe	Phenylalanine
PI	Propidium Iodide
$R_{A,B}$	Production of yield of the compound A from the compound B
RNA	Ribonucleic acid
SCP	Single Cell Protein
sec	Unit for second
SSC	Side Scatter Signal
Thr	Tyrosine
Trp	Tryptophan
TSB	Tryptic Soy Broth
Tyr	Tyrosine
Val	Valine

Introduction and context of the study

Introduction and context of the study

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Due to the fast increase in world population, from 2.6 to 7 billion in last six decades [1] and with an estimation of 9.5 by 2050 [2], many developing countries are facing famine, food insecurity, malnutrition and other diseases [3]. In particular, deficiency of protein is becoming a major problem for human and animals [4]. Indeed, several amino acids found in proteins are essential and cannot be replaced since they are necessary to build up new structural, functional and essential protein [5]. Thus, the demand for protein sources has increased [6]. Between 1960 and 2011, the protein consumption passed from 65 to 80 g/capital/day [2].

Food industries may not be able to meet with the high demands for protein and this is due to the depletion of natural resources, seasonality, plant diseases, climatic contingencies and the low amount of these compounds in available resources [7]. The amount of protein in milk is usually 25 %, while that in soybean is about 35% and in meat about 45% [8]. Therefore, Scientists and Industrials are searching to supplement the available sources of protein with a non-conventional, new and alternative protein sources with interesting amino acids composition combined to a sustainable production [9].

Thanks to biotechnology, microbes are exploited as new sources of protein named “Single Cell Protein” (SCP) [6]. This approach has multiple features, including its independence to seasonal and geographic factors, its high productivity and efficiency in substrate conversion and also the wide variety of raw materials, microorganisms and culture methodologies that can be utilized for these purposes [10]. The term “Single Cell Protein” was introduced in the 1960s to replace “microbial protein” and “petro protein”, giving it a more suitable image [11]. It refers to dried cell extracted from cell culture and used especially in human food or animal feed [3, 12].

Research studies detailed in this PhD manuscript concerns a characterization of the microbial growth dynamics and Single Cell Protein production in response to environmental stress and using different substrate. A bacterium, *Cupriavidus necator*, was used in this aim. The experimental strategy is presented in Figure 1.

The first step consisted in detailing the state of the art in this field. The objective of this part was to define Single Cell Proteins and to list the culture strategies and the factors which can influence their production and composition including environmental parameters, substrate type and microorganism's type. *Cupriavidus necator*, the bacterium used in this thesis, was also presented regarding its biotechnological applications especially its ability to produce high amounts of proteins.

Introduction and context of the study

From the state of the art, it has been decided to focus our study in the investigation of the impact of temperature, pH and the nature of N- and C-sources on SCP production in the bacterium *Cupriavidus necator*.

Considering this first part, cultivations in bioreactor were performed with mono factorial approach. Under well controlled conditions, cultivations were performed using different temperatures values, different pH values and different nitrogen and carbon sources (Figure 1).

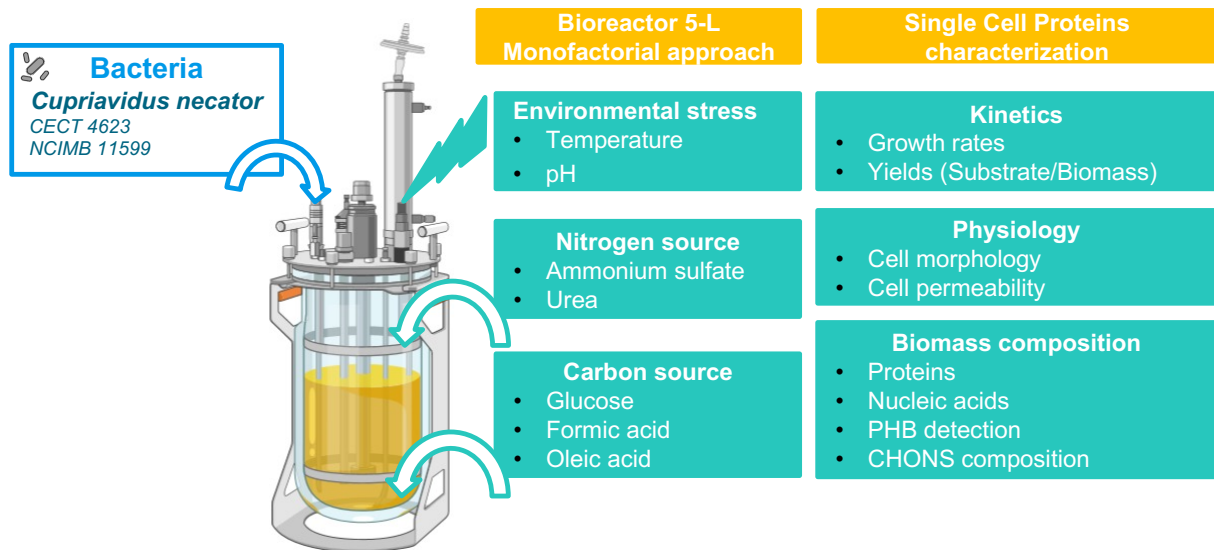


Figure 1 : Experimental strategy of the PhD

The thesis manuscript is presented in four parts:

The first part is dedicated to the literature review. The Single Cell Protein production was presented describing their sources, substrates, compositions, advantages, disadvantages and the factors influencing both their production and their composition. A presentation of the bacterium *Cupriavidus necator* and its biotechnological applications was also presented.

The second part concerns the description of material and methods used during my thesis experiments in order to characterize the SCP production in *C. necator*.

The third part detailed and discussed the experimental results. It is divided into five chapters.

Chapter 1 concerns an optimization of the extraction and quantification of proteins and nucleic acids. On one hand, it presents a comparison between different lysis methods and evaluate their efficiency. On the other hand, it evaluated the quantification methods using different parameters.

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Chapters 2, 3, 4, 5 focused on the characterization of cell growth, morphology and also biomass composition using different temperatures (25, 30, 35 and 40°C), different pH (5, 6, 7 and 8), different nitrogen sources (ammonium sulfate and urea) and different carbon sources (glucose, formic acid and oleic acid), respectively.

Finally, **the last part is devoted to a general conclusion and perspectives.**

Introduction and context of the study

Part I - Literature review

Part I - Literature review

This first part of the manuscript presents literature review:

Single Cell Proteins are defined as dried cell recovered from microbial cell culture of yeasts, bacteria, filamentous fungi or algae, which could be used as ingredients or rich protein-supplements in animal feed and human food [3, 6, 12].

In this context, this part is divided into three sub-chapters.

The first sub-chapter concerns Single Cells Proteins. Indeed, different media (synthetic medium, media derived from agricultural- or industrial by-products) and strategies (i.e., batch, fed batch or continuous modes) can be used depending on objectives. Microbial biomasses contain high amounts of proteins and other intracellular valuable compounds such as fats, carbohydrates, minerals, carotenoids and vitamins. This chemical composition depends on strains, medium composition, culture conditions and downstream process. Many techniques are used to reduce or suppress some drawbacks and make the microbial biomass acceptable and safe for consumption.

The second sub-chapter introduces the strain used in this work, *Cupriavidus necator*, their characteristics especially their biotechnological applications.

Finally, the third sub-chapter presents some conclusions considering the literature and also the objectives of the PhD work.

Chapter I-1: The Single Cell Protein (SCP)

1. The microorganisms for SCP

Many microorganisms can be used for SCP production including, algae, bacteria, molds and yeasts [9].

Mainly bacterial phototrophic strains are recommended as single cell protein sources [13-15], such as *Rhodospseudomonas* [16, 17] , *Rhodobacter* [18] , *Rhodovulum* [19, 20] strains. However, non-photosynthetic bacteria have also been implemented for SCPs production such as *Bacillus* [21], *Cellulomonas* [22], *Streptomyces* [23], *Haloarcula* [24] and *Cupriavidus necator* [25].

Yeasts were used first as an important food supplement. During World War I, German scientists implemented cultures of *Saccharomyces cerevisiae* for SCP production which were consumed in soups and sausages. This group has also established the culture of *Candida arboorea* and *Candida utilis* during World War II as an alternative to foods [26]. Many strains such as *Saccharomyces cerevisiae* with particular characteristics are used to fulfil the objectives of baking and fermentation industries [26].

Added to that, some filamentous fungi strains such as *Fusarium* and *Rhizopus* groups were used during the World War II as a protein source [27, 28].

In East Asian and Central Africa, algae are used as a part of the food diet. *Chlorella*, *Scenedesmus* and *Spirulina*, have been found as the most appropriate species for biomass production for nutritional use [29]. *Spirulina*, the most used algae, has been used for long time as a supplement in protein diet [30].

To choose the relevant microorganism, some criteria should be taken into account: nature of substrate used, performances which includes productivity, yields and growth rate; tolerance to culture conditions; stability and growth during the fermentation and protein properties in the final microbial biomass [7].

2. Substrates for SCP production

2.1. Defined chemical medium

A synthetic medium is composed of a carbon source, a nitrogen source, mineral salts and other microelements and vitamins with an adjusted pH. The defined chemical medium was one of the media type used for Single Cell Protein production [1, 31-35].

2.2. Waste-based substrates

Versatility of some microorganisms toward substrates allows them to uptake low-cost raw material and wastes to produce microbial biomass [27, 36].

The use of biodegradable wastes as substrates for the production of value added products via biotechnological processes can reduce the cultivation costs, their environment effect and can also offer another source of protein [3, 37]. Nevertheless, in order to get access to the metabolizable carbon sources, the use of such substrates requires pretreatments using chemical, enzymatic or physical methods.

The agricultural and industrial by-products that have been used for SCP production will be treated in this part. Table 1 shows the different categories of agricultural and industrial by-products according to Spalvins *et al.* study [38, 39] with substrate and strain examples.

2.2.1. Agricultural by-products

Agriculture waste includes the residue coming from the agriculture production or the food industries. Spalvins *et al.* [38] divided interesting agricultural by-products for SCP production into four categories: mono and disaccharide rich sources, starch rich sources, structural polysaccharides rich sources.

2.2.1.1. Mono- and di-saccharide rich sources

Materials containing mono- and di-saccharides present the most adequate substrates for microorganisms [3]. They almost no need any pre-treatments compared to other substrates with significant SCP yields [38]. Among these particular wastes, there are the residues of sugar, fruit and industries. For instance, the utilization of molasses by a microalga *Scenedesmus acutus* engenders a protein amount of 50 g per 100 g of dry algae [40].

- **Molasses**

Molasses is the residual liquid obtained from sugar or soy industries. It contains a high sugar content (around 50-60 %) with around 10 % nitrogen compounds, 20% fats and 10% minerals [27, 41, 42]. This composition depends on the process conditions.

It is known as good carbon source for SCP production [43]. Indeed, its availability, low cost, composition and also absence of toxins and impurities that can affect the SCP acceptability, make it very attractive [26]. In addition, molasses don't need a pretreatment before their utilization [44].

- **Fruit processing wastes**

The composition of the residues of fruit industries depends on the fruit type and the type or the part of the fruit. For instance, if the waste is the whole fruit, it will be rich on mono- and disaccharides [45].

In juice production industry, wastes can be seeds and peels thus containing more fibers and rich in structural polysaccharides residues [46, 47]. Major cons come from deterioration of fruits due to problems in storage and transport [48].

Table 1 : Agricultural and industrial by-products and their utilization to produce SCP

Source	Category	Example of by-product	Example of strains	References
Agriculture	Mono and disaccharide rich sources	Molasses	<i>Scenedesmus acutus</i>	[40]
		Whey	<i>Bacillus subtilis</i>	[21]
		Banana wastes	<i>Aspergillus niger</i>	[45]
	Starch rich sources	Wheat Bran	<i>Candida utilis, Rhizopus oligosporous</i>	[49]
		Rice bran	<i>Aspergillus flavus</i>	[50]
		Leaf juice	<i>Torula utilis</i>	[51]
	Structural polysaccharides rich sources	Soy bean hull	<i>Bacillus subtilis</i>	[52]
		Potato starch processing	<i>Bacillus licheniformis</i>	[53]
		Orange (pulp, juice, peel)	<i>Rhodococcus opacus</i>	[54]
		Waste Chinese cabbage	<i>Saccharomyces cerevisiae</i>	[55]
Pig (swine) waste		<i>Chlorella vulgaris</i>	[56]	
Brewery's Spent grains		<i>Debaryomyces hansenii</i>	[57]	
Protein or lipid rich sources	Pawn shell waste	<i>Candida species</i>	[58]	
	Wastewaters	<i>Spirulina maxima</i>	[59]	
	Waste capsicum powder	<i>Candida tropicalis</i>	[60]	
	Ram horn	<i>Escherichia coli</i>	[61]	
Industries	Polymer rich sources	Soy bean meal	<i>Bacillus subtilis</i>	[62]
		Sulfite waste liquor	<i>Paecilomyces variotii</i>	[63]
		Waste paper	<i>Candida utilis</i>	[64]
		Lignin residues	<i>Scytalidium acidophilum</i>	[65]
	Carbon compounds	Latex rubber sheet wastewater	<i>Chrysonilia sitophila</i>	[66]
		Methane	<i>Rhodopseudomonas palustris</i>	[67]
		Acetic acid	<i>Methylomonas sp.</i>	[68]
		Formic acid	<i>Bacteria isolated from soil</i>	[69]
		Waste gases	<i>Bacteria isolated from soil</i>	[69]
		Glycerol	<i>Methylococcus capsulatus</i>	[70]
		Gas oil	<i>Yarrowia lipolytica</i>	[71]
	Sources for photosynthetic microorganisms	Methanol	<i>BP yeast</i>	[72]
		Saline sewage effluent	<i>Methylophilus methylotrophus</i>	[73]
Effluents of biogas plants		<i>Chlorella salina</i>	[74]	
Wastewater effluents		<i>Rhodopseudomonas species</i>	[75]	
		<i>Micractinium, Scenedesmus Oocystis, Franceia Euglena</i>	[76]	

- **Dairy residues**

Dairy wastes contain important levels of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) with high amount of fats, oils, phosphorus and nitrogen compounds [77]. They have high levels of lactose making them suitable for SCP production selecting strains able to ferment this sugar [77].

Whey, a by-product from dairy industries and mostly cheese production, contains 4-6 % (w/v) of lactose [43]. In order to produce yeast from whey, in 1956, Fromageries Bel© implemented *Kluveromyces marxianus* [43].

2.2.1.2. Starch rich sources

A considerable part of the agricultural residues includes the products rich in starch such as rice, cereals, maize, or bulb plants [43]. For example, in tropical and sub-tropical area, Cassava, deproteinized leaf juice, or bran is a good and valuable starch source for SCP.

Implementation of a mixed culture (*Candida utilis* with *Rhizopus oligosporus*) with wheat bran engendered a protein amount of 41% of dry matter [49].

Nevertheless, this type of wastes have to be hydrolyzed in order to release the monosaccharides; such pretreatment increases the production cost [38].

2.2.2. Industrial by-products

Industrial wastes include industrial residues from industries, factories or mining and mills process like pigments, chemical liquids, radioactive residue, etc. However, only biodegradable wastes can be utilized for the production of SCP [78].

A number of agricultural residues could have also been mentioned in this part according to the definition of industrial by-products, but in this review, they have been only categorized as agriculture due to the nature of the resource utilized in processing. Thus, this part will summarize the by-products that are not directly in relation with the agriculture production and the food industries.

Spalvins *et al.* [39] categorized industrial by-products in three groups, which are, polymer-rich sources, carbon compounds and sources for photosynthetic microorganisms.

2.2.2.1. Polymer rich sources

Polymers like cellulose, lignin and latex are residues accumulated from many industrial processes (cotton, wood , paper, fuel , latex, etc.) [79]. They are made of repeated unit of D-glucose [79]. These polymers, mainly lignocellulosic ones, are the most abundant industrial by-products [43].

Pretreatments of these substrates must be done mechanically, chemically or enzymatically before their utilization for SCP production increasing [39].

- **Cellulosic wastes**

Cellulose resulted, mainly, from wood, agricultural and municipal wastes [65]. Cellulosic wastes can be used for fermentation after pretreatment [39, 65]. As cellulosic wastes, we can found spent sulfite liquor (SSL), which is a by-product of the sulfite production [39] and hydrolyzed paper waste which contains about 60–70 % sugar including glucose, xylose arabinose, mannose and galactose [65].

- **Lignin waste**

Lignin is one of waste products in the paper manufacturing [39]. It can be used in fermentation using microorganisms able to degrade it [66]. However, a slow digestion and low amounts of protein produced defined the low use of lignin as substrate.

- **Latex waste**

Latex, a complex mixture of compounds derived from plants such *Landolphia* genus and *Hevea brasiliensis*, is utilized to produce latex rubber [80]. The production leads to wastewater rich in formic acid, ammonia, and sodium sulfite. The use of these wastewaters in fermentation is a good alternative to produce SCP with high amounts of protein 65% using *Rhodopseudomonas palustris* [67].

2.2.2.2. Carbon compounds

The organic decomposition produces many compounds such as methane, acetic and formic acids, waste gases and glycerol [81] :

Methane (CH₄) is used for SCP production as carbon source for its low toxicity, selectivity and volatility [68]. The most adequate source of methane for SCP is the natural gas, its methane content is about 85–90 % [68].

Methanol is an industrial by-product from the petrochemical industry. It can be assimilated by many yeast species like *Candida*, *Pichia*, *Hansenula*, and *Torulopsis* [43].

Formic acid contained in industrial wastewaters should be treated by microorganisms able to metabolize it as carbon source [69, 82].

Gas wastes produced from oil and steel refining and some other industries contained carbon monoxide (CO), carbon dioxide (CO₂) and methane (CH₄) in different ratios and can be assimilated by gas-fermenting microorganisms. The utilization of these wastes can reduce emissions of greenhouse gases with value-added products production such as SCP [70].

Glycerol is a by-product of biodiesel production [71]. This residue contains impurities that must be treated before utilization [83, 84]. Glycerol can be utilized as a carbon source for the cultivation of some microorganisms in order to produce SCPs or valuable metabolites [85].

Other carbon compounds can be utilized to produce SCPs including, hexane, propionic acid and capric acid [39].

2.2.2.3. Sources for photosynthetic microorganisms

Cultivated in lagoons or ponds, algae and photosynthetic bacteria are usually used in order to treat liquid wastes reducing the environmental pollution [74-76, 86, 87]. These microorganisms can grow in fresh water or in effluent with high salinity. Depending on strain's choice, for instance, *Chlorella salina* can be cultivated in sewage effluent having a relatively high salinity [74]. High amounts of proteins were obtained using wastewaters by both algae and photosynthetic bacteria. Utilization of *Rhodospseudomonas* species in clarified effluent slurry showed an amount of 60% of dry weight [75].

This part shows that several types of substrates can be used for fermentations in order to produce SCPs. However, the choice of this substrate is very important and have to be done considering many characteristics. Indeed, to choose the suitable raw material, there are many essential considerations:

1. Local availability
2. Pre-treatment expenses
3. Transportation charges
4. Final SCP concentrations in the microbial biomass
5. Efficiency of cultivation conditions
6. Efficiency of SCPs extraction procedure [39]

Combination of residues can be a good approach since the culture medium, in this case, contains essential elements for microbial multiplication [38].

3. Production strategies and Harvesting

3.1. SCP production bioprocesses

The production of SCP is a bioprocess based on the microbial biomass production [3]. It requires for a selected microorganism to furnish optimal nutritional and environmental conditions [88].

3.2. Cultivation methods

According to the substrate state, fermentation can be achieved with three different methods: submerged, semisolid or solid state fermentation [7, 9, 27]. For almost all the SCP studies, the submerged culture was chosen. The substrate used is in liquid form incorporating all nutrients required for microbial growth [89]. To manage the synthesis of high value metabolites, culture

strategies and controlled culture conditions may be applied during the fermentation including controls of dissolved oxygen, pH and temperature [9].

SCP production based on submerged bioprocess can be done using flasks [54, 58, 85], photo bioreactors [90, 91] or bioreactors [9, 92].

Many parameters are mandatory to have an optimal growth, such as temperature, pH, dissolved oxygen, aeration control and monitoring [6, 9]. In order to get high productivity and yield, a suitable system should be applied to maintain the optimal culture conditions during the bioprocess [9].

- Ponds were utilized for microbial growth especially for microalga [56, 87]
- and rarely, biomasses were produced in test tubes for example for photosynthetic bacteria growth [75].
- Erlenmeyer-flask cultures are the most used method to evaluate SCP production. Temperature, incubation time, pH_{initial}, agitation, and medium composition were the parameters that have been mainly evaluated [93].
- In order to study the strain dynamics under controlled conditions, bioreactors are used with different type depending on the study aims. Batches were used to study to evaluate environmental parameters effects such as pH, temperature, inoculum volume [92]. Continuous culture is able to maintain steady state culture with an environment condition in order to enhance productivity for a long period of time [94, 95].

4. Biomass composition

As already specified in the introduction, SCP are defined as “Dried cells extracted from microbial culture”, therefore it is crucial to know the composition of microbial biomass. Furthermore, SCP production performances are expressed either as total biomass production (dry weight) or as total protein production.

Microbial biomass contains, as shown in Table 2, a high amount of protein and other compounds like fats, ash and nucleic acids.

Table 2 : Comparison of chemical composition (%_{DW}) of different microbial biomasses [4, 9, 27]

Source	Proteins	Fats	Nucleic acid	Ash
Algae	40-60	0.7-20	3-8	0.8-10
Bacteria	50-65	1.5-3.0	8-12	0.3-0.7
Fungi	30-45	0.2-0.8	7-10	0.9-14
Yeast	45-55	0.2-0.8	6-12	0.9-14

4.1. Protein extraction

After being harvested, some microorganisms require more treatments to extract proteins from cells. Table 3 presents the main techniques and some examples of strains.

Table 3 : Different methods used for microbial protein extraction

Extraction methods		Studies	Strains
Sonication		[95-98]	<i>Kluyveromyces marxianus</i> <i>Acetobacter diazotrophicus</i> <i>Gluconacetobacter diazotrophicus</i> <i>Methylophilus methylotrophus</i> <i>Deinococcus geothermalis</i>
Cell hydrolysis	using acids - Trichloroacetic acid - Chlorhydric acid	[22] [31, 99]	<i>Cellulomonas biazotea</i> <i>Bacillus brevis</i> <i>Hydrogemonas eutropha</i>
	Using exogenous enzymes	[100]	<i>Kluyveromyces marxianus</i>
NaOH treatment		[101, 102]	<i>Pichia pinus</i> <i>Cupriavidus necator</i>
Heating in alkaline solution		[103]	<i>Bacillus brevis</i>
Digestion with high temperature		[104]	<i>Cryptococcus aureus</i>
Mechanical destruction of cell using mortar and sea sand		[105]	<i>Rhodotorula rubra</i> <i>Pichia pastoris</i> <i>Saccharomyces cerevisiae</i>
Treatment with glass beads		[106, 107]	<i>Saccharomyces cerevisiae</i> <i>Rhodotorula rubra</i> <i>Saccharomyces fibuligera</i> <i>Aspergillus niger</i> <i>Aspergillus oryzae</i> <i>Cupriavidus necator</i>

4.2. Protein quantification

Protein content in microbial biomasses can be estimated by total azote quantification or soluble proteins quantification by colorimetric essays.

4.2.1. Kjeldahl Procedure

This method has been developed by Johann Kjeldahl, in 1883, to estimate nitrogen. The procedure includes three basic steps which are mineralisation using sulphuric acid, neutralization and distillation, and finally a step of titration [108]. This method is not expensive and very acceptable. However, it uses corrosive reagents and it presents lower precision than other methods since it calculates total organic nitrogen and not just protein and a correction factor has to be used to convert total nitrogen into proteins [108].

4.2.2. Colorimetric assays

Colorimetric techniques used for microbial protein quantification include the Biuret assay, the Lowry assay, the Bradford assay and the bicinchoninic acid assay. All of them deals with soluble protein quantification, but protein targets are not the same and thus, quantification results depend on protein composition and on the amino acid profile mainly [109].

- ***Biuret method***

During this assay, a Biuret reaction is done using a Biuret reagent. This reaction is rapid and simple to measure protein concentration but not stable [110]. Interferences are noticed using this method by compounds such as ammonia and glycerol [111].

- ***Lowry method***

It is based on alkaline copper treatment followed with measurement of proteins by reaction with the Folin phenol reagent. To obtain the final colour, two steps are done: the first is the reaction with alkaline copper solution and the second is the reaction of the copper treated protein [112]. This method is recommended for its simplicity, specificity and sensitivity. It is more sensitive than the Biuret reaction. However, it is less stable than the Biuret reaction [112]. Among disadvantages, this method shows interferences by some compounds such as potassium and magnesium ions and carbohydrates [111].

- ***Coomassie blue dye binding or Bradford assay***

Bradford method is based on the reaction between proteins and a dye (Coomassie brilliant) to form a coloured complex measured by spectrophotometry. Indeed, the reagent used for this method, Coomassie Brilliant Blue G-250, passed from red to blue in the presence of proteins. This method has many advantages such as rapidity, reproductivity, sensitivity, linearity and stability of the protein-dye complex colour. However, interferences by nonproteic compounds such as detergents can occurred [111].

- ***Bicinchoninic acid assay***

This assay implemented by using a sodium salt, bicinchoninic acid, as reagent that is able to make a purple complex with cuprous ion under alkaline conditions. This method offers many advantages: simplicity, reagent stability, high sensitivity, protocol flexibility, low protein-to-protein variation and more tolerance toward interfering substances compared to other methods [113]. Table 4 presents examples of techniques used to quantify microbial protein concentration with examples of studies per type of microorganisms.

Table 4 : Different methods used for microbial protein quantification

Type of microorganism	Kjeldahl method	Colorimetric assay		
		Biuret	Lowry	Bradford
Algae	[86, 90, 114]		[115]	
Fungi	[116-118]	[119]	[120]	
Bacteria	[17, 22, 121-126]	[127, 128]	[21, 75, 97, 129, 130]	[24, 54, 96, 131]
Yeasts	[44, 51, 55, 60, 71, 85, 104, 117, 132-143]		[55, 95, 101, 106, 144-149]	[54, 105, 131, 150-152]

4.3. Amino acids composition

The profile of amino acids is an important factor, which has an impact on protein quality. So, a pattern of amino acids has been done to show the required values of the eight essential amino acids in food: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine [153] (Table 5).

Table 5 : Amino Acids scoring pattern from FAO recommendations [153]

Essential Amino Acid	Suggested level (mg/g of protein)
Isoleucine	40
Leucine	70
Lysine	55
Methionine + Cysteine	35
Phenylalanine + Tyrosine	60
Threonine	40
Tryptophan	10
Valine	50

Microbial biomass is usually characterized by a complete amino acid (AA) profile with presence of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) that present an excellent quality of proteins [74].

Table 6 shows the AA profile in different sources of protein and using different substrates and strains compared to other conventional protein sources and FAO standards.

AA-profiles from algae are comparable to animal and vegetal sources of protein and to FAO standards [32, 59]. However, the amounts of lysine and sulfur-containing amino acids (cysteine and methionine) are lower than the recommended amounts [56, 87]. This shortage can be corrected by blending harvested algal cells with other conventional protein sources including legumes and cereals, such as rice or wheat according to Becker and Venkataraman [87].

Bacterial biomass showed high levels of essential and sulfur amino acids compared to the other microorganisms [124].

In the case of photosynthetic bacteria, cysteine, phenylalanine, arginine, valine, leucine and threonine were equal or surpassed other protein sources [124]. Methionine and lysine contents are comparable with recommended values from FAO but lysine contents were lower compared to the proteins from animal origin [75].

For non-photosynthetic strains, they contain essential amino acids with values comparable to FAO reference and casein [22, 99, 148] with a high amount of lysine that can be a good supplement to cereals which are poor in it [99].

The addition of glycine and isoleucine to the basal medium where *Bacillus brevis* was grown, stimulated the protein production but didn't show any difference between amino acids profiles [31].

Fungal cells grown on agriculture wastes showed similar amino acid profiles as soya meal values. Indeed, *Aspergillus niger* and *Fusarium sp.* were adequate to substitute a part of soya meal chickens and pigs feed [154].

Most of the essential amino acid content in yeast biomasses equaled or surpassed the FAO standards [101, 104, 133, 149] with high amounts of threonine and lysine [60, 133, 149, 155]. However, they have a deficiency in sulfuric-containing amino acids which are methionine and cysteine [71, 149].

Table 6 : Essential Amino acid profile of some microorganisms compared with conventional protein sources and the FAO reference pattern
(Amounts comparable or over FAO recommendations in **green**; amounts lower than FAO recommendations in **orange**)

Source		Substrates	Essentials Amino Acids (g AA / 100g protein)									References	
			Trp	Thr	Lys	Met	Cys	Ile	Leu	Phe	Tyr		Val
FAO pattern			1	4	5.5	3.5	NA	4	7	6	NA	5	[156]
Animal Protein	Cow's milk		1.4	4.6	7.8	2.4	0.9	6.4	9.9	4.9	5.1	6.9	[33]
	Beef meat		1.2	4.3	8.4	2.4	1.3	5.2	8	4	3.3	5.5	[33]
	Egg		1.7	5	6.3	3.1	2.3	6.8	9	6	4.4	7.4	[33]
	Fishmeal		NA	2.42	4.94	1.88	1.23	2.8	4.64	2.64	1.52	3.7	[157]
Vegetal protein	Soya beans		1.3	4.4	5.6	1.7	NA	5.3	8.4	5.8	NA	5.2	[56]
	Whole wheat		0.3	2.7	1.9	1.5	1.9	4.2	7	5.5	NA	4.1	[148]
	Wheat flour		NA	2.7	1.9	1.5	NA	4.2	7	5.5	NA	4.1	[75]
	Wheat straw		NA	0.04	NA	0.28	0.32	0.18	0.18	0.26	NA	0.02	[124]
	Groundnut meal		1.1	2.7	3.5	0.9	1.5	4.1	6	5	3.6	4.9	[33]
Source		Substrates	Essentials Amino Acids (g AA / 100g protein)									References	
			Trp	Thr	Lys	Met	Cys	Ile	Leu	Phe	Tyr		Val
Algal Protein	<i>Spirulina maxima</i>	Synthetic medium	1.4	4.56	4.59	1.37	0.43	6.03	8.02	4.97	3.95	6.49	[33]
		Greenway effluent	NA	5.35	4.68	2.1	NA	5.71	9.26	4.45	NA	6.62	[59]
		Cattle manure	1.3	5	3.9	1.4	NA	6.2	9.1	5	4.2	6.2	[86]
		Sewage sludge	1.3	4.5	3.7	2	NA	5.4	8.1	4.3	3.7	6.3	[86]
		Swine manure	1.1	5.2	4.1	2	NA	6.3	9.3	5	4.1	7.2	[86]
		Swine blood slurry	1.2	5.2	3.9	2.1	NA	5.9	8.7	4.7	4	6.9	[86]
		Poultry manure	1.1	4.9	3.9	2.3	NA	5.9	8.8	4.9	3.6	4.9	[86]
	Synthetic medium	1.2	4.8	4.9	2	NA	6.2	8.1	5.2	4.1	5.9	[86]	
	<i>Spirulina platensis</i>	Rural wastes	0.3	6.2	4.8	2.5	0.9	6.7	9.8	5.3	5.3	7.1	[87]
		Synthetic medium	NA	4.19	NA	0.89	2.82	5.94	9.21	5.02	4.29	6.46	[32]
	<i>Chlorella salina</i>	Sewage effluent	1.9	3.4	6.2	NA	NA	1.1	4.2	NA	NA	6.4	[74]
	<i>Chlorella vulgaris</i>	Diluted pig waste	NA	5.3	8	1.8	NA	4.8	9.3	6.6	NA	6.2	[56]
	<i>Scenedesmus acutus</i>	Molasses and/or CO ₂	1.7	4.9	4.6	NA	NA	3.1	7	NA	NA	4.7	[40]

Source		Substrates	Essentials Amino Acids (g AA / 100g protein)										References
			Trp	Thr	Lys	Met	Cys	Ile	Leu	Phe	Tyr	Val	
Bacterial Protein	<i>Hydrogenomonas eutropha</i>	Urea-containing medium	1.05	2.9	3.57	1.54	0.11	2.92	5.44	2.96	2.41	4.08	[99]
		Gas mixture (CO ₂ . H ₂ . O ₂)	1.40	5.25	7.02	2.63	0.56	4.47	8.60	4.42	3.62	6.38	[158]
		Gas mixture (CO ₂ . H ₂ . O ₂)	1.16	5.20	9.02	0.40	0.30	4.50	8.70	3.60	2.40	7.50	[158]
	<i>Pseudomonas saccharophila</i>	Urea-containing medium	NA	5.37	5.73	2.03	0.36	4.14	8.35	3.56	2.32	7.55	[99]
	<i>Raoutella ornithinolytica</i>	Potato residue	NA	10.37	NA	27.25	3.96	7.15	7.15	2.20	NA	5.57	[124]
	<i>Rhodopseudomonas capsulate</i>	Clarified effluent slurry	NA	5.12	5.41	3.23	NA	5.24	8.02	5.23	NA	7.2	[75]
	<i>Rhodopseudomonas palustris</i>	Clarified effluent slurry	NA	4.86	5.2	3.33	NA	4.32	7.23	4.22	NA	6.5	[75]
	<i>Rhodopseudomonas phaeroides</i>	Clarified effluent slurry	NA	5.05	5.6	3	NA	3.85	7.14	4.75	NA	6.55	[75]
	<i>Rhodopseudomonas gelatinosa</i>	Clarified effluent slurry	NA	4.75	4.66	2.88	NA	3.98	7.01	4.8	NA	6.44	[75]
	<i>Rhodopseudomonas acidophila</i>	Clarified effluent slurry	NA	4.82	4.82	3.41	NA	4.43	6.88	4.43	NA	6.88	[75]
	<i>Rhodopseudomonas rubum</i>	Clarified effluent slurry	NA	5.4	4.93	3.05	NA	4.1	6.56	5.12	NA	7.05	[75]
	<i>Rhodopseudomonas tenue</i>	Clarified effluent slurry	NA	4.8	5.05	3.41	NA	4.3	7.72	5.2	NA	7.3	[75]
	<i>Bacillus brevis</i>	Basal medium (BM)	0.8	7.2	10.9	2.9	0.5	6	8.5	5.8	5.1	10.4	[31]
		BM supplemented with AA	0.6	6.6	8.9	2.7	0.1	6.3	8.9	6.0	5.2	9.5	[31]
	<i>Bacillus sp.</i>	Methanol/mineral salt	NA	3.2	4.28	1.10	0.18	2.12	4.48	2.48	2.86	3.54	[92]
	<i>Methylobacterium extorquens</i>	Synthetic medium	NA	NA	2.5	0.88	0.34	1.72	3.32	2.03	1.44	2.75	[34]
	<i>Brevibacterium factofermentum</i>	Synthetic medium	0.6	2.8	3.6	0.9	0.4	2.6	4.3	2.3	1.6	3.6	[35]
	<i>Bacterium glutamaticum</i>	Synthetic medium	0.3	3.0	2.3	0.9	0.2	2.2	4.9	1.9	1.3	2.9	[35]
<i>Cellulomonas biazotea</i>	Kallar glass	NA	2.5	3.7	1.9	5.5	3.2	5.9	3.6	1.5	4.5	[22]	
<i>Clostridium autoethanogenum</i>	Synthetic medium	NA	3.81	8.43	2.25	1.02	5.69	6.58	3.46	3.03	5.45	[157]	
Source		Substrates	Essentials Amino Acids (g AA / 100g protein)										References
			Trp	Thr	Lys	Met	Cys	Ile	Leu	Phe	Tyr	Val	
Fungal protein	<i>Aspergillus niger</i>	Synthetic media	NA	5.0	5.9	2.6	1.1	4.2	5.7	3.8	2.1	5.2	[1]
		Agriculture wastes	NA	4.4	5.0	1.9	1.0	4.0	6.5	4.0	4.0	5.8	[154]
	<i>Morchella crassipes</i>	-	NA	3.0	3.5	1.0	0.4	2.9	5.6	1.9	1.5	3.0	[1]
	<i>Paecilomyces variotii</i>	-	NA	4.6	6.4	1.5	1.1	4.3	6.9	3.7	1.2	5.1	[1]
	<i>Penicillium notatum</i>	-	NA	3.6	4.0	1.0	NA	3.2	5.5	3.0	1.3	3.9	[11]
	<i>Fusarium sp.</i>	Agriculture wastes	NA	10	4.5	1.9	2.2	3.8	5.0	5.9	3.0	5.8	[154]

Source		Substrates	Essentials Amino Acids (g AA / 100g protein)									References	
			Trp	Thr	Lys	Met	Cys	Ile	Leu	Phe	Tyr		Val
Yeast protein	<i>Candida utilis</i>	Salad oil manufacturing wastewater	NA	4.7	7.8	1	NA	4.1	7.9	3.4	NA	4	[133]
		Waste capsicum powder A	NA	4.5	7.6	1.1	NA	4.3	7.5	4.2	NA	4.6	[60]
		Waste capsicum powder B	NA	4.3	7	1	NA	4.1	6.9	3.6	NA	4.7	[60]
		Forestry by-products	0.62	2.35	3.45	0.58	NA	2.31	3.42	2.10	1.67	2.79	[159]
		Sugar cane bagasse	NA	2.5	6.8	0.8	0.7	3.9	6.1	3.5	NA	4.8	[148]
	<i>Candida lipolytica</i>	Neutralization paste	0.9	6	6.5	1.3	1.3	4.2	6.7	6.4	11.5	5.3	[160]
	<i>Candida curvata</i>	Neutralization paste	1.1	5	7	1.3	1.3	4.3	7.7	4.5	7.1	5.5	[160]
	<i>Candida langeronii</i>	Sugar cane bagasse	NA	5.1	7.8	0.75	0.7	4.1	6.5	3.5	NA	4.5	[148]
	<i>Candida rugosa</i>	Neutralization paste	1.6	6	8.7	0.9	1.6	4.2	6.7	5.1	8	5.5	[160]
	<i>Candida tropicalis</i>	Sugar cane bagasse	NA	4.98	7.22	1.6	NA	3.99	6.45	3.55	NA	4.76	[148]
	<i>Yarrowia lipolytica</i>	Pure glycerol	0.58	3.93	5.61	NA	NA	2.92	5.03	NA	NA	3.88	[71]
		raw glycerol	0.7	5.39	8.26	NA	NA	4.31	6.94	NA	NA	5.79	[71]
		Oat bran	NA	2.08	1.83	0.47	NA	1.39	3.40	1.38	NA	1.77	[161]
		Rye bran	0.35	2.31	2.93	0.87	NA	1.91	3.38	1.80	NA	2.36	[161]
		Rye straw	0.45	1.89	3.10	0.52	NA	1.91	3.45	1.79	NA	2.25	[161]
	<i>Kluyveromyces marxianus</i>	Inulin	NA	5.9	6.3	2.4	NA	4.4	6.5	3.8	NA	5.6	[162]
	<i>Kluyveromyces marxianus</i>	Deproteinized whey concentrates	NA	7.45	NA	0.95	NA	5.07	7.62	3.67	2.45	6.89	[163]
	<i>Kluyveromyces marxianus</i>	Deproteinized sour whey	NA	6.94	NA	0.77	NA	5.48	7.74	3.58	2.5	7.5	[163]
	<i>Kluyveromyces marxianus</i>	Pineapple plant residue											[164]
	<i>Rhodotorula pilimanae</i>	Neutralization paste	1.1	5	6	1.6	1.6	3.7	7.7	6.9	7.9	5.2	[160]
<i>Geotrichum candidum</i>	Neutralization paste	1.2	6.2	6.5	1	1.1	4.6	6.9	6.5	10.5	5.6	[160]	
<i>Pichia pinus</i>	Methanol	NA	2.37	4.48	2.03	7.55	3.02	8.10	4.40	5.3	3.5	[101]	
	Mango peel extract	NA	1.28	4.7	2.83	7.70	6.32	9.05	4.56	5.62	2.09	[101]	
<i>Saccharomyces fragilis</i>	Coconut water	NA	3.97	8.47	1.73	0.43	4.81	5.7	3.9	2.78	4.72	[149]	
<i>Cryptococcus aureus</i>	Artichoke extract	NA	4.74	12.6	3.13	3.11	5.46	9.09	3.16	2.7	7.17	[104]	

5. Factors influencing SCP production and composition

Many parameters are important, and can have an impact on SCP production and their chemical composition. SCP production performances are expressed either as biomass production and/or as total protein production. Most of the studies deal with mono-factorial approaches but some works describe the use of multifactorial approaches using statistical experimental designs. Among these factors, we can mention substrate choice, strain type and the environmental parameters.

5.1. Medium composition

In the literature, the substrate was evaluated as an important factor for cell growth and/or SCP production. Many studies aimed to evaluate the use of different agricultural or industrial residuals or different substrates compositions using different carbon sources, nitrogen sources or C/N ratio, etc.

5.1.1. Complex substrates

Considering the literature, two types of studies were done using complex substrates. Generally, it is an evaluation of the use of different agricultural/industrial wastes as substrates. However, sometimes, some studies deal with the addition of carbon or nitrogen sources, to this type of substrates. Examples of these studies are presented below considering the type of the microorganisms used.

Bacteria

In order to evaluate the utilization of agro-waste as potential low-cost substrate for protein production, Mahan *et al.* [54] use two strains of *Rhodococcus opacus* grown on orange wastes (pulp, juice and peel), lemon wastes (pulp, juice and peel) and corn stover (stalks, leaves and cob) effluents. After 96 hours of fermentation in flasks, Cell Dry Weight (CDW) was similar on corn stover effluent, better for strain-1 on lemon residues (3.28 g/l) and better for strain-2 on orange residues (2.96 g/l) (Table 7) [54]. The use of different sources of wastes did not really affect the growth of this bacteria. In addition, this oleaginous bacterium showed slightly different amounts of protein when growing on different agricultural wastes. The values estimated by the Bradford protein assay for strain 2 were 45, 42 and 47% of dry weight, using lemon waste, orange waste and corn stover effluent respectively (Table 7) [54].

Table 7 : Production of SCP using two strains of *Rhodococcus opacus* in different agro wastes [54]

Strain	Agricultural waste	Cell dry weight (g/l)	Protein content (%)
<i>R. opacus</i> 1	Lemon waste	3.28	52.1
	orange waste	2.32	56.9
	Corn stover effluent	2.20	52.7
<i>R. opacus</i> 2	Lemon waste	2.22	45.8
	orange waste	2.96	42.2
	Corn stover effluent	2.66	47.0

Streptomyces strains are well-known regarding their ability to degrade lignocellulose. In this context, Iyo and Antai [121] used two strains of *Streptomyces* in order to improve the protein content in two lignocellulose-based substrates: sugarcane bagasse and groundnut hull lignocelluloses. The cultivation was done in semi-solid-state culture on conical flasks. Estimated by micro Kjeldahl method, the amounts of protein were higher in sugarcane bagasse for both strains. *Streptomyces viridosporus* presented a biomass containing 21% of crude protein [121].

Gomashe *et al.*, [21] investigated the use of liquid whey for SCP production in conical flask from *Bacillus subtilis*. They chose five liquid wheys from five different markets. An estimation of protein content was done by Lowry’s method. Single Cell Protein production was higher in sample 4, followed by sample 3, sample 2 and then sample 1 and sample 5 with 0.32; 0.28; 0.24 and 0.16 mg/ml of sample, respectively [21].

Fungi

Both cell growth and protein content were evaluated by Khan and Dahot [165]. The first part of the study was dedicated to a screening of waste as substrate for SCP. Indeed, they evaluated the effect of agricultural wastes, pre-treated with sulfuric acid, as carbon sources for *Penicillium expansum* when grown on flasks for 240 hours at 28°C. Rice husk showed the maximum biomass (1.64 g/l) and the highest protein percentage (18.25 %_{DW}) (Table 8).

Table 8 : Effect of different agricultural waste on SCP production by *Penicillium expansum* [165]

Agricultural wastes	Biomass (g/l)	Protein content (%)
Sugar cane Baggase	0.317	15.53
Orange peel	0.314	9.89
Wheat straw	0.319	6.48
Cotton seeds	0.421	16.66
Cajanus cajan seeds	0.534	10.94
Castor been	0.328	11.62
Rice husk	1.640	18.25

Treated rice husk was then supplemented with 1% of pure sugars in order to study their effect on SCP production by *Penicillium expansum* grown on the same conditions than the screening experiment. The maximum cell biomass (5.107 g/l) and the highest protein content (30.10 %) were obtained when supplementation was done with 1% of sucrose (Table 9).

Table 9 : Effect of the use of different pure sugars with pre-treated rice husk on SCP production from *Penicillium expansum* [165]

Pure sugar	Biomass (g/l)	Protein content (%)
Mannose	3.125	24.50
Glucose	4.953	28.20
Fructose	4.022	24.93
Galactose	4.844	26.25
Maltose	4.808	25.00
Lactose	2.988	26.60
Sucrose	5.107	30.10
Starch	4.448	28.00
Cellulose	1.287	26.20
Molasses	4.377	25.37

Yeast

Khan *et al.*, [117] did a comparative study, with different fruit wastes, to produce SCPs by using *Saccharomyces cerevisiae* grown on petri plates. Table 10 shows the amounts of protein estimated by the Kjeldahl Method. The highest value of the crude protein (58,62 %_{DW}) was obtained when using banana skin [117].

Table 10 : SCP production by *Saccharomyces cerevisiae* grown on various fruit wastes [117]

Substrate	Crude protein (% _{DW})
Banana skin	58.62
Rind of pomegranate	54.28
Apple waste	50.86
Mango waste	39.98
Sweet orange peel	26.26

An evaluation of growth and protein production abilities was done by Kurcz *et al.* [85]. *Candida utilis* was grown, in conical flasks, on potato wastewater with different percentages of glycerol [0-25% (v/v)] as carbon source. The growth curve showed that high concentrations of glycerol (above 10%) inhibit the growth yield. However, the highest yield was with a percentage of 5%

of glycerol, around 7.5 g/l after 24h of fermentation, 18 g/l after 48h and 34 g.l⁻¹ after 72h. That can be probably due to the osmotic pressure of the culture medium, that decreased the microorganism vitality [85]. Estimated by the Kjeldahl method, the protein amounts in biomass decreased when the percentage of glycerol increased. However, the cultivation medium containing only potato waste water generated the highest values with 49% and 42 % (Table 11) [85].

Table 11 : Protein percentages in *Candida utilis* biomass grown on potato waste water with different supplementation in glycerol [85]

Culture medium	Protein content (% _{DW})	
	After 48h fermentation	After 72h fermentation
Potato wastewater	48.9 ± 0.7	41.7 ± 0.3
Potato wastewater + 5%	40.8 ± 0.8	36.7 ± 0.5
Potato wastewater + 10%	35.1 ± 0.8	32.4 ± 0.6
Potato wastewater + 15%	35.7 ± 1.1	36.4 ± 0.9
Potato wastewater + 20%	33.7 ± 0.9	35.8 ± 0.6
Potato wastewater + 25%	28.4 ± 1.2	29.7 ± 1.0

The objective of Drzymała *et al.* study [161] was to use rye straw, rye bran and oat bran hydrolysates as substrates for *Yarrowia lipolytica* growth. In flask cultures, this yeast was able to grow on these medium with protein contents between 30 and 44.5 % DW [161].

Kurcz *et al.* [85] produced biomass from *Candida utilis* in different medium containing potato waste water with a percentage of glycerol ranged between 0 and 25%. Added to substrate variation, both growth and protein content were determined after different incubation time, 48 and 72 hours. Values were different. For instance, *Candida utilis* cultivated in potato wastewater without glycerol, showed 49% of protein after 48 hours and 42% of protein after 72 hours [85]. The author explains that with the probability of synthesis of other compounds from a part of the glycerol assimilated by the microorganism [85].

Rages and Haider [166] study aimed to evaluate SCP through *Candida lipolytica* using olive fruit residuals with different nitrogen sources concentrations, in conical flask cultures. The nitrogen source chosen was peptone and the concentrations tested were 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 %. The protein content was measured after a cell lysis step via a ceramic mortar.

Table 12 shows that the protein production decreased with the increase of peptone concentration. Indeed, the highest amount of protein (64%) was obtained with a medium

containing 0.4% of peptone. However, when the nitrogen source concentration increased from 0.4 to 0.7%, the SCP accumulation was inhibited [166].

Table 12 : Peptone concentration effect on protein content in *Candida lipolytica* biomass grown in olive fruit residuals [166].

Peptone concentration (gm/100 ml)	Protein content (% _{DW})
0.2	60
0.3	59
0.4	64
0.5	41
0.6	38
0.7	35

5.1.2. Carbon source

To evaluate the use of different carbon sources, Sakarika *et al.*, [167] compared biomass and protein productions from pure microorganisms (*Cupriavidus necator*, *Methylobacterium*, *Corynebacterium glutamicum*, *Yarrowia lipolytica* and *Komagataella phaffii*) and mixed cultures using two substrates: formate and acetate. 1L-Erlenmeyer flasks containing 0.4 liter of culture were used and samples were harvested in the stationary phases [167].

Growth rates were higher on acetate (between 0.15 and 0.41 h⁻¹) than formate (between 0.061 and 0.29 h⁻¹) at pH 7. Protein content was higher in stationary phases and was function of growth rates. Indeed, the protein content increased when the growth rate decreased. Depending on microorganisms and substrates, the obtained results were between 18 and 82%_{DW} (Figure 2) [167].

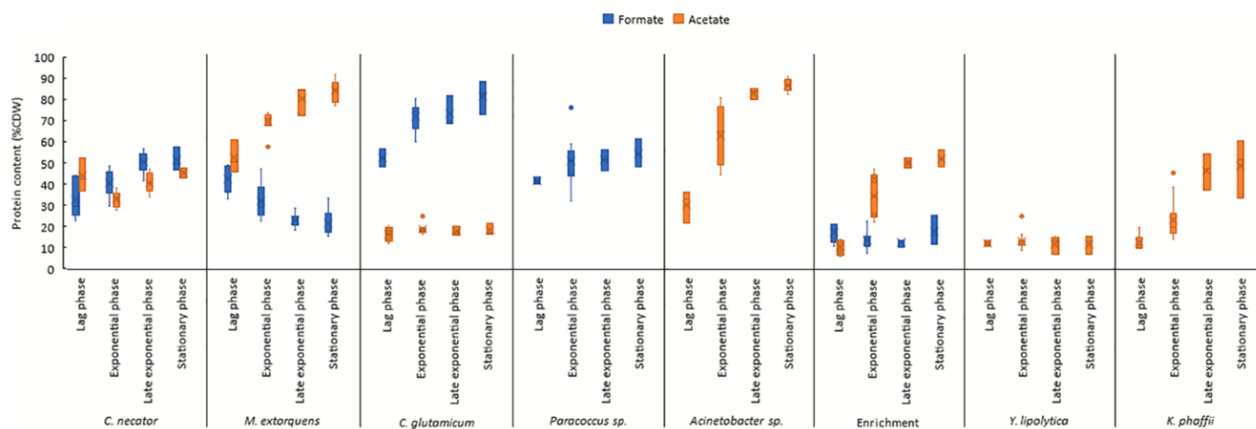


Figure 2 : Protein yields of the different microorganisms grown in batch mode, on acetate or formate at pH 7 [167]

5.1.3. Nitrogen source

Some researchers were interested on studying the nitrogen source form as a promoting factor for SCP production.

Alsudani [168] investigated the nitrogen sources (urea, ammonium sulfate, ammonium phosphate and ammonium chloride) effect on the fungal strain, *Trichoderma reesei*, grown in synthetic media in Erlenmeyer flasks. Dry weights and protein amounts were measured using the Lowry method [112]. The use of ammonium phosphate had the highest values with 2.83 ± 0.10 g/100 ml for dry weights and 1.05 ± 0.01 g/100 ml for protein content (Table 13).

Table 13 : Nitrogenous sources effect on dry weights and protein content for *Trichoderma reesei* [112]

Nitrogen source	Dry weight (g/100 ml)	Protein content (g/100 ml)
Urea	2.46 ± 0.30	0.81 ± 0.02
Ammonium sulfate	2.57 ± 0.25	0.94 ± 0.02
Ammonium phosphate	2.83 ± 0.10	1.05 ± 0.01
Ammonium chloride	2.45 ± 0.20	0.83 ± 0.03

Taran and Bakhtiyari [24], studied the effect of some factors on SCP production by a halophilic microorganism grown on glucose as carbon source, in order to optimize the bioprocess. Among the factors tested, we found the type of nitrogen source (NH_4Cl , yeast extract, peptone, tryptone) and nitrogen concentration (0.1; 0.2; 0.4; 0.8 % w/v) [24]. A multifactorial strategy was applied, the fermentation assays were done in Erlenmeyer flasks and protein amounts were estimated by the Bradford method after a digestion of the pellet using freeze-thaw cycles (at -20°C and 37°C). Tryptone was the best nitrogen sources for protein synthesis with values ranged between 54 and 56 % [24]. The concentration of 0.8 (w/v) gave the best performance leading to a protein contents of 55 and 56 % [24].

Shaku *et al.* [103] investigated the effect of some organic nitrogen sources (yeast extract from 0 to 5% and polypeptone from 0 to 3%) on growth and protein production by *Bacillus brevis*. The increasing amount of yeast extract in the medium increased the cell growth ($\text{OD} = 8$ at 0.5 %) but not the protein production (0.1 mg/ml at 0.5%). However, an increasing amount of polypeptone had improved both bacterial growth ($\text{OD} = 4,5$ at 3%) and protein accumulation (4 mg/ml at 3%) [103].

A study with *C. necator* was also done using different forms of inorganic sources of nitrogen (ammonium sulfate, nitrate, nitrite and urea) [169]. The results showed the growth was higher using ammonium. Indeed, the maximal growth rates values were 0.26 h^{-1} against 0.18 h^{-1} with

urea and 0.14 h⁻¹ with nitrate [169]. The ammonium sulfate suggest also a higher protein production with 83%_{DW} against 75% with nitrate and 65% with urea [169]. According to the article, this could be caused by the higher C/N ratio during the urea hydrolysis. Indeed, a high concentration of CO₂ caused high percentages of intracellular lipids and carbohydrates and low percentage of proteins [169].

The study of Zhao *et al.* [60] aimed to improve the growth of *Candida utilis*. Capsicum powder waste was supplemented with different nitrogen sources including organic sources (corn steep powder, yeast extract, soybean meal, and peptone) and inorganic sources (ammonium sulfate, ammonium chloride and urea). The biomass production using only the waste capsicum powder was 14.2 g/l. However, the results of this study showed a highest biomass concentration of 20.7 ± 0.21 g/l with addition of corn steep powder and a lowest one (12.7 ± 0.10 g/l) with addition of urea (Table 14) [60]. The authors claimed that the high temperature of capsicum's drying could broke down some nutriment. The corn steep powder was the key element since it is a rich source of growth factors including vitamins, minerals, amino acids and nitrogen. However, urea, due to its low nitrogen content, slow down the cell growth [60].

Table 14 : Effect of the addition of different nitrogen sources on *Candida utilis* growth [60]

Nitrogen sources		Cell mass (g/l)
Waste capsicum powder without addition of nitrogen source		14.2 ± 0.12
Organic sources	Corn steep powder	20.7 ± 0.21
	Yeast extract	18.4 ± 0.16
	Soybean meal	14.3 ± 0.10
	Peptone	16.3 ± 0.12
Inorganic sources	Ammonium sulfate	16.2 ± 0.18
	Ammonium chloride	15.3 ± 0.15
	Urea	12.7 ± 0.10

5.1.4. C:N Ratio

The C:N ratio impact was also investigated in some studies.

With the objective of reducing the oil in the salad oil manufacturing wastewater, this substrate has been used for *Candida utilis*. To improve both growth yield and protein content, Zheng *et al.*, [133] made a series of flask cultures of *Candida utilis* using different N:C ratio (0, 1:25, 1:10, 1:08, 1:06, 1:05, 1:04, 1:03). After a period of 24 hours, the highest growth was obtained with a ratio of 1:05 with a 3,2 g.l⁻¹ of cell mass (Figure 3) [133].

The protein amounts, estimated by the Kjeldahl method, were from 49% to 18% (Figure 1). The protein value was stable at N:C ratio between 1:05 and 1:08 but decreased gradually from

1:08 to 0 (Figure 3) [133]. Considering the three studied parameters (Oil reduction efficiency, protein content and cell mass), a N:C ratio between 1:06 and 1:08 can be recommended.

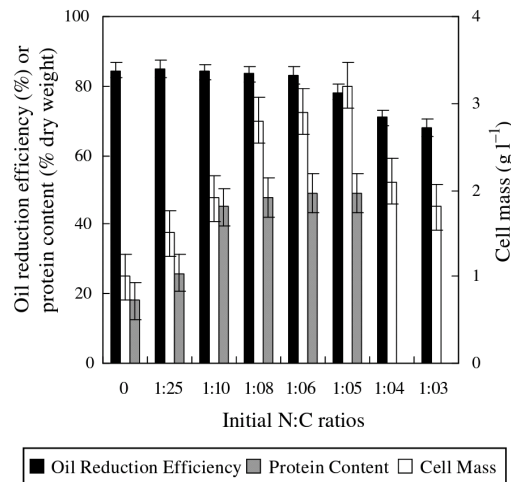


Figure 3 : The effect of the N:C ratio in the initial medium on growth, protein content and oil reduction [133]

In order to determine the best conditions of SCP production with *Saccharomyces cerevisiae*, Mujdalipah and Putri [170] tried different ratio of pineapple skin and rice washing water and different fermentation times. Using Biuret method, the best ratio was 1:2 in 56 hours with 0.4752 g of cells and protein content of 289.08 ppm [170].

Cao *et al.*, study [171] aimed to investigate the effects of C/N ratio in media and light-oxygen condition on SCP production from photosynthetic bacteria. As showed on Figure 4, lower C/N (5 and 15) and the light-aerobic condition were more favorable not only for biomass production but also for protein production [171].

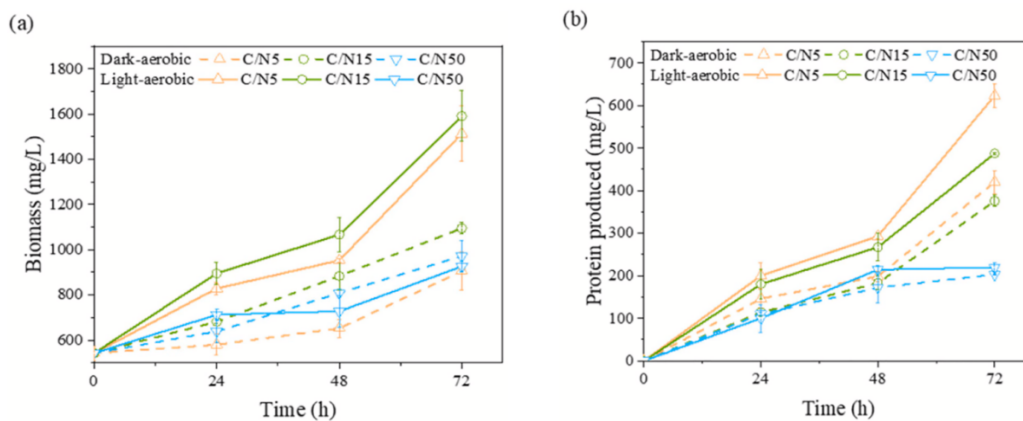


Figure 4 : The effect of the C:N ratio and light-oxygen condition in biomass (a) and proteins (b) produced by photosynthetic bacteria [171]

As a conclusion, many approaches were used in order to evaluate SCP media. On one hand, the use of wastes from agriculture or from food industries with or without carbon and nitrogen supplementations were evaluated and compared. On the other hand, protein production from different carbon sources, nitrogen sources or different C:N ratios were investigated.

Almost all the studies were performed using flask cultures. They studied, often, both biomass concentration and protein content based on the dry weight but discussed only the final points of the cultures.

The protein quantification was done using colorimetric assays (Bradford, Lowry or Biuret) or a total nitrogen determination method (Kjeldahl).

Depending on the nature of wastes used, their use on SCP production didn't show always high protein amounts. However, the supplementation and the use of different carbon and nitrogen forms were promising factors to evaluate SCP. For instance, waste media's supplementations with different concentrations of glycerol or peptone show a real effect on protein content percentage in the final biomass. The same result was also obtained using different forms of nitrogen, organic or inorganic. However, a generic conclusion cannot be drawn because it is very dependent on the microorganisms chosen.

5.2. Strains

The choice of strain is one of the most studied factors. According to their work objectives, researchers evaluated the ability of different types of microorganisms for SCP production. For instance, to evaluate a new low-cost substrate as SCP substrate, they chose strains from different microorganism types (algae, photosynthetic and non-photosynthetic bacteria, yeast and fungi). For optimization studies based on growth performances and protein content or for reducing environmental impact, same types of microorganisms are mostly implemented.

Algae

Renault *et al.*, [115] investigated the chemical composition of twelve algae strains grown under batch fermentation and standard conditions (pH8.6, temperature 25°C). 18 watt cool-white fluorescent lights illuminated the cultures from the bottom with an illumination cycle 12h-light/12h-dark. Protein content was accessed by, the Folin-Lowry method after lyophilisation of cells. Chlorophyceae class showed protein accumulation ranged between 27.4 and 66.9 % of DW and *Chlorella* strains had the lowest content with 27.4 and 30.7 %. For Bacillariophyceae family, biomasses showed 19.9-36.2% of proteins with higher content using the two *Nitzschia* strains 34.9 and 36.2% (Table 15) [115].

Table 15 : Protein content of twelve micro algal biomasses [115]

Alga class	Strain	Protein content (% _{DW})
Bacillariophyceae	<i>Melosira sp.</i>	21.8
	<i>Navicula sp.</i>	19.9
	<i>Nitzschia closterium</i>	34.9
	<i>Nitzschia frustulum</i>	36.2
Chlorophyceae	<i>Ankistrodesmus sp.</i>	60.4
	<i>Chlamydomonas sp.</i>	66.9
	<i>Chlorella sp.</i> (1)	30.7
	<i>Chlorella sp.</i> (2)	27.4
	<i>Dunaliella salina</i>	40.3
	<i>Scenedesmus dimorphus</i>	50.4
	<i>Scenedesmus quadricauda</i>	54.4
	<i>Selenastrum sp.</i>	52.3

Fungi

Trying to produce SCP from *Trichoderma* species, Alsudani [168] compared four strains (*T. hamatum*, *T. viride*, *T. reesei* and *T. harzianum*) grown on synthetic media (Czapek-dox broth medium) in Erlenmeyer flasks. Both dry weights and proteins amounts were measured. *Trichoderma reesei* gave the highest dry weight value (2.15 g/100 mL), but no significant difference in protein production (estimated with the Lowry method) between *Trichoderma reesei* and *Trichoderma viride* was obtained, 0.94 and 0.92 g/100 mL, respectively (Table 16) [168].

Table 16 : Dry weight and protein content of four *Trichoderma* strains [168]

<i>Trichoderma</i> strains	Dry weight (g/100 ml)	Protein amount (g/100 ml)
<i>T. harzianum</i>	1.64 ± 0.20	0.72 ± 0.02
<i>T. viride</i>	1.88 ± 0.15	0.92 ± 0.03
<i>T. reesei</i>	2.15 ± 0.10	0.94 ± 0.01
<i>T. hamatum</i>	1.96 ± 0.20	0.81 ± 0.02

Bacteria

Vrati [75] evaluated the growth of different photosynthetic bacteria on effluents of biogas plant added to the SCP composition quality. After fermentation in tubes, the *Rhodospseudomonas* species presented slightly different results (Table 17). *Rps. capsulata* was found to show maximum final biomass with 4.56 g/l [75]. Protein contents were estimated with the Lowry method after hydrolyzing the cells with NaOH. The protein amounts were ranged between 40 and 61 %_{DW}. *Rps. capsulata* had the highest percentage [75].

Table 17 : Cell mass and protein content of different photosynthetic bacteria grown on clarified of biogas plant [75].

Strain	Cell mass (g/l)	Protein amount (% _{DW})
<i>Rps. capsulata</i>	4.56	61.25
<i>Rps. palustris</i>	4.04	60.00
<i>Rps. sphaeroides</i>	3.02	57.50
<i>Rps. gelatinosa</i>	3.24	50.00
<i>Rps. acidophila</i>	2.75	51.25
<i>Rps. rubrum</i>	4.16	46.25
<i>Rps. tenue</i>	4.06	40.00

Spanoghe *et al.*, [172] tested growth and protein production from three photohydrogenotrophic purple bacteria (*Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodospseudomonas palustris*). After flask cultures under photohydrogenotrophic cultures, these strains showed promising growth rates. *Rhodobacter capsulatus* gave the highest value with $2.7 \pm 0.12 \text{ d}^{-1}$ against around 0.3 d^{-1} for the other strains (Figure 5) [172]. Regarding proteins, they were obtained with a modified Lowry method and expressed as fractions of the biomass (%TSS). *Rhodobacter capsulatus* had also the highest value on protein 2.9 g protein/g H₂ (Figure 5) [172].

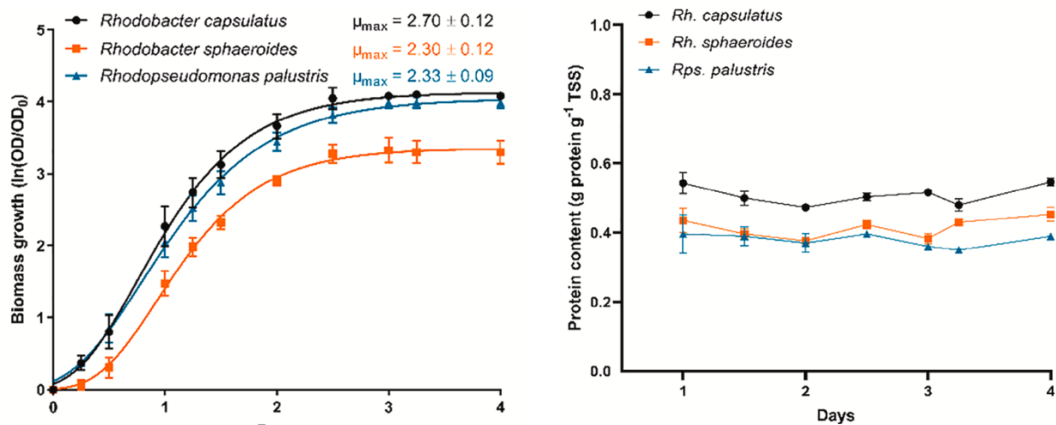


Figure 5 : Biomass growth and protein production using photohydrogenotrophic purple bacteria [172]

The aim of Kiessling and Askbrandt [35] study was to evaluate two bacterial biomasses, *Brevibacterium factofermentum* and *Bacterium glutamaticum* that were used to replace fish meal on protein diets for adults. Indeed, they did compare the nutritional characteristics of these two strains with fish meal that has the most balanced amino acid profile. The total protein value was not very different using these two actinobacteria strains, it was 70,6 %_{DW} for *Brevibacterium factofermentum* and 68,7%_{DW} for *Bacterium glutamaticum* [35].

Yeasts

Choi *et al.*, [55] investigated the possibility of microbial biomass production using a juice from waste Chinese cabbage as substrate. To evaluate, at the same time, biomass production and the protein content, they studied four yeast species, *Candida utilis*, *Pichia stipitis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* [55]. At the end of the fermentation, it was found that the cell concentrations ranged between 6.2 and 10.4 g/l, with a higher growth for *P. stipitis* (Table 18) [55]. On the other hand, they used Lowry method for protein quantification. *K. marxianus* produced more protein (44%_{DW}) against 43, 36 and 35 using *C. utilis*, *P. stipitis*, *S. cerevisiae*, respectively (Table 18) [55].

Table 18 : Cell mass and protein content of microbial biomasses of four yeast strains using cabbage juice medium [55]

Yeast strains	Cell mass (g/l)	Protein amount (% _{DW})
<i>Candida utilis</i>	6.2 ± 0.07	43 ± 0.36
<i>Pichia stipitis</i>	10.4 ± 0.20	36 ± 0.65
<i>Kluyveromyces marxianus</i>	7.1 ± 0.06	44 ± 0.38
<i>Saccharomyces cerevisiae</i>	7.4 ± 0.06	35 ± 0.45

To evaluate Waste Capsicum Powder as substrates, Zhao *et al.* [60] studied the SCPs production and chemical composition of the final biomass using suitable strains for SCP production, *Candida utilis*, *Candida tropicalis* and *Saccharomyces cerevisiae* [60]. *C. utilis* presented the highest biomass concentration and also the highest protein content in both media [60]. They were, respectively, 14,2 g/l and 48% using the first WCP (Table 19) [60]. Zhao *et al.*, [60] concluded that could be due to the ability of this strain to grown on a variety of carbon sources.

Table 19 : Cell mass and protein content for four yeast strains grown on waste capsicum powders [60]

Strain	Waste capsicum powder 1		Waste capsicum powder 2	
	Cell mass (g/l)	Protein (% _{DW})	Cell mass (g/l)	Protein (% _{DW})
<i>Candida utilis</i>	14.2 ± 0.12	48.2 ± 0.33	11.7 ± 0.10	40.9 ± 0.32
<i>Candida tropicalis</i>	11.6 ± 0.07	46.5 ± 0.38	8.5 ± 0.16	38.2 ± 0.26
<i>Saccharomyces cerevisiae</i> 1	9.6 ± 0.08	25.4 ± 0.36	7.4 ± 0.15	29.5 ± 0.43
<i>Saccharomyces cerevisiae</i> 2	8.2 ± 0.11	40.5 ± 0.41	7.1 ± 0.08	36.1 ± 0.51

Rhishipal and Philip [58] work was aimed to test marine yeast SCP production by using prawn-shell waste. Two *Candida* species, two *Rhodotorula* species and also a mixed culture were tested on flask cultures. The protein contents were estimated by the Lowry method after drying the cells at 80°C for 16h. The protein amounts obtained were ranged between 60 and 70% (Table 20) [58]. The slightly higher amount was when using the Strain 2 of *Candida* (Table 20) [58]. From this study, they showed that prawn shell waste was an efficient substrate for SCP production from marine yeasts [58].

Table 20 : Protein content in SCP using marine yeasts grown on prawn shell waste [58]

Strain	Protein content (%_{DW})
C1 : <i>Candida</i>	65.2 ± 3.1
C2 : <i>Candida</i>	70.4 ± 2.8
R1 : <i>Rhodotorula</i>	62.9 ± 3.6
R2 : <i>Rhodotorula</i>	60.6 ± 2.1
Mixed culture (C1+C2+R1+R2)	69.3 ± 3.6

Patelski *et al.*, [143] investigated the use of sugar beet pulp as a substrate for SCP production. Fermentation was done using five yeast strains (*Trichosporon cutaneum*, *Candida tropicalis*, *Pichia stipitis*, *Candida guilliermondii* and *Saccharomyces cerevisiae*) grown in shake flasks for 48 hours at 30°C. *C. tropicalis* and *P. stipitis* had the highest biomass content with 12 g_{DW}/l against a lowest biomass observed for *C. guilliermondii* cultivated with urea as a nitrogen source with 6.3 g_{DW}/l (Figure 6). The cell yield depends, so, on the strain and its ability to assimilate various nitrogen sources. Indeed, *T. cutaneum*, *P. stipitis* and *S. cerevisiae* were able to assimilate the ammonium ions and urea, however, *C. guilliermondii* and *C. tropicalis* were not able to assimilate urea as nitrogen source [143]. Crude protein content was also evaluated after 48 hours of culture. The highest protein levels (51.1 - 53.8%), estimated by Kjeldahl method, were observed using *C. tropicalis* and the lowest one (42.3 %) using *T. cutaneum* with diammonium phosphate as nitrogen additive [143] (Figure 6).

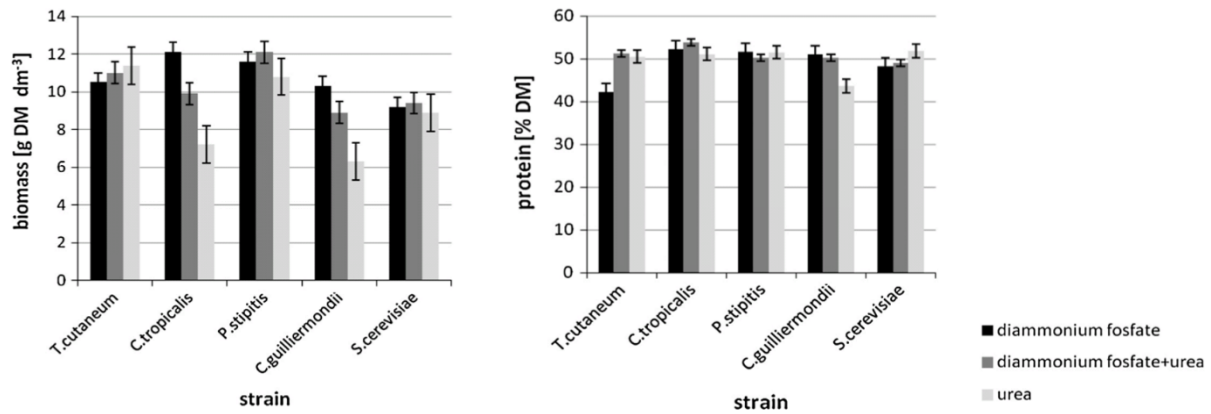


Figure 6 : Cells concentration and protein content after 48 hours of fermentation using five yeast strains grown on sugar beet pulp with various nitrogen supplementations [143].

In conclusion, for the same type of microorganism (algae, fungi, bacteria or yeasts), the choice of the strain had an impact on growth and protein production. Thus, the choice of the microorganism is very important to produce Single Cell Proteins and many studies were done to evaluate its production.

Studies are generally performed using flask cultures with both biomass and protein content measurements. However, we can report that only the final points were compared and discussed. Thus, we could not have an idea of the dynamics of the productions. Lowry assay was mainly used to quantify the protein amounts for strains' evaluation.

5.3. Environmental parameters

Mono-factorial and/or multifactorial approaches have been implemented in order to study the impact of environmental parameters on SCP production (biomass production and composition). These parameters will be presented depending on the microorganism nature.

Algae

Regarding the temperature effect, Kosaric *et al.* [59] studied multiplication of *Spirulina maxima* under high temperatures using Erlenmeyer flask culture. It grew actively in the first 4 days and then started to die. The optimum growth was found between 30 and 40°C, however, it was inhibited at 10°C and 45°C.

Bacteria

The aim of the study of Abdul *et al.* [124] was to determine the optimal production using different environmental conditions for *Raoutella ornithinolytica* cultivation in potato residue using conical flasks. They studied the effect of pH, temperature, inoculum size and culture duration based on mono-factorial approach (variation of 1 factor at a time). The optimal SCP

dry weight (obtained by the Kjeldahl method) were observed at pH 8.5, temperature of 50°C, 0.5 ml/100ml as inoculum volume and an incubation time of 72 hours (Table 21). The highest SCP concentrations were respectively 2.5; 0.775; 1.132; 5.209 and 5.207 g/l (Table 21) [124]. In this study, they use a mono-factorial analysis, but a multifactorial conclusion. This can lead to errors since they didn't investigate interactions between the factors studied.

Emtiazi *et al.*, [96] used a nitrogen fixing bacteria, *Gluconacetobacter diazotrophicus* known also with the name of *Acetobacter diazotrophicus* in order to convert wastes and produce high-value products such as SCP. The impact of different pH (5 and 7) on the biomass production was evaluated based on a mono-factorial approach. The bacteria showed the highest growth at pH 5 with an OD of 1.4 after 4 days of fermentation [96].

Table 21 : Evaluation of environmental parameters to determine optimal growth using *Raoutella ornithinolytica* in potato residue [124]

Environment parameter	SCP dry weight (g/100ml media)	
pH	4.5	0.5
	5.5	1
	6.5	1.5
	7.5	2
	8.5	2.5
Temperature (°C)	25	0.140
	30	0.652
	40	0.538
	45	0.674
	50	0.775
	55	0.644
Inoculum volume (ml/100ml)	0.5	1.132
	1	0.775
	1.5	0.880
	2	0.683
	2.5	0.644
	3	0.434
Incubation time (hour)	24	2.763
	48	4.065
	72	5.207
	96	3.596
	120	2.869

Fungi

Jaganmohan *et al.*, [173] produced SCPs with *Aspergillus terreus* using cheap energy sources through submerged fermentation in conical flask. Optimization of fermentation conditions was the aim of this study. They studied two environmental factors separately, pH (from 4 to 7) and temperature (from 25 to 45°C). After 5 days of incubation in room temperature, the highest cell mass (90mg) was found using pH 5.5 (10mg) and a temperature of 35°C (10mg). On the other hand, the highest protein content was found at pH 5.5 (3mg) and a temperature of 35°C (35mg) [173]. As a conclusion, the optimal parameters were the same for both biomass and protein production.

Nigam [174] studied the cultivation of four strains of fungi, *Trichoderma viride*, *Trichoderma reesei*, *Fusarium oxysporus*, *Chaetomium cellulolyticum*, on sugar beet pulp and molasses using flasks. The impact of different parameters on protein production (measured with the Kjeldahl method) such as inoculum volume was quantified. Indeed, the protein amount in *Trichoderma reesei* biomass had increased by increasing the volumes of inoculum (from 10 to 50 ml of inoculum/100 g pulp). The most efficient inoculum volume was 50-ml in the third day (29%) as 10-ml inoculum in the fifth day of the submerged fermentation [174].

Yeast

Kot *et al.*, [140] focused on the effect of pH. They studied the effect of initial pH on the growth of *Rhodotorula glutinis* and also on the final protein content. The yeast was grown on potato waste water and glycerol. After 72h of fermentation in flask, they determined that the initial pH (pH_i) affected the growth of the strain. Table 22 shows the results of the study. Indeed, a low initial pH (2) inhibited the growth with a concentration of 0.7 ± 0.1 g/l and the highest biomass yield was obtained with pH_i 5 (19.4 ± 0.6 g/l) (Table 22) [140]. The biomass didn't show a large variation in the protein values obtained with the Kjeldahl method, ranged from 39.5 and 41.3 % [140]. According to this study, pH is not a main factor affecting protein amounts.

Table 22 : The effect of initial pH on the growth of *Rhodotorula glutinis* and the protein content [140]

Initial pH	Biomass concentration (g/l)	Protein content (%DW)
2	0.7 ± 0.1	-
3	13 ± 1.5	41.2 ± 1.9
4	17.3 ± 0,8	40.4 ± 1.3
5	19.4 ± 0.6	40.5 ± 2.0
6	18 ± 0.5	39.9 ± 2.2
7	17.2 ± 1.4	39.5 ± 1.1

The aim of Gao *et al.*, [104] study was to optimize cultivation conditions using a marine yeast, *Cryptococcus aureus*, and inulin from artichoke as substrate for protein accumulation.

Temperature and initial pH were studied, based on a sequential mono-factorial approach. They started with studying the temperature influence at pH 5 using seawater containing 0.6 g/l of Jerusalem artichoke extract and 0.4 g/l of the hydrolysate of soybean cake. The temperature values ranged between 23 and 37°C. After 3 days of fermentation in bioreactor, 28°C was found as the best temperature leading to the highest protein content, obtained with the Kjeldahl method, 3.5 g/l of medium (Figure 7). pH effect was then investigated with the suitable temperature (28°C). Initial pH tested was ranged from 3 to 8 and the highest protein content (3.6 g/l) was estimated at pH6 (Figure 7).

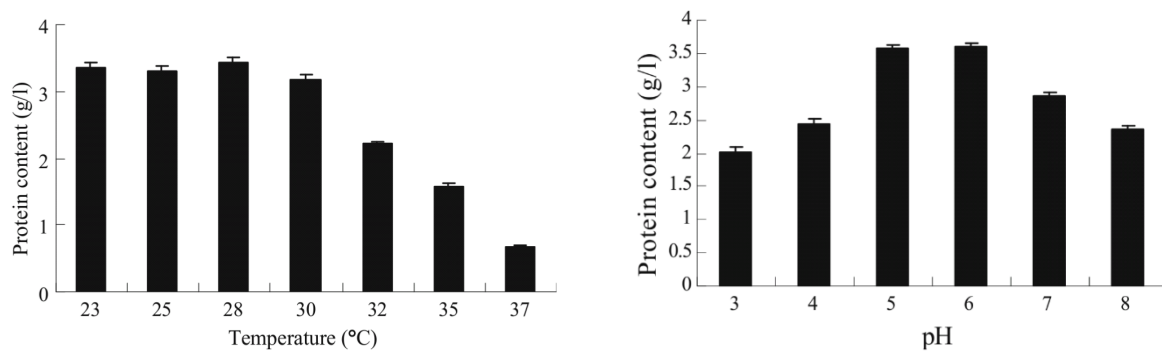


Figure 7 : Effect of temperature and pH on SCP production from *Cryptococcus aureus* [104]

Hongpattarakere and H-Kittikun [146] investigated the optimization of SCP production from *Schanniomyces castellii* using cassava starch as carbon source in conical flasks cultures. A monofactorial study was done for four cultural parameters: inoculum size (1-3 %, v/v), initial pH (2.9-6.8), temperature (24-37 °C) and shaker speed (100-250 rev/min).

For cell growth, the highest value was observed with 5% (v/v) of inoculum, pH5, 25°C and 250 rev/min with 5.27, 5.37, 5.24, and 5.24 g/l, respectively. Protein content, measured by the Lowry method, showed a higher amount (26-28%) using 7% of inoculum, pH4, 24/34°C and 200/250 rpm. However, the lowest values (0.86 g/l and 6 %) was found when the incubation temperature was raised above 34°C (Table 23) [146].

Table 23 : Effect of cultural conditions on biomass yield and protein accumulation using *Schanniomyces castellii* grown on cassava starch [146]

Factor		Cell dry mass (g/l)	Protein content (% _{DW})
Inoculum size (%, v/v)	1	3.0	22
	3	4.2	24
	5	5.27	26
	7	5.17	27
	10	5.21	26
Initial pH	2.9	0.41	9
	4.0	5.02	26
	5.0	5.37	25
	6.0	4.69	24
	6.8	4.63	23
Temperature (°C)	24	5.21	26
	28	5.24	25
	34	5.05	26
	37	0.86	6
Shaker speed (rev/min)	100	4.86	24
	150	5.18	26
	200	5.14	28
	250	5.24	28

Putra *et al* [175] studied the performance of *Saccharomyces cerevisiae* grown on date extracts as substrate using erlen flask culture. The effect of pH was evaluated from 4.5 to 5.6. The pH was uncontrolled for some cultures and controlled for others.

At uncontrolled pH, the final cell yield obtained was similar between pH 5 and 5.6. In controlled pH, the microbial growth was lower (Table 24) [175].

Table 24 : Effect of pH on *Saccharomyces cerevisiae* growth [175]

Initial pH	Final pH	Final cell mass (g/L)	Cell mass yield (g/g)
4.5	3.5	7.98	0.180
4.8	3.7	9.21	0.198
5.0	3.7	10.43	0.238
5.3	3.9	10.00	0.223
5.6	4.0	10.34	0.225
4.5*	4.5	5.60	0.159
5.0*	5.0	5.85	0.147
5.3*	5.3	5.35	0.130

*Controlled pH

Rages and Haider [166] study aimed to evaluate SCP through *Candida lipolytica* using olive fruit residuals in conical flask cultures. The protein content was measured after crushing the cells using a ceramic mortar. The initial pH value effect on protein production was investigated

in this study. As showed in Table 25, a value above 5.5 decrease the protein production (by the Lowry method) from 70 to 58 %_{DW} at pH 7.5 [166].

Table 25 : Effect of pH on *Candida lipolytica* growth and protein production [166]

Initial pH	Final pH	Protein (%_{DW})
5	6.17	71
5.5	6.36	70
6	6.76	65
6.5	6.84	55
7	7.24	59
7.5	8.15	58

Therefore, many environmental parameters were investigated in order to evaluate Single Cell Protein. Almost all the studies were done using flask cultures. They evaluated biomass concentration and/or protein content on the dry weight. The protein content was principally quantified via the total nitrogen method, Kjeldahl. However, there is no quantification of the dynamics to understand the production among the different phases of the culture. Only the final points were mentioned.

Among the environmental parameters discussed, temperature and pH were the most studied parameters. Their effects on SCP production were very dependent on the strain choice. Thus, we could not have a global conclusion of their impact.

As a global conclusion for this part, many factors could affect biomass growth and/or protein production including media composition, strain choice and environmental parameters. However, many limits could be mentioned, such as the use of non-controlled Erlenmeyer-flask cultures for almost all the studies and the absence of dynamics details on Single Cell Protein production along the cultures.

6. Drawbacks of SCP

As shown before, the amount of proteins in SCPs can range between 60 and 82% of dry cell weight which can contribute at a beneficial parity of essential amino acids [176].

Despite of this high protein content and the other environmental and production advantages, using the microbial biomass as SCP can generate some drawbacks listed below.

6.1. Nucleic acids

High nucleic acid concentration in microbial biomass can be at the origin of many health-oriented problems. Indeed, an increase in the blood of uric acid amount coming from the

degradation of the nucleic acid causes many health problems [4]. Among these problems, we can notice difficult digestion, dermic affections, gastro-intestinal disturbances and particularly kidney diseases by the formation of stone or gout [10]. Consequently, the intake of purines should be limited to 2g per day [177].

6.2. Toxins

Some strains of bacteria and fungi produced toxins during their multiplication exerting toxic complications on humans and animals.

6.2.1. Mycotoxins

Aflatoxins, ochratoxins and trichothecenes are the most important mycotoxins. They are generally produced by the *Aspergillus* and *Penicillium* stains [178]. Minimum amounts of mycotoxins are able to produce allergies, skins troubles and many other health disorders.

Aflatoxins can cause many health problems from immunity diseases to death. Aflatoxins B1, B2, G1, G2, M1 and M2 are the major forms and the most puissant carcinogen is Aflatoxin B1 [179]. Among the ochratoxin family, ochratoxin A is the most plentiful and undesirable. It may cause destruction of liver and kidneys [180]. Added to aflatoxins and mycotoxins, trichothecenes engender dermatologic and hematologic problems [181].

6.2.2. Bacterial toxins

Bacteria can produce two types of toxins: endotoxins and exotoxins. Endotoxins are generated by gram-negative bacteria. They are lipopolysaccharides that liberated upon lysis and which are the major compound of the cell envelope [182]. This compound is are related to many health diseases such as diabetes, neurological problems and gut inflammation and liver damage [183]. The lipid A portion is responsible for the toxicity [184]. Exotoxins such as enterotoxin, erythrogenic toxin, alpha-toxin, and neurotoxin are caused by gram-positive bacteria. Those toxins can engender generalized symptoms and lesions [6].

6.3. Non-digestible cell-wall

A problem of presence of cell wall which is non-digestible, in case of algae, yeast and fungi may be noticed. Indeed, humans and other non-ruminants cannot digest the cellulose of the algal wall because of the lack the cellulolytic enzyme in his organism [156].

7. SCP processing for food

To maintain international safety standards, some quality controls procedures must be done before using the final product as a food source [27]. Thus, cell walls and toxic compounds should be removed and nucleic acid must be reduced to acceptable values [177].

7.1. Nucleic acid reduction

Nucleic acids, such as purine, should be reduced in SCPs [3]. Chemical and enzymatic methods can be used for that [9]. They include treatment with NaOH or NaCl (10%), thermal shock and activation of endogenous nucleases in the final biomass [185].

Abou-Zeid *et al.*, [186] studied the effect of protein extraction with NaOH on the nucleic acid content of yeast biomass which passed from 7.03 %_{DW} to 1.5 %_{DW}. They also presented the effects of heat shock process (temperature and duration) on the reduction of nucleic acid which reached 69.42 %_{DW} at 70°C for 80s. A third method, NaCl treatment, was applied and the best concentration of NaCl was 4%, which caused 34.2% reduction of RNA [186].

7.2. Mycotoxins' reduction

Treatments have been focused principally on aflatoxins. Ammonization is the most successful method tested since it can decrease the toxicity level by 99%. Nowadays, molecular biology techniques are utilized in order to isolate the aflatoxins pathway from biomass such as probing, transcript mapping, expression libraries, cloning, sequencing, gene disruption, and chromosome walking [6].

7.3. Bacterial toxins reduction

Present in soluble form in the medium, exotoxins can be simply removed. Formaldehyde, diluted acids, alcohol (50%) and high temperatures (>60°C) can convert these toxins into nontoxic toxoids which are beneficial to immunization or denature them [6].

However, endotoxins removal is more difficult since they are part of cellular constituents of gram-negative bacteria, thus, to prevent their formation, engineering techniques should be applied be modified or suppressed [6].

7.4. Liberation of cell protein by destruction of indigestible cell wall

The digestibility is a crucial criterion of using SCP as food ingredient. The destruction of the cell wall can be done by enzymatic or mechanical methods.

The enzymatic digestion requires endogenous or exogenous enzymes of other microorganisms or autolytic enzymes under the influence of organic solvents (chloroform, alcohol, ethyl acetate, etc.) and mineral salts (NaCl, etc.) For example, good results were shown with NaCl (2-10 %) and ethanol (1-9 %) application on yeast biomasses [187].

The mechanical cell disintegration can be processed by grinding, crumbling, crushing, ultrasonification, and pressure homogenization [187].

8. Comparison of SCP sources

Biomasses produced from different unicellular microorganisms presented different characteristics and compositions. These features could be classified as advantages and

drawbacks. Table 26 summarizes them. The comparison between the four types was based, principally, on cell growth and chemical composition of biomass.

Table 26 : Comparison of different SCP sources [3, 9, 40, 156, 188]

SCP Source	Advantages	Drawbacks
Algae	<ul style="list-style-type: none"> • Simple cultivation, • effective utilization of solar energy, • faster growth, • high protein content, • high nutrient content [40] 	<ul style="list-style-type: none"> • dark green color • fishy smell • non-digestible cell wall [156]
Bacteria	<ul style="list-style-type: none"> • Rapid growth, • short generation times, • Capable of growing on a variety of raw material. [9] 	<ul style="list-style-type: none"> • Harvesting of cells is difficult and costly because of their low density and small size • High nucleic acid content compared to fungi and yeasts
Fungi	<ul style="list-style-type: none"> • Ease of harvesting [188] 	<ul style="list-style-type: none"> • Lower growth rates • Lower protein content • Acceptability [188]
Yeasts	<ul style="list-style-type: none"> • Ease of harvesting • High lysine content • Can grow at acidic pH • Familiarity and acceptability [9] • Absence of toxic and carcinogenic compounds [188] 	<ul style="list-style-type: none"> • lower growth rates compared to bacteria • lower protein content than bacteria • lower methionine content than bacteria [3]

9. SCP as food and feed

SCPs derived from algae, bacteria, filamentous fungi, yeasts or mixed cultures can be utilized for both human consumption and animal feed. Table 27 shows examples of application categories with examples of strains utilization for food or feed.

Table 27 : Possibilities of application for SCPs [3]

Human consumption	Animal feed
<ul style="list-style-type: none"> • As emulsifying aids • As aroma carriers • As vitamin carriers • To improve the nutritive value of baked products • In soups • In ready-to-serve meals • In diet recipes 	<ul style="list-style-type: none"> • Fattening calves • Feed for laying hens • Fattening poultry • Fattening pigs • Fish breeding • Feed off domestic animals

Nowadays, many companies developed their own food and feed products which are based on microbial biomass. Table 28 presents some examples of these products.

Table 28 : Examples of microbial food and feed products [189, 190]

Type of microorganism	Strain	Product name
Algae	<i>Aurantiochytrium limacinum</i>	All-G Rich
	<i>Arthrospira sp.</i>	Spirulina
Bacteria	<i>Methylococcus capsulatus</i>	FeedKind
	<i>Methylobacterium extorquens</i>	KnipBio Meal
	<i>Methylophilus methylotrophus</i>	Pruteen
	<i>Methylococcus capsulatus</i>	UniProtein
	<i>Cupriavidus necator</i>	Novo-Nutrients Solar Foods Kiverdi
Filamentous fungus	<i>Fusarium venenatum</i>	Quorn
Yeast	<i>Pichia pastoris</i>	Provesta

Chapter I-2: *Cupriavidus necator*1. *Cupriavidus necator* bacterium

Cupriavidus necator is a gram negative, non-pathogenic, facultative chemo-lithoautotrophic bacterium belonging to the beta-proteobacteria class [191]. Indeed, when growing in autotrophic conditions, it is able to use molecular hydrogen as an oxidizable energy source [192, 193]. So, it has the ability to grow under both heterotrophic and autotrophic conditions (Figure 8) with the ability to produce PolyhydroxyButyricAcid (PHB) granules [194].

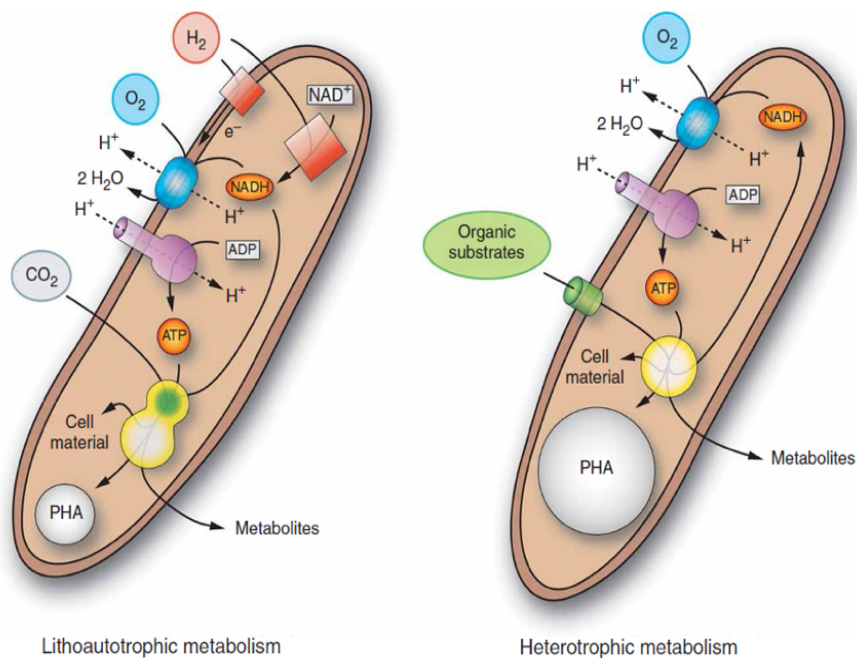


Figure 8 : Aspects of lithoautotrophic and heterotrophic metabolism of *Cupriavidus necator*. (yellow circle : central metabolism, yellow/green circle : the Calvin-Benson-Bassham cycle, red squares : the two energy-conserving hydrogenases, gray circles : PolyHydroxyAlkanoates (PHA) storage granules) [194]

The phylogenetic classification of this bacterium has been modified many times. Since mid-2004, it was identified as “*Cupriavidus necator*” [195]. Previously, this bacterium was identified as: *Hydrogenomonas eutropha* (between 1958 and 1969) [196], *Alcaligenes eutrophus* (between 1969 and 1995) [192], *Ralstonia eutropha* (between 1995 and 2004) [197] and *Wautersia eutropha* (from the beginning of 2004 to mid-2004) [198].

2. *C. necator* metabolism

- **Heterotrophic metabolism**

Many carbon sources can be assimilated by *Cupriavidus necator* including organic acids (formate, fumarate, succinate, aspartate, malate,...), fatty acids (palmitic acid, oleic acid, linoleic acid, ...), aromatic compounds, some carbohydrates (fructose, xylose,...) and some alcohol and polyols [194, 199-202]. Wild strains of *Cupriavidus necator* are able to assimilate fructose [203] but not glucose, mannose and ribose [200]. They have not the phosphofructokinase to convert Fructose-6-Phosphate to Fructose-1,6-diphosphate [203], therefore the Entner-Doudoroff pathway is used (EDD and EDA, Figure 9). Some spontaneous mutants' strains are able to consume glucose such as DSM 545 and CECT4623 [204, 205].

- **Autotrophic metabolism**

Calvin-Benson-Bassham (CBB) cycle allows the fixation of carbon dioxide in *C. necator* [194] and Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) is the enzyme which catalyzes the CO₂ accumulation by the microorganism.

C. necator can use formate, as organo-autotrophic source, since it contains formate dehydrogenase in its genome [206].

Figure 9 shows a schematic presentation of *C. necator* central metabolism with also the formic acid and the oleic acid pathways that will be used during this PhD work.

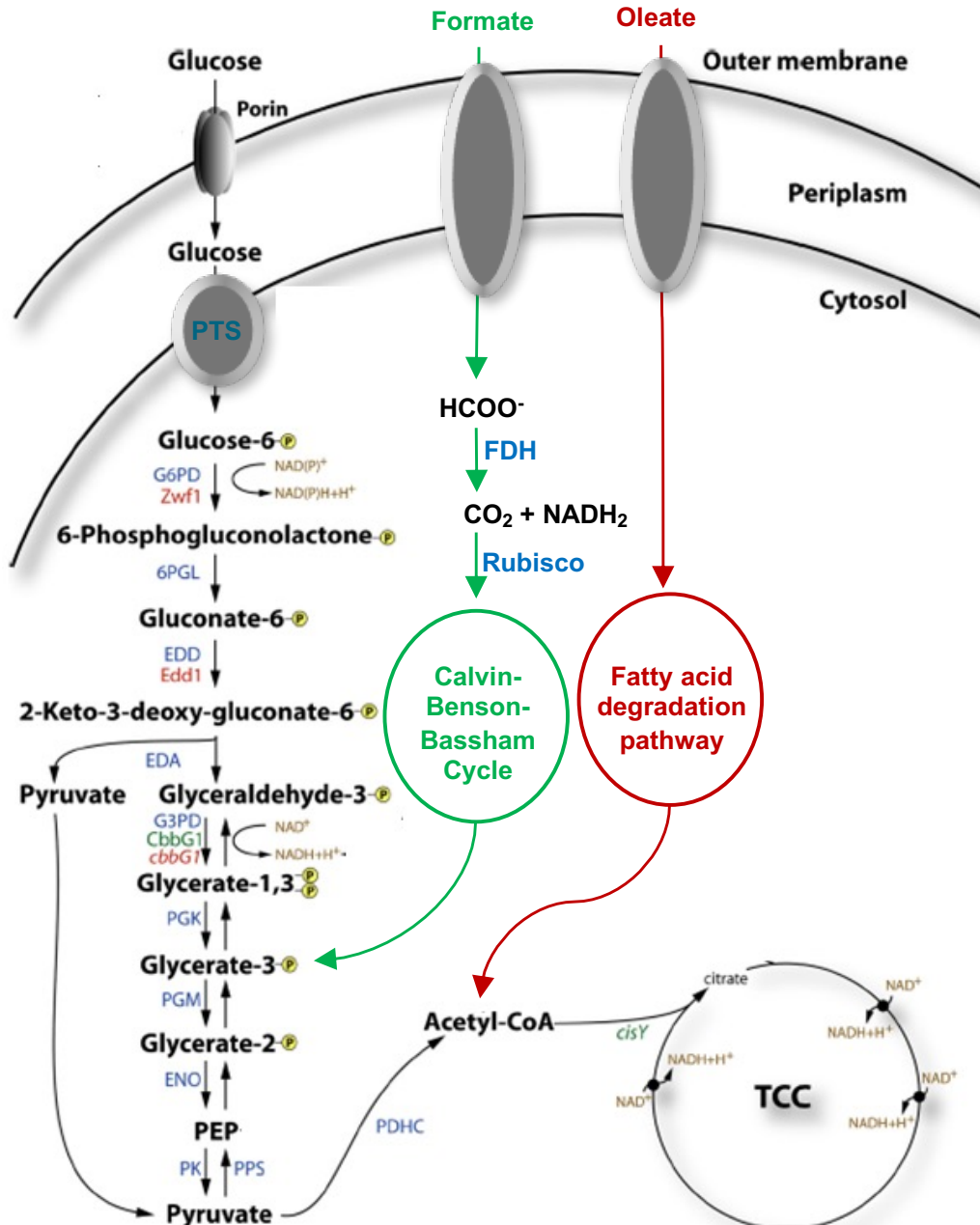


Figure 9 : Schematic presentation of the **central metabolism** of *Cupriavidus necator* including **formic acid** and **oleic acid** pathways [204, 207].

Abbreviations:

Porin (outer-membrane protein); PTS (Phospho Transferase System); G6PD/Zwf1 (glucose-6-phosphate 1-dehydrogenase); 6PGL (6-phosphogluconolactonase); EDD/Edd1 (phosphogluconate dehydratase); EDA (2-keto-3-deoxygluconate-6-phosphate aldolase); G3PD/CbbG1/cbbG1 (glyceraldehyde-3-phosphate dehydrogenase); PGM (phosphoglycerate mutase); ENO (enolase); PK (pyruvate kinase); PPS (phosphoenolpyruvate kinase); PDHC (pyruvate dehydrogenase multienzyme complex); TCC (citric acid cycle = Krebs cycle); FDH (formate dehydrogenase); G2-P (glyceraldehyde 2-phosphate); G3-P (glyceraldehyde 3-phosphate); Rubisco (ribulosebiphosphate carboxylase).

3. *C. necator* in biotechnology

3.1. PolyHydroxyAlkanoates (PHA) biosynthesis

Cupriavidus necator was found able to synthesize bioplastics which are PolyHydroxyAlkanoates (PHAs). They present an alternative to synthetic plastics due to their durability and thermal stability [25]. Among companies that utilized this bacterium for PHA production, we found ICI (United Kingdom), Monsanto (USA) and Tianjin Northern Food (China). According to its carbon number, PHA presents three major groups: short chain length (C3-C5), medium chain length (C6-C14) and long chain length [25, 208] (Figure 10).

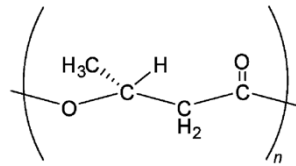


Figure 10 : Chemical structure of PHA (n : number of repeating unit of PHB) [25]

Poly-(3-hydroxybutyrate) (PHB) (Figure 11) is the most known type of PHA [209]. PolyHydroxyAlkanoates PHB properties have attracted researcher's interest [210]. Many studies had the objective to investigate the PHB production in *C. necator* using limitation condition such as nitrogen [102, 211], oxygen [211-213], substrates [199, 212-216] and carbon/nitrogen ratio [217, 218] and/or environmental stress factors [219-223].

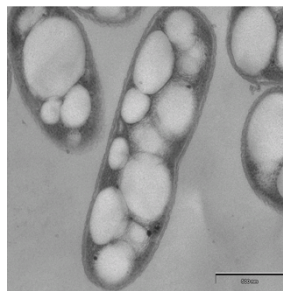


Figure 11 : Microscopic image of PHB granules in *C. necator* (0.5 μm) [224]

3.2. Single Cell Proteins production

In the 1970s, *Cupriavidus necator* was found as a good candidate for SCP production for human consumption and animal feed [210]. Generally, bacterial cells contain at least 50 %_{DW} of protein [225]. Foster and Litchfield [226] published the first study of using *C. necator* as SCP source. The final biomass contained 74% of protein using CO₂ as substrate which is a high content

compared to other microorganisms that were around 50% [226]. Table 29 compared *C. necator* chemical composition with other microorganisms.

Table 29 : Chemical composition of *C. necator* cultivated on CO₂ in comparison with and other microorganisms [25, 99, 226]

	<i>C. necator</i>	Bacteria	Yeast	Fungi	Algae
Protein (%_{DW})	74	50-65	45-55	30-45	40-60
Nitrogen (%)	12-14	11.5-12.5	7.5-8.5	5-8	7.5-10
Fat (g/100g_{DW})	9	8-10	1-8.1	1.3-4.4	3-16
Nucleic acid (%_{DW})	7-8	8-12	6-12	7-10	3-8
Ash (%_{DW})	1.73	3-7	5-9.5	9-14	8-10

Most of the older studies focus on the protein production using *C. necator* grown under autotrophic conditions [99, 158, 212, 224, 227-229]. Some studies investigated the effect of different factors on growth and protein production [167, 169, 212].

In autotrophic cultures, a study performed in continuous mode investigated the effects of the dilution rate (0.1; 0.2; 0.3 h⁻¹) and also of H₂/O₂ ratio of gas-mixture feeding system (0.2; 0.4; 0.6, at a dilution rate of 0.1 h⁻¹) on cell composition including proteins and nucleic acids [212]. The results showed that increasing the dilution rate from 0.1 to 0.3 h⁻¹ increased the protein content (measured with modified Biuret method) from 62 to 80%_{DW} and also the nucleic acid (extracted with perchloric acid and quantified with optical density) percentage from 9 to 15%_{DW}. Regarding the H₂/O₂ ratio, the protein content decreased from 80 to 60%_{DW} when the ratio passed from 0.2 to 0.6. However, no variation on nucleic acid content was obtained (12%_{DW}) [212].

Sakarika *et al.* [167] compared protein production from different microorganisms using different CO₂ sourced substrates using Erlenmeyer-flask cultures. They showed that *C. necator* is a promising bacterium for protein production on formate and acetate with amounts between 40 and 60%_{DW} using the Markwell method [167].

Another study of Yang *et al.* [169] also studied the effect of ammonium concentration and nitrogen form used on *C. necator* (335) growth and protein production in autotrophic conditions using glass bottles' cultures. Indeed, the cell growth was inhibited at high ammonium concentrations and urea was the best nitrogen form for growth [169]. Added to that, the biomass was lyophilized and then, the crude protein amounts were measured using the BCA method (Pierce™ BCA protein assay kit). The protein content was higher with ammonium (83.4 ± 3.8%) followed by nitrate (74.9 ± 4.7%) and urea (65.3 ± 2.8%).

Regarding the protein composition, the amino acid profiles suggest that *Hydrogenomonas eutropha* may be potentially used as a protein supplement [99]. Indeed, the amino acid profile was equal or surpassed animal and vegetal proteins (Table 6). Compared to the other microorganisms, lysine content were higher between 7 and 9 g/100 g of protein (Table 6) [99, 158, 226].

As already detailed in the first chapter, lipopolysaccharide secretion is one of the drawbacks in gram-negative bacteria, including *Cupriavidus necator*. Indeed, these endotoxins are included in the cell-envelope structure leading to many health diseases [182, 183]. Thus, production of feed or food from gram negative bacteria is worrying [230, 231]. These endotoxins are in general purified with PHA during their extraction [232]. Thus, the PHA extraction guaranty an endotoxin-free biomass. Some studies that are interested on PHA production from *Cupriavidus necator*, investigated its removal from the bioplastics, which became an important biotechnological concern. For instance, Gahlawat *et al.*, [232] compared the use of different alkalis (KOH and NH₄OH and NaOH) to remove this undesirable compound [232].

However, these compounds haven't been evaluated in Single Cell Proteins' papers, yet.

Chapter I-3: Conclusions and objectives of the PhD work

Research for new sources of protein is a major concern of world wide agricultural policies in order to be able to fulfill protein needs of the growing world population. Production of Single Cell Protein (SCP) is a potentially alternative source of protein for food and feed since it shows many interesting features such as its independence to seasonality, global warming, and environmental pollution such as pesticides.

The **first chapter** of this part described Single Cell Proteins: definition, microorganisms, substrates, production strategies, biomass composition, factor influencing growth and protein production and some drawbacks.

A high amount of protein is obtained using algae, fungi, bacteria and yeast, which exceed 50% of the biomass dry weight. Thus, microorganisms are a promising source for alternative protein production. In addition, they present a complete amino acids profile with presence of all the essential amino acids.

The essential amino acids amounts are equal or surpassed vegetal and animal proteins. Many parameters and cultural conditions could promote variations in biomass quantity and composition. Indeed, using agricultural/industrial residuals or defined chemical media, many studies investigated the effect of substrate nature, media composition, strain type, environment parameters, etc. in SCP production.

Generally, these investigations were performed in Erlenmeyer-flask cultures not in bioreactor, under well controlled parameters. In addition, there is no description of the dynamics of protein production along the cultures in these publications. Often, only the final values were presented and discussed. Also, it is difficult to compare these works because the extraction and the quantification methods of proteins are not always the same. For instance, regarding protein quantification, colorimetric assays or total nitrogen assays could be used. The results were also often dependent on the strain which is a very important factor to fix in a SCP study.

Despite the good chemical composition (especially protein amounts), using the microbial biomass as SCP can generate some drawbacks such as toxins production by fungi or gram-negative bacteria, non-digestible cell wall and the high amounts of nucleic acids. Thus, some procedures were tried in order to maintain international safety standards in microbial proteins. Indeed, cell walls and toxic compounds should be removed and nucleic acid must be reduced to acceptable values. However, there is no article investigating the evaluation of the amounts of these compounds in complementary of Single Cell Protein production, yet.

The **second chapter** of this part focused on *Cupriavidus necator*, a facultative chemolithoautotrophic bacterium. Its metabolism and its biotechnological applications were presented. Indeed, it can grow in both heterotrophic and autotrophic conditions thanks to its specific metabolism. *C. necator* was also known as a promising candidate for biotechnology applications. For Single Cell Protein production studies, *Cupriavidus necator* gave high protein amounts reaching 80% of the dry weight with a complete and an interesting amino acid profile. However, only few studies were investigated in this context, compared to Poly-(3-hydroxybutyrate) papers. In addition, almost all SCP studies were investigated using autotrophic conditions.

Some drawbacks related to *C. necator* biomass were also detailed. Endotoxins could be produced because of its gram-negative cell structure. Added to that, the nucleic acid amount presents a problem that should be reduced to an acceptable value for consumption.

C. necator is a good candidate for Single Cell Protein production since it gives more than 70%_{DW} of proteins. However, we should be aware about the nucleic acid amounts and the endotoxins production.

Considering the factors influencing Single Cell Proteins production and *Cupriavidus necator* characteristics, the scientific questions during my thesis, will be the following:

- What are the optimized methods for protein and nucleic acids extraction and quantification in *Cupriavidus necator*?
- What are the impacts of environmental parameters on the dynamics of growth and protein production using *Cupriavidus necator*?
- What are the impacts of substrate composition on the dynamics of growth and protein production using *Cupriavidus necator*?

Part II - Materials and Methods

Part II - Materials and Methods

This chapter describes experimental materials and methods used for experiments. It deals with the chosen strain, the culture media used and the chosen culture conditions. In addition, it presents the analytical methods used to quantify the biological dynamics of *C. necator*. Also, biochemical methods of extraction and quantification of proteins and nucleic acids were described.

1. Strain

The strain used in this study is *Cupriavidus necator* CECT4623 (NCIMB 11599) [215]. This strain is a H16 derivate glucose utilizing mutant [205]. Growth conditions of the *C. necator* on minimum medium are often reported at a temperature of 30 °C and a pH 7 [107, 233, 234].

2. Culture media

Two types of growth media were used for *C. necator* cell cultures: rich (TSB) and mineral media (MIT and FAME). All reagents used for medium preparation were of the highest grade commercially available.

2.1. Rich media

The Tryptic Soy Broth TSB medium (*Becto, Dickson and Company, Le Pont de Claix, France*) is used for colony isolation (solid form) and also for cellular revivification (liquid form). Its composition is described in Table 30. It was prepared with osmosis water and then sterilized by autoclaving (121 °C, 20 min).

Table 30 : Composition of the rich media TSB

Compound	Composition (g/l)	
	TSB (liquid)	TSB (solid)
Tryptic Soy Broth	27.5	27.5
Agar	0	20

2.2. Minimum media

2.2.1. Stock solution preparation

Concentrated stock solutions of each compound were prepared separately and sterilized by autoclaving (121 °C, 20 min). However, Na₂HPO₄ stock solution and Ammonium Iron (III) Citrate (28%) solution (and urea if used) were sterilized by filtering through a 0.22 µm syringe-fitted filter.

For culture using oleic acid as a carbon source, a mixture was elaborated using sterilized compounds (20 volumes of oleic acid, 80 volumes of osmosis water and 0.5 volume of tween 40). The mixture is, then, sonicated with a sonicator (Qsonica, Newtown, USA) equipped with a probe of 6mm of diameter to create a homogenous emulsion (Figure 12). Indeed, the mixture was subjected to 3 sonication cycles: 60 seconds of pulse followed by 60 seconds of rest (amplitude 32%).

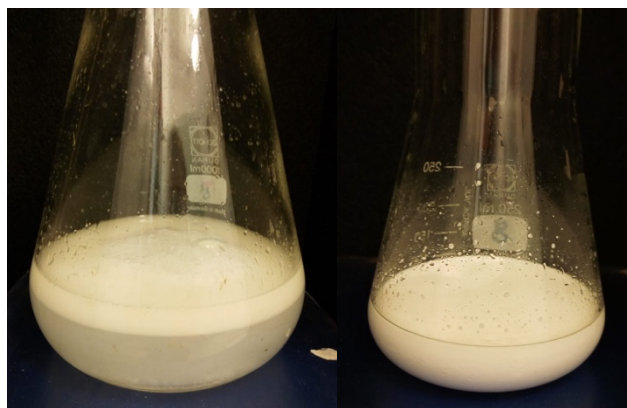


Figure 12: Oleic acid mixture before and after sonication

2.2.2. MIT medium

An optimized mineral medium developed at MIT for *Cupriavidus* was used for flask cultures because of its higher buffering capacity (Table 31). To prepare the MIT medium, stock solutions were mixed in a sterile Schott flask containing sterile osmosis water according to the composition presented in Table 31. pH reached a value of 6.8 due to phosphate buffer used ($\text{Na}_2\text{HPO}_4 / \text{NaH}_2\text{PO}_4$). A biomass concentration of 4 $\text{g}_{\text{CDW}}/\text{l}$ can be reached, based on the amount of nitrogen in this medium.

2.2.3. FAME medium

The mineral FAME medium developed in our team was used for all bioreactor fermentations to reach higher cell concentration (design for 10 $\text{g}_{\text{CDW}}/\text{L}$). It was prepared as described in Table 32. In bioreactor, compounds were mixed in osmosis water according to the composition presented in Table 32. The pH was adjusted to 7 using potassium hydroxide solution (4M) and the mixture was sterilized by autoclaving (121°C, 20 min). After cooling, oligo-elements and glucose (and urea if used) stock solutions were added sterilely. A biomass of 10 $\text{g}_{\text{CDW}}/\text{l}$ can be reached.

Table 31 : Composition of the mineral medium MIT

	Compounds	Concentration (g/l)
Oligo-elements	NaH ₂ PO ₄ , 2 H ₂ O	5.2
	Na ₂ HPO ₄ , 12 H ₂ O	11.6
	K ₂ SO ₄	0.45
	NaOH	0.04
	MgSO ₄ , 7 H ₂ O	0.8
	CaCl ₂ , 2 H ₂ O	0.08
Trace solution	FeSO ₄ ·7H ₂ O	0.0150
	MnSO ₄ ·H ₂ O	0.0024
	ZnSO ₄ ·7H ₂ O	0.0024
	CuSO ₄ ·5H ₂ O	0.0005
Carbon source	Glucose	10
	Or Formic Acid + Glucose (*)	6 and 1 respectively
	Or Oleic Acid mixture (**)	5
Nitrogen source	NH ₄ Cl	1
	Or Urea (***)	1.4

(*) preculture for formic acid culture
 (**) preculture for oleic acid culture
 (***) preculture for urea culture

Table 32 : Composition of the minimum medium FAME

	Compounds	Concentration (g/l)
Macro-elements	(NH ₄) ₂ SO ₄	2.8
	Or Urea (***)	2.8
	MgSO ₄ ·7H ₂ O	0.75
	NitriloTriacetic Acid	0.285
Oligo-elements	CaCl ₂ ·2H ₂ O	0.015
Rich phosphate solution	Na ₂ HPO ₄ ·12H ₂ O	1.568
	KH ₂ PO ₄	0.263
Element solution	H ₃ BO ₃	0.0009
	CoCl ₂ ·6H ₂ O	0.0006
	ZnSO ₄ ·7H ₂ O	0.0003
	MnCl ₂ ·4H ₂ O	0.000009
	Na ₂ MoO ₄ ·2H ₂ O	0.000009
	CuSO ₄ ·5H ₂ O	0.00003
	NiCl ₂ ·6H ₂ O	0.00006
Ammonium Iron (III) Citrate (28%) solution		0.09
Carbon source	Glucose	20
	Or formic acid (*)	1
	Or oleic acid mixture (**)	10

(*) medium for formic acid culture
 (**) medium for oleic acid culture
 (***) medium for urea culture

3. Culture conditions

3.1. Glycerol cell stock preparation

At the beginning of the thesis work, *C. necator* cell frozen stocks were prepared as follows: From a TSB agar plate of the *C. necator* strain, a single colony was picked and transferred into a 50 mL sterile baffled shake flask containing 4.8 mL of TSB medium. After incubation (24 h, 30 °C, 120 rpm), this culture was used to inoculate 43.2 ml of the same medium in 1l-baffled shake flask. The culture was then incubated (24 h, 30 °C, and 120 rpm). The culture (48ml) was mixed to 16 ml of pure sterile glycerol solution to reach a final concentration of 30% (v/v). Finally, the prepared stock culture was aliquoted into sterile cryotubes (0.8 mL each) and stored at -80 °C.

Sterilization of glycerol and TSB medium was carried out by autoclaving (121 °C, 20 min).

All the experiments presented in the manuscript were performed with cells from the same batch of glycerol stock.

3.2. Erlenmeyer Flask cultures

For each culture, one glycerol stock kept at -80°C was plated on TSB plates and incubated at 30 °C for 48h. One isolated colony was used to inoculate a 50 mL flask culture containing 5 ml of TSB medium, which was grown for 24 h at 30 °C and 150 rpm. Subsequently, a second subculture at 10% (v/v) was carried out using 30 ml MIT mineral medium MIT. Finally, cells arising from the second subculture were used to inoculate at 10 % (v/v) a last subculture in a culture flask containing 300 ml of the MIT mineral medium. The two final pre-cultures were incubated at 30 °C for 12h at 150 rpm (Figure 13).

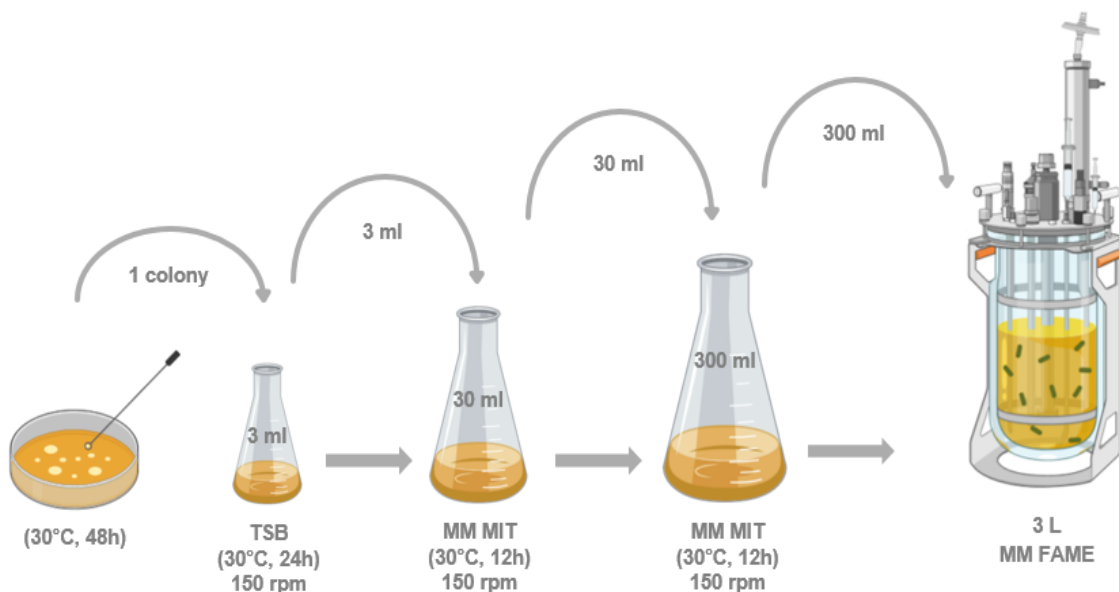


Figure 13 : Precultures scheme for bioreactor culture (from Biorender)

3.3. Cultures in bioreactor

Figure 14 presents a bioreactor setup model used during my PhD cultures including bioreactor assembly, control and regulation systems and gas analysis monitor system.



Figure 14 : Bioreactor set-up

3.3.1. Experimental setup

Bioreactor cultures were conducted in a 5L stainless-steel stirred tank bioreactor (*BIOSTAT® Bplus, Sartorius, Germany*). The working volume was 3 liters.

Both stirred tank types were fitted with two six-bladed Rushton impellers and four equally spaced baffles.

The system is equipped with control systems of:

- pH (*pressurized gel-filled pH electrode, Mettler Toledo, Columbus, OH, USA*)
- Temperature
- Partial pressure of dioxygen (pO_2) (*optical oxygen sensor InPro 6860i, Mettler Toledo, Columbus, OH, USA*)
- Agitation rate
- Aeration flow rate

MFCS/win 2.1 software was used for data acquisition of the online measurement during fermentation. Each culture was operated and automatically controlled with the BIOSTAT® Bplus control tower.

3.3.2. Control and regulations

The pH probe was calibrated before sterilization using standard buffer solutions at pH4 and 7. The pH value was maintained by automatic addition of an ammoniac solution (14.13 mol/l).

- For urea culture, both KOH solution (at 16 mol/l) and a H₃PO₄ solution (3.65 mol/l) were used.
- For formic acid culture, a solution of formic acid was prepared in an ammoniac solution (14.13mol/l) at a concentration of 25.97 mole/l for pH control.

Temperature was regulated by fluid circulation in the jacket of the reactor.

Temperature and pH control systems consist of probe and electrode linked to PID controller modules.

Calibration of the dissolved oxygen probe was carried out after sterilization at a pO₂ of 0% then at 100 % in the bioreactor, after bubbling with pure Nitrogen and air, respectively.

In order to ensure highly aerobic conditions throughout the cultures, a partial oxygen pressure (pO₂) higher than 30% of saturation was maintained manually or automatically using regulation cascades.

During the cultures, agitation and aeration flow rates were increased from 300 to 1000 rpm and from 0.3 to 1.4 l/min respectively, depending on culture conditions.

3.3.3. Bioreactor cultures

The mineral medium FAME (composed only of the macro-elements) was sterilized in the bioreactor by autoclaving (20 min, 121 °C). After cooling, the medium was completed (Table 32): a pre-sterilized phosphate saline stock solution is added via a septum using a sterile syringe. For batch cultures, the carbon source was injected into the bioreactor using a stainless-steel cannula. For the pH-controlled fed batch (pH-stat), an initial volume of formic acid solution was added via a stainless-steel cannula and then formic acid was added automatically via the pH regulation pump to maintain the pH constant during the cultivation [206].

To avoid foam formation during the culture, an antifoam (Polypropylene Glycol, PPG) was added manually. To ensure sterility, the incoming gas crossed a PTFE membrane filter of 0.2 µm pore size (Midisart®, Sartorius).

A 300 ml inoculum from the last preculture step (Figure 13), was used to inoculate the bioreactor at an initial biomass concentration between 0.2 and 0.4 g_{CDW}/l by means a stainless-steel cannula.

3.3.4. Gas analysis

During fermentation cultures, a fermentation gas monitor system (*LumaSense Technologies Europe*) measures inlet and outlet gas compositions. It is composed of a multipoint sampler 1309 (*INNOVA 1309, LuminaSense Technology Europe, Frankfurt, Germany*) combined with a gas analyzer (*INNOVA 1313, LuminaSense Technology Europe, Frankfurt, Germany*). Regular and precise measurements of both oxygen (O₂) and carbon dioxide (CO₂) molar fractions are achieved by magneto-acoustic and photo-acoustic spectroscopic methods. Gas analysis data were recorded every minute throughout fermentations with the *BZ 6003* software. The gas analyzer was verified using a standard gas mixture composed of 5% CO₂ and 10% O₂.

3.3.5. Sampling procedure description

Generally, broth samples were harvested at approximately 2-3h intervals all along the fermentation.

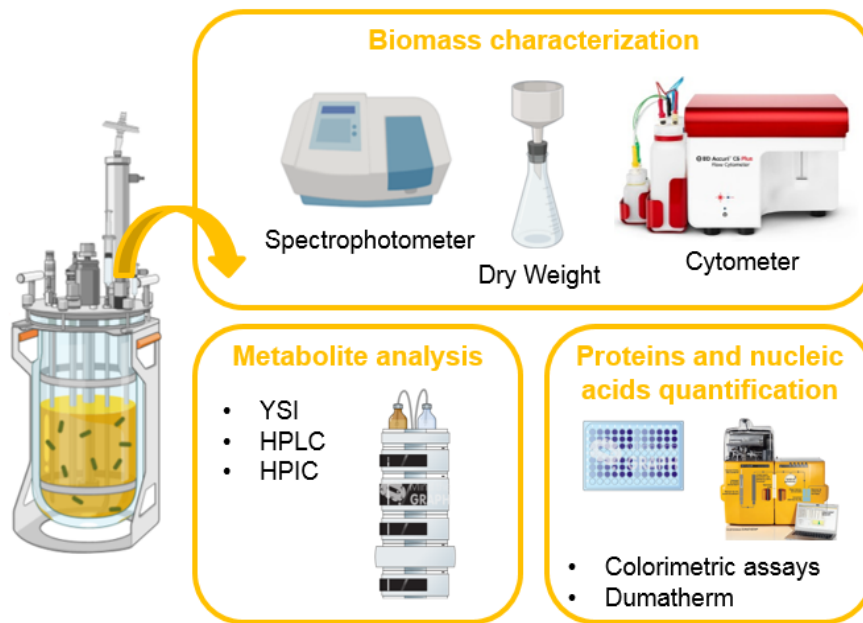


Figure 15 : Off-line analysis during bioreactors cultures (from Biorender)

Samples were taken aseptically from the bioreactor (Figure 15). Off-line analysis was achieved on culture samples. A sample was initially taken for optical density (OD), cell dry weight (DCW), cell viability and PHB detection by flow cytometry and extracellular metabolite (glucose/organic acid/fatty acid) quantification by HPLC. Then, considering the OD value, another sample was taken for proteins and nucleic acids quantification.

4. Analytical techniques

4.1. Biomass characterization

4.1.1. Biomass concentration

4.1.1.1. Optical density

To monitor biomass growth, optical density was measured at 600 nm (OD_{600nm}) using a visible spectrophotometer (*DR3900, Hachlange, Loveland, Colorado, USA*) with a 2 mm path length absorption cell (*Hellma, Darmstadt, Germany*). To be within the linear working range of the spectrophotometer for the absorbance reading (between 0.1 and 0.6 absorbance unit), culture samples were diluted when necessary.

To avoid interferences of oleic acid on OD measurements, 1 volume of isopropanol was added to 1 volume of cell culture in order to dissolve oleic acid in alcohol. After centrifugation, 1 volume of osmosis water was added to the pellet. This sample was used for OD measurement.

4.1.1.2. Dry cell weight

Dry Cell weight (DCW, in g/l) was determined by thermogravimetry. A volume of culture medium (containing as least 10 mg of cells equivalent dried mass) was harvested and filtrated through a 0.2 μm pore-size pre-dried and pre-weighed polyamide membrane (*Sartorius, Göttingen, Germany*) with a vacuum pump. The membrane was then washed with distilled water in order to remove traces of salts, and then dried in an oven (*Heraeus, France*) to a constant weight at 60°C under partial vacuum (200 mmHg) for 48h. The dry biomass concentration (g/l) was then calculated from the difference of weights of the filter over the sample volume. Based on the correlation, 1 unit of OD_{600nm} corresponds to 1.5 or 2 g_{CDW}/l .

For the culture using oleic acid as a carbon source, dry weights were quantified after drying cell pellets with Genevac (*EZ-2 Series, Warminster, United Kingdom*) after a washing step with isopropanol.

4.1.1.3. C.H.O.N.S elemental analysis

- **Sample preparation**

Cells, collected at the end of every culture, were dried using Genevac (*EZ-2 Series, Warminster, United Kingdom*) then analyzed in order to determine the elemental composition of the biomass C.H.O.N.S). This analysis was performed by the technological platform Toulouse White Biotechnology (UMS-TWB). 10 mg was the quantity of dry cell needed to the biomass elemental composition.

- **Quantification principle**

The Carbon (C), Hydrogen (H) and Nitrogen (N) elements were detected by combustion of the sample in an oxygen (O_2)/ helium (He) gaseous flow at 1050 °C.

C, H and N interact with O₂ as follows: C → CO₂; H → H₂O; N → N_xO_y

The gaseous nitrogen oxide (N_xO_y) is reduced to N₂ at 450°C in presence of copper (Cu). Then, the combustion products (CO₂, H₂O and N₂) were measured by gas chromatography.

The Oxygen (O) was measured through the *pyrolysis* of the *sample*, over carbon in a nitrogen flow, at 1120°C: O + C → CO. The carbon monoxide (CO) was *fractionated on a chromatographic column* and quantified by *a specific infrared detector*.

4.1.2. Flow cytometry

Cell permeability (measured as Propidium Iodide (PI) permeability) was monitored by flow cytometry. In addition, a detection of presence or not of intracellular PHB was done using fluorescent Nile Red (NR) dye.

4.1.2.1. Flow cytometer principle

Flow cytometry is a laser-based device enabling the characterization of single-cells from light scattering and fluorescence analysis. Individual cells pass in single-file through a focused fluid stream. Scattered light and fluorescence emissions generated by every single-cell pathing through the excitation lasers are collected by detectors and transmitted to a computer for data analysis by suitable software.

As shows Figure 16, a flow cytometer is composed of : a light source, a flow cell, a hydraulic fluidic system, several optical filters, detectors and a data processing unit [235-239].

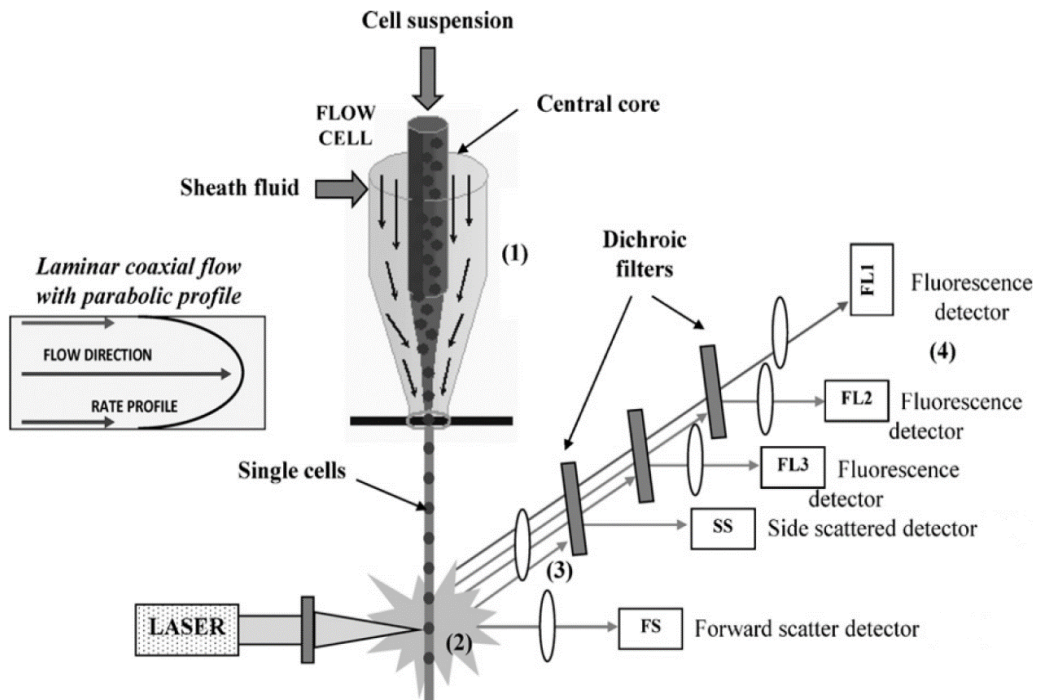


Figure 16: Simplified representation of a common flow cytometer setup [239].

BD Accuri C6[®] flow cytometer (*BD Biosciences BD Biosciences, Franklin Lakes, NJ, USA*) was used for these studies. The device was equipped with blue (488 nm) and red (640 nm) excitation lasers. Light scatter was collected from two angles: forward (FSC: 0° ± 13°) and side (SSC: 90° ± 13°) scatters. FSC signal is correlated to cell size and SSC signal is correlated to granularity. Fluorescence intensity was measured by four detectors Photomultipliers/PMT): FL1 (533/30 nm), FL2 (585/40 nm), FL3 (>670 nm), and FL4 (675/25 nm) bandpass or long pass optical filter/photomultiplier detector systems. The optical bench of the BD Accuri C6[®] flow cytometer is shown in Figure 17.

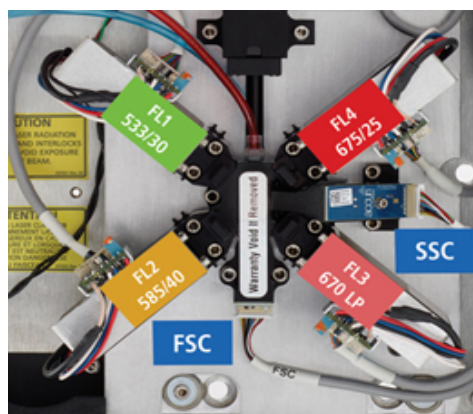


Figure 17: BD Accuri C6[®] optical configuration

4.1.2.2. Characteristics of the fluorescent molecules studied

In order to have additional information about the physiological status of cells and the biomass composition, fluorescent dyes were used.

Propidium iodide (PI) (*Molecular probes, Invitrogen, USA*) was used for the quantification of permeable cells and Nile Red (NR) (*Molecular probes, Invitrogen, USA*) was used for PHB production detection. The signal was collected in the FL3 channel for the two dyes. Table 33 summarized the spectral properties of the fluorescent molecules.

Table 33: Characteristics of the fluorescent molecules used

Dye	Excitation wavelength (λ_{max}) (nm)	Emission wavelength (λ_{max}) (nm)	Emission detector
Propidium Iodide (PI)	490	635	FL3
Nile Red (NR)	559	635	FL3

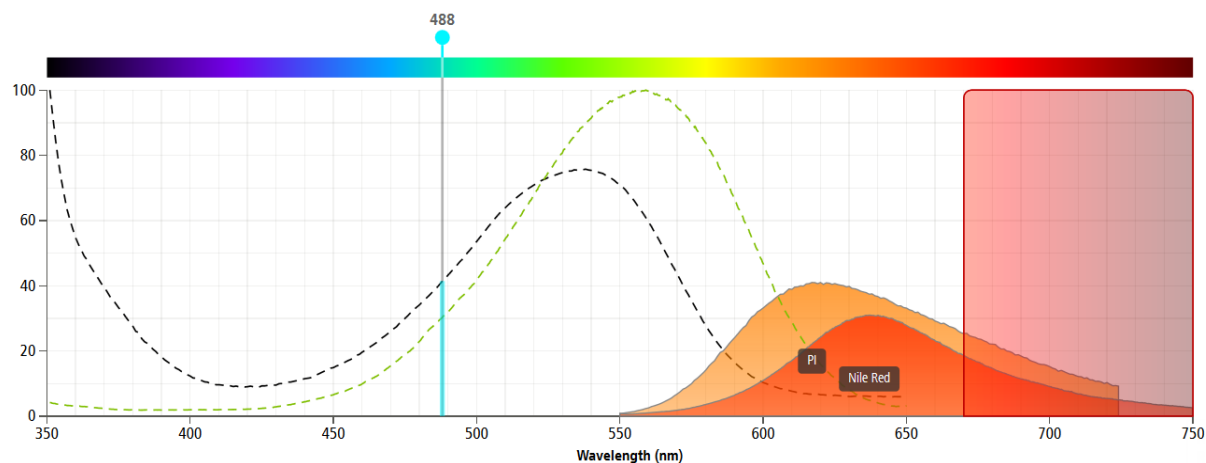


Figure 18: Excitation and emission spectrum of PI and NR, as enabled by the flow cytometry Accuri C6 with excitation by the blue laser. (**Orange curve**, PI emission spectra; **green dotted curve**, PI excitation spectra; **Red curve**, NR emission spectra; **black dotted curve**, NR excitation spectra; **red rectangle**, FL3 emission filter range; (from BD Spectrum Viewer)).

4.1.2.3. Experimental protocol for cell permeability and PHB detection

Two culture samples were diluted in filtered NaCl solution (0.9 %) to obtain a cell concentration of 10^6 cells/ml.

The first sample was stained with 20 μ L of the commercial solution at 1 mg/l PI and incubated 20 minutes in dark at room temperature. As a control, a 100 % dead-cell sample was used by incubating cells in 70 % isopropanol at room temperature for 1 hour.

The second sample was stained with 1 μ l of the commercial solution at 1 mg/ml Nile Red and incubated 30 minutes in dark at room temperature (adapted from Li and Wilkins study [240]). A PHB⁺ sample (from a flask culture producing PHB under N deficiency, at 0.5 and 0.33 g/l NH₄Cl) was used as a control. Samples were carried out at the slow flow-rate (14 μ l/min) until 20,000 events. FSC signal and SSC signal were used as trigger channels with thresholds of 12,000 and 2,000 respectively.

The PI and the NR dyes were collected at the FL3 channel (examples in Figure 19). The BD Accuri CFlow® software was used for data acquisition and FlowJo software (Becton Dickinson, Sparks, MD, USA) was used for data processing. Indeed, single cells (named singulet) with similar cell size and granularity were first selected, based on the FSC-Height vs FSC-Area (488 nm laser) plot and the SSC-Height vs SSC-Area (488 nm laser) plot. Then, single cells were sorted depending on their fluorescence intensity based on the histogram of the fluorescence. For Nile red staining, single cell (singulet) treatment was based only on the FSC-Height vs FSC-Area plot due to the impact of the Nile Red dye on SSC-A and SSC-H signals.

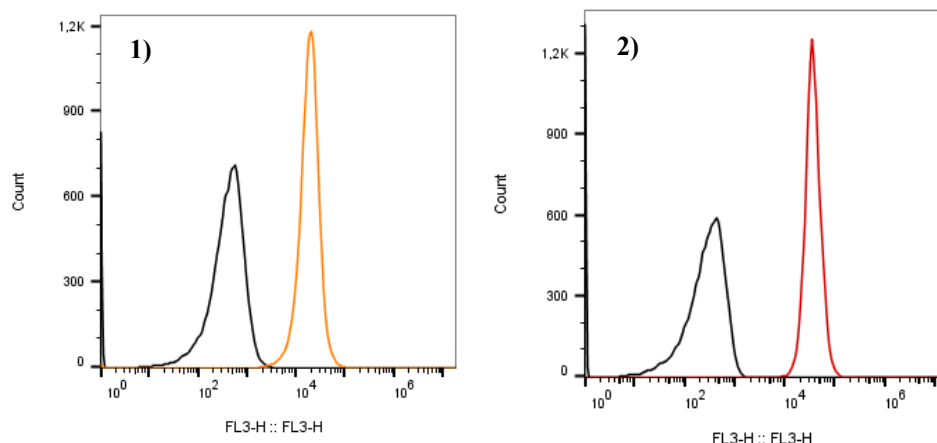


Figure 19 : Example of cytograms (before and after fluorescent molecules application) (1) dead cells (PI⁻; PI⁺); 2) cells producing PHB (NR⁻; NR⁺)

4.2. Determination of glucose, organic acids, ammonium and fatty acids concentrations

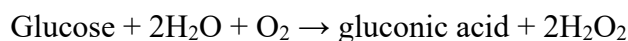
First, 1.5 ml broth samples were centrifuged (*MiniSpin, Eppendorf, USA*) in Eppendorf tubes at 13,400 rpm for 6 min. Then, supernatants were filtered on Minisart filters with 0.2 µm pore diameter polyamide membranes (*Sartorius, Germany*). Finally, supernatant samples were diluted (when needed) with Milli-Q grade water (18.2 mΩ-cm resistance). Samples were processed following this protocol for all chromatographic and biochemical analysis.

4.2.1. Biochemical analysis for glucose: YSI

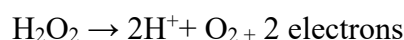
When glucose was used as carbon source, its concentrations were analyzed using an *YSI Model 2700 analyzer (Yellow Springs Instruments, Yellow Springs, Ohio, USA)* during the fermentation.

4.2.1.1. Principle

The measurement is done by the detection of hydrogen peroxide (H₂O₂) liberated during the conversion of glucose into gluconic acid using an immobilized glucose oxidase:



Then, the hydrogen peroxide produced is oxidized at a platinum electrode to produce electrons which generate an electrical current proportional to the glucose concentration.



4.2.1.2. Experimental protocol

Before assays, the instrument is calibrated using a commercial calibration standard (2.50 g/l glucose) (*YSI 2776 Standard, Yellow Springs, Ohio, USA*). Supernatants have to be diluted in

order to enter in the linear range (between 0 and 2.50 g/l). A direct reading of the glucose concentration was provided (*expressed* in g/l).

4.2.2. High Performance Liquid Chromatography: HPLC

During cultures, the carbon source concentrations were obtained with the high-performance liquid chromatography (HPLC). Supernatants were thawed at room temperature, diluted (if required) with Milli-Q grade water (18.2 mΩ-cm resistance) and filtered with 0.2 μm pore diameter polyamide membranes (*Minisart filters, SARTORIUS, Germany*).

4.2.2.1. Equipment description and operating conditions

The device used was a Dionex UltiMate 3000 HPLC system (*California, USA*) and was instrumented with:

- an autosampler (Dionex Ultimate Autosampler UHPLC+ focused)
- a pump (Ultimate 3000 Pump)
- a pre-column (Micro-Guard IG Cation H, BIO-RAD)
- a column Aminex HPX-87H⁺ (Bio-RAd, US)
- an UV detector (Ultimate 3000 Photodiode Array Detector)
- a refractometer (Shodex RI-101)
- A software system for data acquisition and processing (Chromeleon, Chromatography Management System version 6.80).

Sulfuric acid (H₂SO₄) at 5 mM was used as eluent under isocratic conditions in order to separate the compounds of interest on the column. The flow rate of the mobile phase was set at 0.5 ml/min. The column temperature was set at 50 °C and the processed samples were kept in the autosampler at 8°C. The injected sample volume was 20 μL and the running time of the chromatographic method was 35 min.

4.2.2.2. Experimental protocol

Using a gauged flask, a standard stock solution containing the compounds studied was prepared with Milli-Q water. The standards used for metabolite quantification were citrate, glucose, pyruvate, succinate, lactate, formate, acetate and ethanol. The stock solution was composed of 25 g/l of glucose, 5 g/l of each organic acid, 5 g/l ethanol. Dilutions (4:5; 3:5; 2:5 and 1/5 v/v) were made from this stock solution in order to establish a 6-point calibration curve. Table 34 presents the appropriate detector, the retention time and the concentration range for each compound.

Table 34 : Retention times and concentration ranges of the compounds quantified with HPLC

Compound	Retention time (min)	
	RI	UV
Citrate	09.969	09.603
Glucose	11.084	-
Pyruvate	11.691	11.328
Succinate	14.436	14.070
Lactate	15.611	15.247
Formate	17.100	16.500
Acetate	18.519	18.165
Ethanol	26.550	-

In order to check quality of analysis and system stability, calibration standards were injected at the beginning and at the end of the sequence analysis. Added to that, one standard was injected after every 5 samples.

4.2.3. High Performance Ionic Chromatography: HPIC

For the culture using urea as a nitrogen source, the concentration of ammonium in the culture medium was quantified through ionic chromatography by conductimetry. Supernatant samples were diluted with Milli-Q grade water (18.2 mΩ-cm resistance) in order to reduce the ionic strength (salts of the culture media) of the broth sample that could interfere with the sensitivity of the analysis, and filtered with 0.2 µm pore diameter polyamide membranes (*SARTORIUS, Germany*).

4.2.3.1. Equipment description and operating conditions

An ICS2100 system (*Dionex, USA*) equipped with a CD conductivity detector was used.

It is implemented with:

- an autosampler (AS autosampler, Dionex).
- a pump (SP, Dionex).
- a pre column IonPac™ CG16 (RFIC™, 3x50mm),
- a column IonPac™ CS16 (RFIC™, 3x250mm)
- an ion suppressor CERS 500 (2 mm, Thermo Scientific, USA)
- a heated conductivity cell detector DS (Dionex, USA),
- a software system for data acquisition and processing (Chromeleon, Chromatography Management System version 6.80).

Quantification of ammonium on the IonPac™ column was done using a 30 mM MetaneSulfonic Acid (MSA) eluent under isocratic conditions. The flow rate of the MSA (mobile phase) was fixed at 0.36 ml/min. The temperature of the column and the HPIC autosampler were 40°C and

8°C, respectively. The injected sample volume was 10 µL and the running time of a sample was 25 min. The current in the ion suppressor was fixed at 40 mA.

4.2.3.2. Experimental protocol

A standard stock solution of ammoniac was made at a concentration of 16.87 mg/l. From this solution, many dilutions were prepared (1:2, 1:4, 1:8, 1:20 and 1:40 v/v) to generate a 6-point calibration curve (a second-degree polynomial). Table 35 shows the retention time and the concentration range for ammonium using this method.

Table 35 : Retention time and concentration range of ammonium quantified by HPIC

Compound	Retention time (min)	Concentration range (mg/l)
NH ₄ ⁺	8.707	0.42-16.87

Calibration standards were added at the beginning and at the end of the sequence analysis to verify quality of analysis and system stability. Added to that, one standard solution was injected after every 10 samples of the sequence.

5. Biochemical assays

In order to quantify proteins and nucleic acid amounts, cell lysis treatment is needed in order to have access to the intracellular content. Different methods were evaluated and compared. A complete description of the optimization is presented in Part III-Chapter1. Herein, only the optimized ones will be presented.

5.1. Cell lysis methods

5.1.1. FastPrep-24™ beating grinder and lysis system

The FastPrep-24™ 5G bead beating grinder (*MP biomedical, Solon, OH, USA*) and lysis system is a versatile sample disruption instrument designed to quickly and efficiently homogenize, grind, or lyse biological samples.

Cell pellets of 16-20 mg_{DW} of dry cells were thawed on ice, then resuspended in 800µl of buffer. This volume is then transferred in 2ml screw top plastic vial containing 0.6 g of 0.1 mm glass beads (*Sigma, Darmstadt, Germany*). The cells were lysed using FastPrep-24™5G (*MP Biomedicals, Solon, OH, USA*) at 6 m/s for 40s. Three cycles of Fast-Prep with 5 min rest in between were carried out. Cell debris was removed by microcentrifugation (*MiniSpin, Eppendorf, USA*) (14300 rpm, 10 min) (adapted from [107]).

5.1.2. NaOH treatment

16 to 20 mg for dried cells were resuspended in 5.5 ml of filtrated milliQ water. Cell lysis was performed by addition of 1 ml of NaOH 10 % (w/v) and incubation at 100°C for 5 min. After cooling, the samples were centrifuged at 8000 rpm for 10 min in order to remove cell debris (adapted from [102]).

5.2. Protein and nucleic acid quantifications

5.2.1. Bicinchoninic acid method for soluble proteins quantification

Supernatant coming from NaOH treatment was used to quantify solubilized proteins using a commercial colorimetric assay kit: Bicinchoninic acid (BC Assay: Protein Assay Kit, Interchim, France).

This assay includes two steps:

- Chelation of copper (Cu^{2+}) with protein in an alkaline environment forming a blue-colored complex.
- Reaction between the BCA reagent with the cuprous cation (Cu^+) producing a purple color.

The high optical absorbance of the final complex allows to measure this reaction at 562nm. Here, an absorbance microplate reader (BioTek® Instruments, Winooski, USA) was used. Protein concentrations were calculated using a reference curve for BSA standard protein (Figure 20).

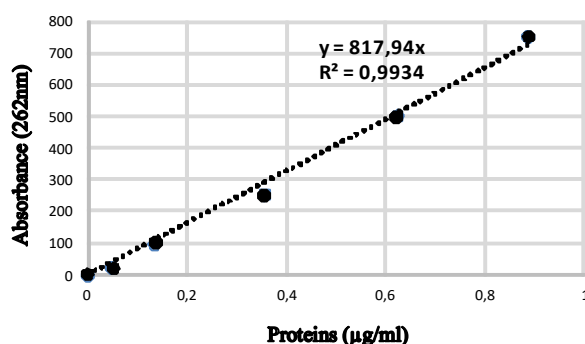


Figure 20 : Example of Standard curves for BCA protein quantitation

5.2.2. RiboGreen and PicoGreen method for Nucleic acids quantification

Using cell lysates from FastPrep-24™ extraction, RNA and DNA content were quantified using commercial colorimetric assays kits: RiboGreen (Quant-iT™ RiboGreen® RNA Reagent and Kit, Molecular probes, UK) and PicoGreen (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Molecular probes, UK), respectively.

The quantification is done with the reaction of the sample with Quant-iT™ RiboGreen® RNA and Quant-iT™ PicoGreen® DNA reagents [241, 242]. These reactions allow to quantify low RNA and DNA contents using a reference curve of five standards (Figure 21). Here, a fluorescence microplate reader (BioTek® Instruments, Winooski, USA) was used using fluorescein excitation/emission wavelengths (485/525) after 5 minutes of incubation.

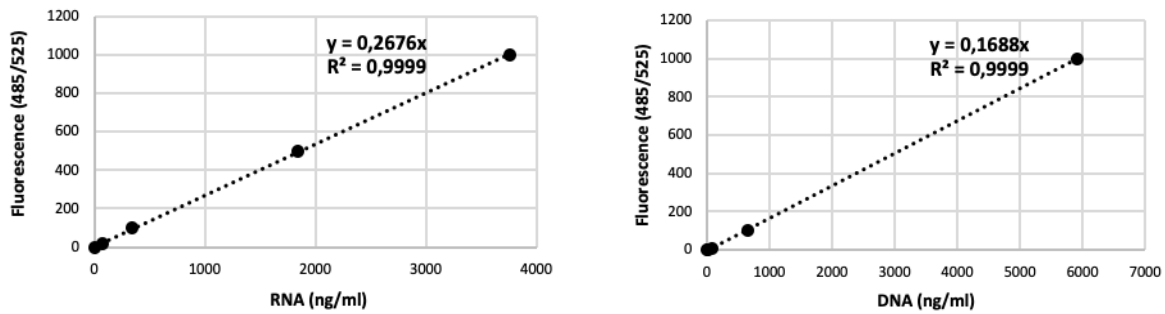


Figure 21 : Example of Standard curves for RNA and DNA quantitation

5.2.3. Dumas method for total protein quantification

A pellet of 5mg_{DW} was dried using Genevac (EZ-2 Series, Warminster, United Kingdom). Total nitrogen amount was measured using the Dumatherm (C. Gerhardt GMBH & Co, Königswinter, Germany) allowing the quantification of total proteins, using 6.25 as a multiplication factor [243]. In general, a factor of 6.25 was used to convert total nitrogen on total protein in SCP studies for algae (*Spirulina maxima* [86], *Spirulina platensis* [114],...), fungi (*Penicillium expansum* [165], *Aspergillus awamori* [116],...), yeasts (*Candida utilis* [85], *Candida lipolytica* [132], *Kuyveromyces marxianus* [55],...) and bacteria (*Cellulomonas biazotea* [22], *Raoutella ornithinolytica* [124], ...).

The Dumas method use Helium gas, and it is performed in five steps (Figure 22):

- Step 1: Combustion at 1030°C
- Step 2: Reduction of NOX to N_2 , under O_2 flux
- Step 3: Condensation to eliminate H_2O
- Step 4: CO_2 elimination
- Step 5: Detection of the signal (in percentages of N_2).

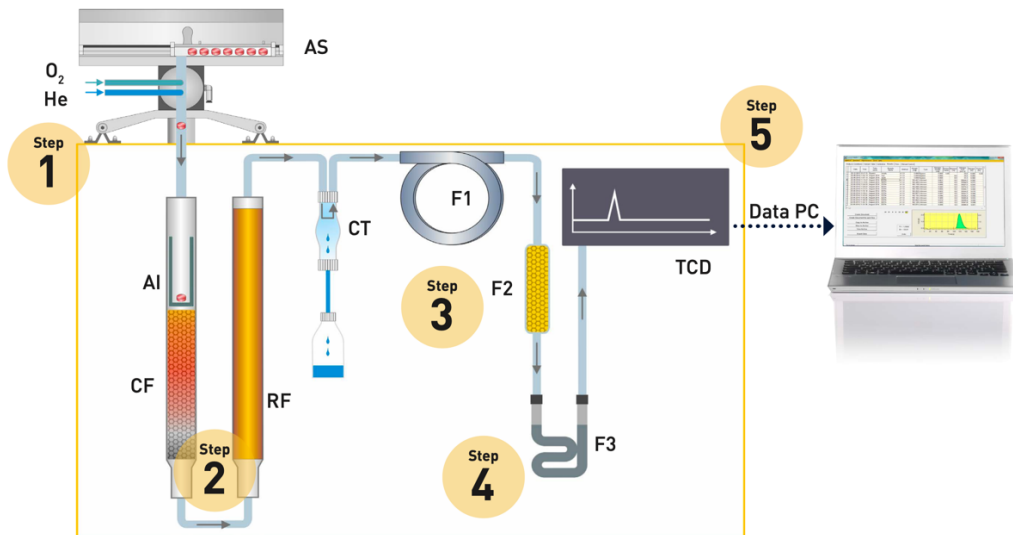


Figure 22 : The DUMATHERM process

5.3. Urea colorimetric detection

The Urea Nitrogen Colorimetric Detection Kit (Live technologies Corporation, Frederick, USA) is designed to quantify ureic nitrogen in medium by a reaction with two acidic reagents. The mixture is incubated for 30 minutes in room temperature. The absorbance is read at 450 nm using a microplate reader (BioTek® Instruments, Winooski, USA). The ureic nitrogen quantification is then calculated using a reference curve (Figure 23) as recommended by the supplier.

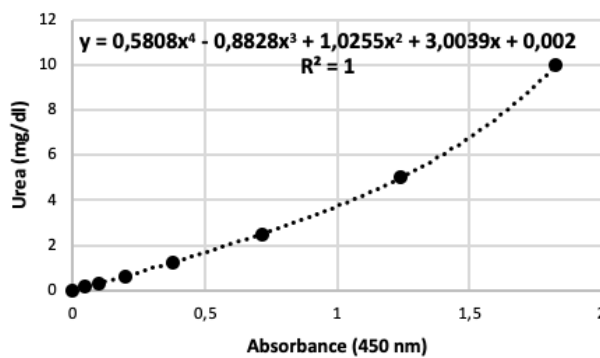


Figure 23 : Standard curves for urea quantitation

6. Data treatment

6.1. Statistical analyses on specific growth rates and yields

Specific growth rates were determined as being the slope of $\ln(\text{OD})=f(t)$. The error on the specific growth rate was calculated as the standard deviation of the slope.

For the yield determination, the state variables were plotted pairwise in a scatter plot within the considered period of the culture. A linear regression was applied to determine the yield (as the slope) and the error (as the standard deviation of the slope).

6.2. Statistical analyses on biomass characterization

Concentrations of metabolites and biomass are given as the mean value of independent analyses. Measurement of proteins and nucleic acids was performed on triplicates for every culture point.

6.3. Normality of distribution with BoxPlot representation

An empirical distribution is presented by a graphical tool, which are the Boxplots (Figure 24):

- The first quartile (25th percentile) represents the bottom of the BoxPlot.
- The third quartile (75th percentile) represents the top of the BoxPlot.
- The median = the second quartile (50th percentile) is represented by a line inside the box.

Thus, a symmetric boxplot (the median is in the middle of the box with same length whiskers) could be considered to be normally distributed [244, 245].

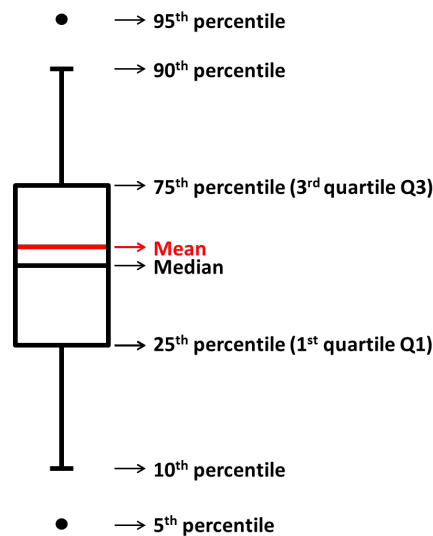


Figure 24: Boxplot representation [245]

Part III - Results and discussions

Chapter III-1: Development and validation of methodologies for extraction and quantification SCP and nucleic acid content in *Cupriavidus necator*

Chapter III-1: Development and validation of methodologies for extraction and quantification SCP and nucleic acid content in *Cupriavidus necator*

1. Introduction

This chapter describes the development of optimized extraction methods for quantification of proteins and nucleic acids within the biomass, which is one of the key steps to response to the scientific questions of the PhD. These quantifications are crucial to understand the impact of environmental factors on both the quantity and the composition of the produced Single Cell Protein by *C. necator*. Many cell lysis methods are described in literature in order to extract protein and/or nucleic acids from cells with the objective to quantify SCP composition. These methods can be divided into two types, chemical or physical ones. Among chemical methods, cell autolysis using endogenous or exogenous enzymes [100], acid cell hydrolysis (using trichloroacetic acid [22], chlorhydric acid [31, 99]) or heating in alkaline solution [103] (sodium hydroxide (NaOH) treatment [101, 102]), were reported. In addition, enzymatic digestion at high temperature [104], sonication [95-98], destruction using mortar and sea sand [105] and breaking with glass beads [106, 107] were used as physical methods. Those methods were used for both bacteria (such as *Cryptococcus Aureus* [104], *Bacillus brevis* [31, 103], *Cellulomonas biazotea* [22], *Acetobacter diazotrophicus*, *Gluconacetobacter diazotrophicus*, *Methylophilus methylotrophus*, *Deinococcus geothermalis* [95-98]) and yeasts (such as *Saccharomyces cerevisiae*, *Saccharomyces fibuligera*, *Rhodotorula rubra*, *Pichia pastoris*, *Pichia pinus*, *Kluyveromyces marxianus* [105-107]). The bacterium *Cupriavidus necator* was studied in the literature using cell hydrolysis with chlorhydric acid [99], sodium hydroxide treatment [102] and treatment with glass beads [107].

In this chapter, different methods were evaluated in order to obtain optimized protocols for protein, RNA and DNA extractions at the lab-scale level. Chemical and/or physical treatments were investigated. Specific parameters were also varied and compared. Quantification methods were chosen such as bicinchoninic acid (BCA) analysis, PicoGreen and RiboGreen (explained in **Part II**) and some of them were also modified in order to propose optimal protocols.

2. Results and discussion

2.1. Evaluation of cell lysis methods for protein and nucleic acid extraction

• **Initial protocols**

Three initial protocols were selected from literature because there were used for bacteria and for some of them especially with *C. necator* : FastPrep-24™ beating grinder [106, 107],

sonication [98] and sodium hydroxide treatment [101, 102]. The operating parameters for these three methods are presented in Table 36.

Table 36 : operating parameters used in selected extraction method

Methods		Parameters
Physical	FastPrep-24TM system	Beads diameter: 0,1 mm Quantity of beads: 0,6 g Number of cycles: 3 Speed: 6 m/s Time: 40 sec Time between two cycles: 300 sec (samples in ice)
	sonication	Probe diameter: 6mm Amplitude: 32% Number of cycles: 30 cycles Pulse time: 20 sec Time between two cycles = 20 sec
Chemical	sodium hydroxide	NaOH concentration: 10% w/v Incubation temperature: 100°C Incubation duration: 5 min

- **Optimization approaches**

Based on the previous protocols, each one was modified and tested separately to find the optimal parameters to use either for protein or nucleic acid quantification. Trying to find the optimized protocol, parameter values were fixed considering their impact for cell extraction and according to some preliminary assays. For the FastPrep system, the number of cycles was seen as an important parameter to investigate for cell lysis performance, then 3 and 6 cycles were chosen. For the NaOH treatment, a two-factorial approach was investigated using both NaOH concentration (10, 15 and 20 % w/v) and the incubation time (5 and 10 min) at 100°C. In addition, sonication was evaluated alone and in combination with the most relevant NaOH treatment to evaluate if sonication could improve alkaline treatment.

A volume of cellular biomass was aliquoted at the end of a flask culture into samples of 16 mg of dried biomass to ensure that all methods were developed on the same average cell sample. The experimental design is detailed in Table 37 using different codes for better understanding on graphs.

Table 37 : Experimental design for Extraction methods evaluation

Method		FastPrep parameters						NaOH parameters		Sonicator parameters			
		Beads diameter (mm)	Quantity of beads (g)	Number of cycles	Speed (m/s)	Time (s)	Time between 2 cycles (s)	[NaOH] (%w/v)	Time of incubation (min)	Number of cycles	Pulse duration (sec)	Downtime (sec)	Amplitude (%)
Physical	B3	0.1	0.6	3	6	40	300						
	B6	0.1	0.6	6	6	40	300						
	S30/20									30	20	20	32
Chemical	Na10/5							10	5				
	Na10/10							10	10				
	Na15/5							15	5				
	Na15/10							15	10				
	Na20/5							20	5				
	Na20/10							20	10				
Na10/5_S30/20								10	5	30	20	20	32

Abbreviations

Ba (B: beads treatment; a: number of cycles)

Sb/c (S: sonication treatment; b: number of cycles; c: pulse duration)

Nad/e (Na: NaOH treatment; d: NaOH concentration; e: incubation time)

- **Evaluation of the extraction method**

Using cell lysates obtained from extraction methods detailed in Table 37, soluble protein, RNA and DNA contents were quantified, in triplicates, using commercial colorimetric assay kits according to manufacturing instructions. Quantifications of protein concentrations were made with Bicinchoninic acid (BC Assay: Protein Assay Kit, Interchim, France), RiboGreen (Quant-iT™ RiboGreen® RNA Reagent and Kit, Molecular probes, UK) and PicoGreen (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Molecular probes, UK) were used to quantify DNA and RNA concentrations respectively.

Quantification results for proteins, RNA and DNA are presented in Figure 25.

Regarding protein extraction

Results for extracted proteins are presented in Figure 25-A. The efficiency of protein extraction is impacted by the method and more precisely extraction efficiency is different depending on whether we use a chemical or a physical method.

FastPrep system (B3 and B6) led to the lowest protein percentages $15.63 \pm 1.49\%_{DW}$ using 3 cycles (B3) and $13.82 \pm 2.11\%_{DW}$ using 6 cycles (B6), respectively.

Sonication (S30/20) allowed obtaining a better extraction percentage ($25\%_{DW}$). But, clearly, chemical treatments (Na) led to the highest ones with a maximum value ($34.06 \pm 1.81\%_{DW}$) obtained using a concentration of NaOH 10% (w/v) with an incubation time of 5 minutes compared to the other conditions. However, the increase of both NaOH concentration and incubation time decreased slightly the protein extraction. For instance, after 5 minutes of incubation, cells treated with NaOH at 15% and 20% (w/v) presented amounts of $31.54 \pm 1.44\%_{DW}$ and $28.83 \pm 0.97\%_{DW}$, respectively. In addition, increasing the incubation time to 10 minutes decreased the protein amount to $31.04 \pm 1.99\%_{DW}$ at a concentration of 10% w/v. Our results show also that adding sonication to the chemical treatment (Na10/5_S30/20) did not permit increasing the extraction efficiency and a slight decrease in protein percentage was obtained from 34.06 ± 1.81 to $30.80 \pm 1.18\%_{DW}$.

Therefore, as a conclusion, the chemical method (Na10/5) using a NaOH concentration of 10% during 5 minutes incubation time alone was found to be the optimal one to extract proteins.

Regarding RNA and DNA extraction

Figure 25-B shows the results of RNA quantification. The FastPrep system (B3 and B6) shows comparable percentages with $5.52 \pm 0.46\%_{DW}$ après 3 cycles against $5.22 \pm 0.25\%_{DW}$ after 6

cycles. The sonication (S30/20) had slightly the same effect with $5.20 \pm 0.17 \%_{\text{DW}}$. The different protocols using NaOH treatment (Na) showed lower percentages (around $3 \%_{\text{DW}}$) as well as the combination of NaOH treatment with sonication (Na10/5_S30/20) ($2.15 \pm 0.24 \%_{\text{DW}}$).

Figure 25-C presents the results for DNA quantification. Using respectively 3 and 6 cycles, the FastPrep lysis system showed higher percentages, with 2.16 ± 0.25 and $2.21 \pm 0.23 \%_{\text{DW}}$. Sonication had slightly lower value with $2.07 \pm 0.12 \%_{\text{DW}}$. Very weak percentages (around $0.25 \%_{\text{DW}}$) were obtained using NaOH treatment alone as well as in combination with sonication ($0.29 \pm 0.05 \%_{\text{DW}}$). The Na20/10 is not significantly different from them too with a DNA content of $0.3 \pm 0.1 \%_{\text{DW}}$.

The low percentages of RNA and DNA obtained by the chemical treatments could be explained by the effect of NaOH solution which seems to degrade nucleic acids. Indeed, NaOH treatment was used in the literature in order to reduce nucleic acids amounts in SCPs to acceptable values. For example, Abou-Zeid et al., [186] study had the objective of reducing nucleic acid amounts in SCP produced by *Candida lipolytica* in gas oil. The most suitable method found to extract proteins was the sodium hydroxide treatment (10% w/v NaOH, 5 min incubation at 100°C). However, this protocol removed 75% of the RNA and 81% of the DNA [186].

Figure 25-B and C results show that, in the tested conditions, physical methods are found as the optimal ones to extract both RNA and DNA contents.

In conclusion, chemical protocols presented a better protein extraction yield and the physical treatments were better for nucleic acid extraction. Thus, the validated protocols were:

- **for protein extraction: NaOH treatment (10%w/v, 5min at 100°C) (Na10/5)**
- **for nucleic acid extraction: FastPrep system (3 cycles of lysis) (B3).**

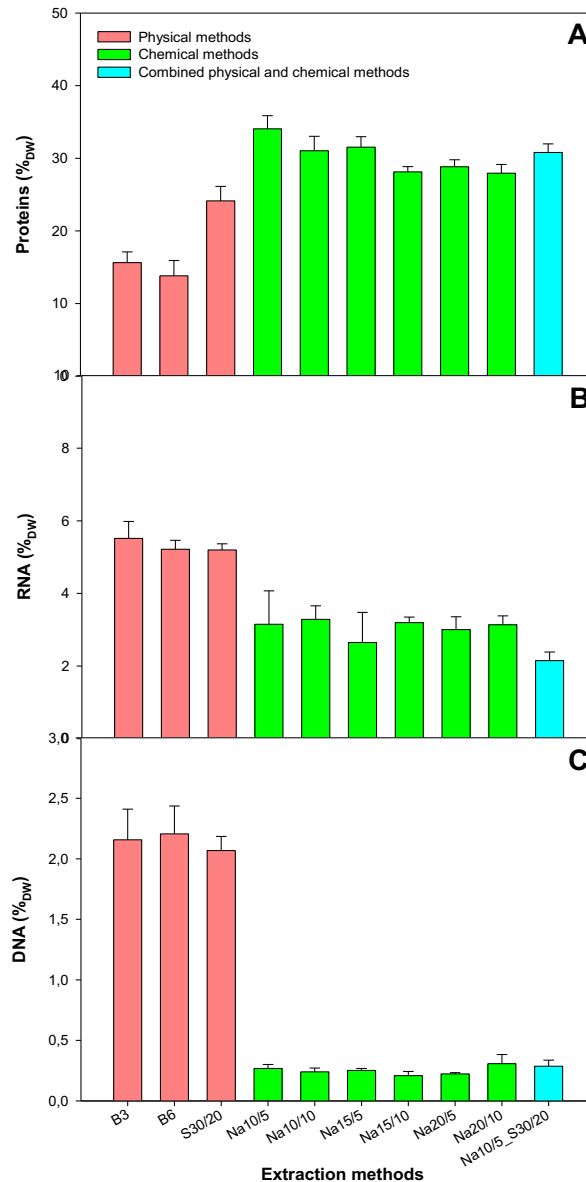


Figure 25 : Protein, RNA and DNA quantification using the extraction protocols listed in **Table 37**

- **Impact of the quantity of biomass**

In order to verify if the impact of the cell weight of the sample had an impact on the performances, three different weights were used 8, 16 and 24 mg (in triplicates). Figure 26 compares protein and nucleic acid extraction results using 8, 16 and 24 mg of cells. For proteins, the percentages obtained were 30.69 ± 0.83 , 35.70 ± 1.64 and 27.81 ± 0.73 %_{DW}, respectively. For RNA, the results were comparable for 16 mg (5.33 ± 0.24 %_{DW}) and 8mg (5.21 ± 0.38 %_{DW}) and slightly lower with 24mg (4.94 ± 0.24 %_{DW}). For DNA extraction 1.33 ± 0.23 %_{DW}, 1.80 ± 0.11 %_{DW} and 1.63 ± 0.04 %_{DW} were the values with 8, 16 and 24 mg of cells, respectively. Therefore, 16 mg of cell was found as the efficient extraction cell weight regarding the established protocols.

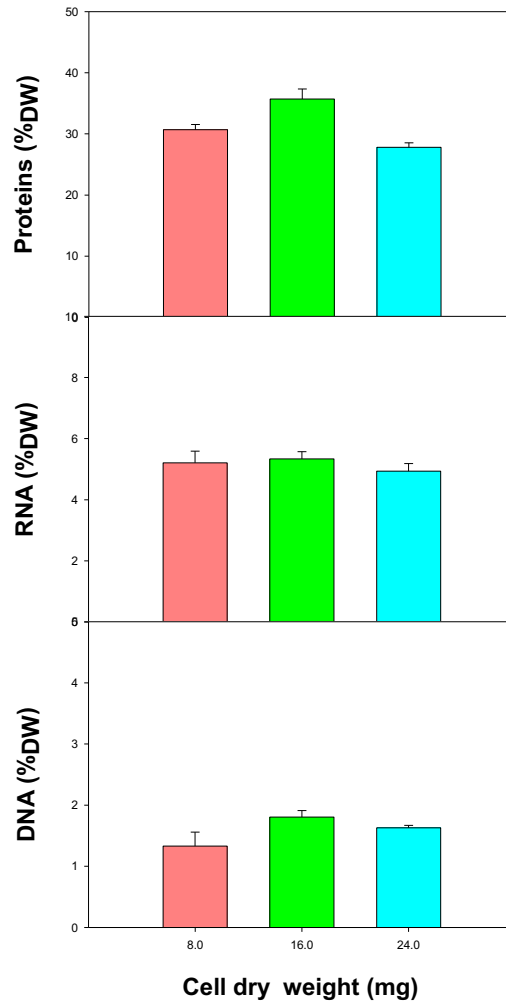


Figure 26 : Impact of cell weight on protein and nucleic acid extractions

- **Protocol validation and reproducibility study**

Six different samples were lysed independently using the two protocols validated before in order to verify the reproducibility (Figure 27). Table 38 presents the validated protocols.

Soluble proteins, RNA and DNA extracted showed average values of 34.77 ± 0.25 %_{DW}, 5.2 ± 0.4 %_{DW} and 1.75 ± 0.20 %_{DW}, respectively resulting in errors of 0.7 % for proteins, 7 % for RNA and 11 % for DNA, thus the repeatability was considered as acceptable.

The protein values obtained with these validated protocols are still inferior to the values presented on the literature for bacteria in general. Indeed, in the literature, the proteins percentages were between 50 and 65 %_{DW} and the nucleic acids amounts were 8-12 %_{DW} [4, 9, 27].

Indeed, in those studies, a quantification of the total proteins was done through the Kjeldahl method which is a method quantifying total nitrogen content. Here our method allowed the quantification of the NaOH-solubilized proteins which may likely not represent all the cellular

proteins. Therefore, a complementary method using Dumas’s technology which quantifies total nitrogen like the Kjeldahl method will be also used in this work and discussed throughout the manuscript. For nucleic acids, the values were ranged between 6.3 and 7.5 %_{DW} using the FastPrep system as extraction method.

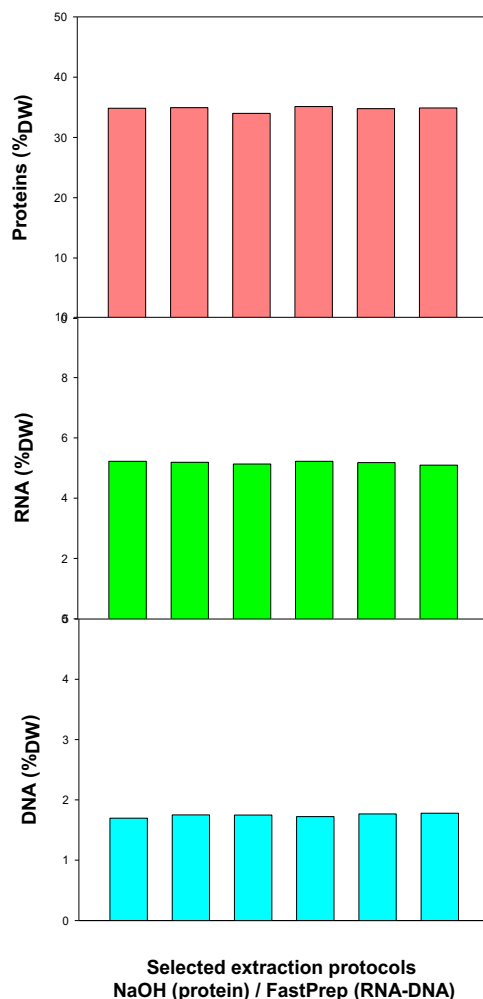


Figure 27 : Protein and nucleic acid quantifications to validate the two developed protocols

Table 38 : Optimized protocols for proteins and nucleic acids extractions in *Cupriavidus necator*

Method of extraction	Cell weight (mg)	FastPrep parameters				NaOH parameters	
		Number of cycles	Speed (m/s)	Time (s)	Time between 2 cycles (s)	[NaOH] (%w/v)	Time of incubation (min)
Protein	16					10	5
RNA	16	3	6	40	300		
DNA	16	3	6	40	300		

2.2. Evaluation of the methods for protein and nucleic acid quantifications

In the previous part, only one quantification method was presented in order to simplify the discussion. Indeed, the optimized protocols of quantification were used for nucleic acids (Table 38). However, for protein quantification, the previous results were not obtained with the optimized protocol, the samples were diluted only by a factor 5.

2.2.1. BCA protein assay

In order to test interferences between BCA reagent and NaOH solution used for protein extraction, different sample dilutions were implemented (1/2, 1/5, 1/10, 1/15, 1/20) in triplicates using samples from two different bioreactor cultures. Figure 28 shows that increasing the dilution factor increases the protein percentages. For the first culture, the protein percentages obtained were 19.68 ± 1.40 , 27.33 ± 0.74 , 35.66 ± 0.76 %_{DW} using the dilution factors of 2, 5 and 10, respectively (bars D2 (1), D5 (1), and D10 (1) on Figure 28). However, the same value was obtained with the factors 10, 15 and 20 (around 35%_{DW}) suggesting that there is no longer any interference effect from ten times dilution. The duplicates using samples from another culture (bars D2 (2), D5 (2), D10 (2), D15 (2) and D20 (2) on Figure 28) led to the same results and conclusion.

The dilution 1/10 is found as the optimal dilution for protein quantification by BCA method after NaOH extraction protocol.

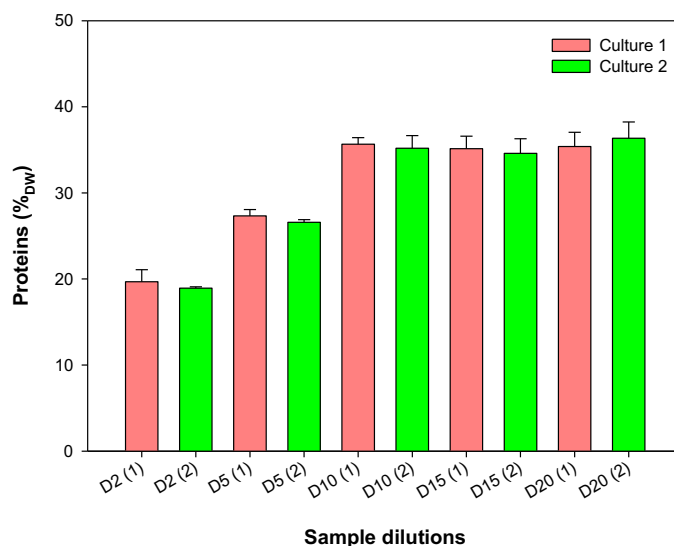


Figure 28 : Protein quantification using different sample dilutions for two different erlen flask cultures

2.2.2. RiboGreen and PicoGreen nucleic acids assays

Microplate reader sensitivity (25, 50, 75, 100 and 125) and incubation time (2 and 9 minutes) were the parameters used in order to choose the reading conditions and to verify the stability of the measurement. For instance, RNA measurement was investigated varying these two factors (Figure 29). Using different conditions, RNA values were similar (around 5% DW). For sensitivity equal to 25, 100 and 125 the signal was too low or too high (saturated) that's why the fluorescence could not be measured. Considering the values presented in Figure 29 (*i.e.*, sensitivity 50 and 75), both microplate reader sensitivity and the incubation time had no effect on fluorescence measurements. For DNA, the measurement could be done only at a sensitivity of 50. Studying the incubation time, the figure showed no impact with a similar value, around 2.2 %_{DW}.

Sensitivity of 50 for the microplate reader and 2 min for the incubation time were found as the optimal conditions for nucleic acid quantification for RiboGreen and PicoGreen assays after beads treatment protocol.

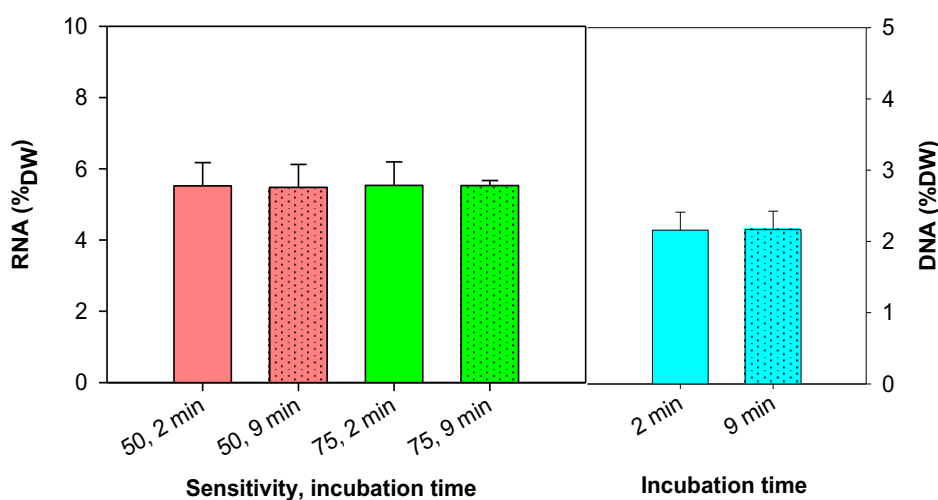


Figure 29 : RNA and DNA quantifications using different conditions

Table 39 presents the protocol validated for both proteins and nucleic acids quantifications.

Table 39 : Optimized protocols for protein and nucleic acid quantifications in *Cupriavidus necator*

Compounds	Extraction method	BCA assay	RiboGreen / PicoGreen assays	
		Sample dilution	Microplate reader sensitivity	Incubation time
Protein	NaOH (Na10/5)	10	50	2
RNA	FastPrep (B3)		50	2
DNA	FastPrep (B3)		50	2

3. Conclusion

The aim of this chapter was to evaluate proteins and nucleic acid extraction and quantification methods in order to obtain optimized protocols for further biomass characterization.

To optimize the extraction methods both physical and chemical methods were compared. Beads treatment and sonication were used as physical protocols and sodium hydroxide treatment with different concentrations and incubation time were used as chemical methods. Added to that, a combining of bead treatment and NaOH treatment was tested. As a result, the sodium hydroxide treatment (10% w/v; 5 min) was the most efficient for protein extraction and the beads treatment with 3 cycles of FastPrep was found as the best protocol for RNA and DNA extraction.

On the other hand, an optimization of the quantification protocols was done. For the Bicinchoninic acid assay for protein quantification, the sample should be diluted with a factor of 10 in order to minimize the interferences between the kit reagent and the NaOH solution used for the extraction. For both RNA and DNA quantification with the RiboGreen and the PicoGreen assays, the microplate reader sensitivity should be at 50 and the sample should be read after 2 minutes of incubation.

As a result, the optimized protocols for both extraction and quantification for proteins, DNA and RNA are presented in Table 40. These protocols will be used in the next chapters in order to characterize the biomass composition.

Table 40 : Optimized protocols for proteins and nucleic acids extractions and quantifications in *Cupriavidus necator*

Compound	Extraction			Quantification	
Proteins	<i>NaOH treatment</i>			<i>BCA assay</i>	
	NaOH (% w/v)	Incubation time (min)		Sample dilution	
	10	5		10	
Nucleic acids	<i>FastPrep treatment</i>			<i>RiboGreen and PicoGreen assays</i>	
	Number of cycles	Speed (m/s)	Time (sec)	Microplate reader sensitivity	Incubation time (min)
	3	6	40	50	2

**Chapter III-2: Impact of the
temperature on Single Cell Protein
production and composition in
*Cupriavidus necator***

Chapter III-2: Impact of the temperature on Single Cell Protein production and composition in *Cupriavidus necator*

1. Introduction

This chapter investigates the impact of using different temperatures on Single Cell Proteins (SCP) production by *C. necator* through the characterization of cell growth, SCP production and biomass composition by *C. necator*. In literature, studies regarding growth and/or protein production using different temperatures were reported only on some algae (*Spirulina maxima* [59]), bacteria (*Raoutella ornithinolytica* [124]), fungi (*Aspergillus terreus* [173]) and yeasts (*Cryptococcus aureus* [104] and *Schawanniomyces castellii* [146]). However, no study with *C. necator* was reported evaluating protein production under temperature stress.

In this study, four batch cultures of *C. necator* were performed in well-controlled bioreactors under the same operating conditions (glucose as carbon source, ammonium sulfate as nitrogen source, pH7). Inoculum were prepared in Erlenmeyer flask cultures under the same conditions including the temperature (at 30°C). However, the temperature value had changed from one batch culture to another. The tested values were 25, 30, 35 and 40°C. During the batch cultures, the temperature value was kept constant thanks to the regulation system.

In this chapter, growth parameters, cell morphology, SCP production, composition and elemental composition of the biomass were evaluated and discussed with regard to the literature studies.

2. Results and discussion

2.1. Growth dynamics and viability

Cell growth

Growth kinetics and substrate consumption of *C. necator* were analyzed and compared during batch cultures. Figure 30 presents the evolution of the biomass concentration as well as glucose concentration over time for cultures carried out at the temperatures of 25, 30, 35 and 40°C. All kinetics followed an exponential growth. From a macroscopic point of view, with an initial glucose concentration between 16 and 18 g/l, a biomass concentration of 8g/l was obtained at the end of the culture. No organic acid and other metabolites were detected in the culture supernatants. So, based on these data, glucose was converted into biomass and carbon dioxide mainly.

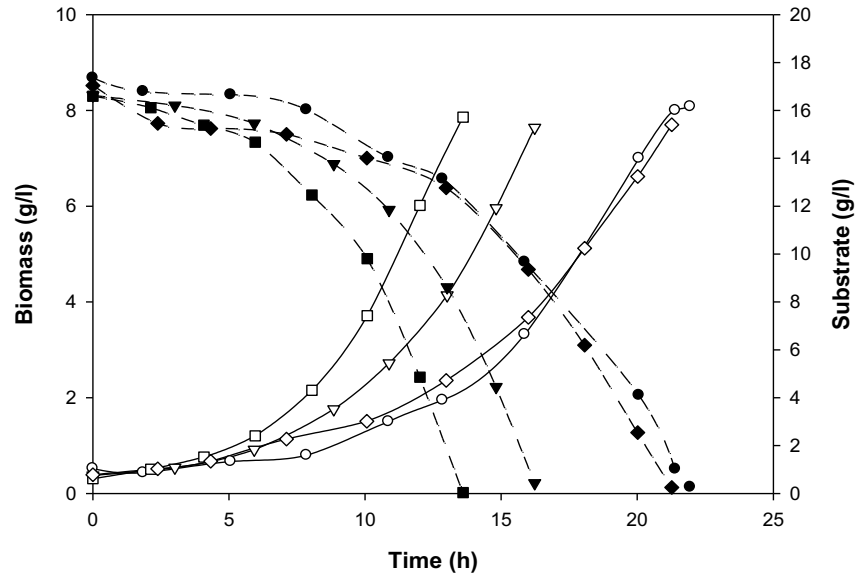


Figure 30 : Evolution of the concentration of biomass (solid line) and glucose (dotted line) over time for each tested temperature: 25°C (●○), 30°C (▲△), 35°C (■□) and 40°C (◇◆).

Both growth rates and biomass yields were calculated and presented in Table 41. At 30 °C, which is usually the optimal growth temperature of *C. necator* reported in the literature, the maximal growth rate value was $0.21 \pm 0.01 \text{ h}^{-1}$. The maximal growth rate reached a higher value ($0.25 \pm 0.01 \text{ h}^{-1}$) at 35 °C. However, its value was lower (0.14 h^{-1}) at 25°C and 40 °C. Our results also showed that no significant difference was detected in biomass yields between cultures. Indeed, the values were at 0.45 ± 0.04 ; 0.46 ± 0.01 ; 0.47 ± 0.02 and $0.43 \pm 0.05 \text{ gx/g}_s$ at 25, 30, 35 and 40 °C, respectively.

As a result, the maximal growth was obtained at 35°C and the lower was at 25°C and 40°C. The temperature had an effect on *C. necator* growth.

Table 41 : Growth rate and biomass yield values depending on temperature

Temperature (°C)	$\mu \text{ (h}^{-1}\text{)}$	$R_{x,s} \text{ (g}_x\text{/g}_s\text{)}$
25	0.14 ± 0.01	0.45 ± 0.04
30	0.21 ± 0.01	0.46 ± 0.01
35	0.25 ± 0.01	0.47 ± 0.02
40	0.143 ± 0.004	0.43 ± 0.05

In the literature, in the case of *C. necator*, the optimal temperature used was always 30°C [158, 167, 169, 199, 201, 209, 215, 228, 246, 247]. However, in our study, 35°C gave the optimal growth rate. This result could be explained by the difference between the strain used in this

work and in the other papers (ATCC17699=H16, LMG1199, Z1, ATCC17697, DSM 531, DSM 541 and DSM 545).

In SCP papers, regarding the effect of the temperature on cell growth, same effect was obtained using other microorganisms. Indeed, the use of higher and/or lower temperature than the optimal temperature known inhibit the cells' growth, for example, using *Spirulina maxima* [59], *Raoutella ornithinolytica* [124], *Aspergillus terreus* [173], *Cryptococcus aureus* [41] and *Schanniomyces castellii* [146].

Cell morphology and viability

Flow cytometry measurements were made during the cultivations at different temperatures to investigate the impact of temperature on cells' morphology.

Figure 31 shows the evolution of both cell size (FSC channel) and granularity (SSC channel) distributions during the four batch cultures cultivated at 25, 30, 35 and 40°C.

For the cell size (FSC) evaluation, the profiles were slightly the same along the culture at 25, 30 and 35°C. However, at 40°C, the cell size increased overtime.

Regarding the granularity (SSC), the profile at 25°C was stable during the culture. However, observing the profiles at the other temperatures, the cells changed in granularity.

Considering the literature, the enlargement of cell size and the changes observed in cell granularity is generally related to the production of PHB granules [217, 248]. That statement was reinforced at 40°C with a microscopic analysis. Indeed, as presents Figure 32, granules production was observed during the culture.

As a conclusion, depending on the temperature cell morphology has changed. Cells tend to elongate (FSC) at 40°C and tend to change in granularity (SSC) at a temperature above 25°C.

Cell viability was also quantified through membrane permeability by Propidium Iodide (PI) labelling during fermentations. Lower percentages of cell death were observed. Indeed, IP-permeable population did not exceed around 2% and 5% at 25°C and 40°C, respectively. The percentage was higher at 40°C but it was still a low value (going from 0.9% to 4.56%).

There was no loss in membrane permeability and microbial cells seemed to keep intact which could reveal a good adaptation of the strain to the different temperature values used.

Part III - Results and discussions

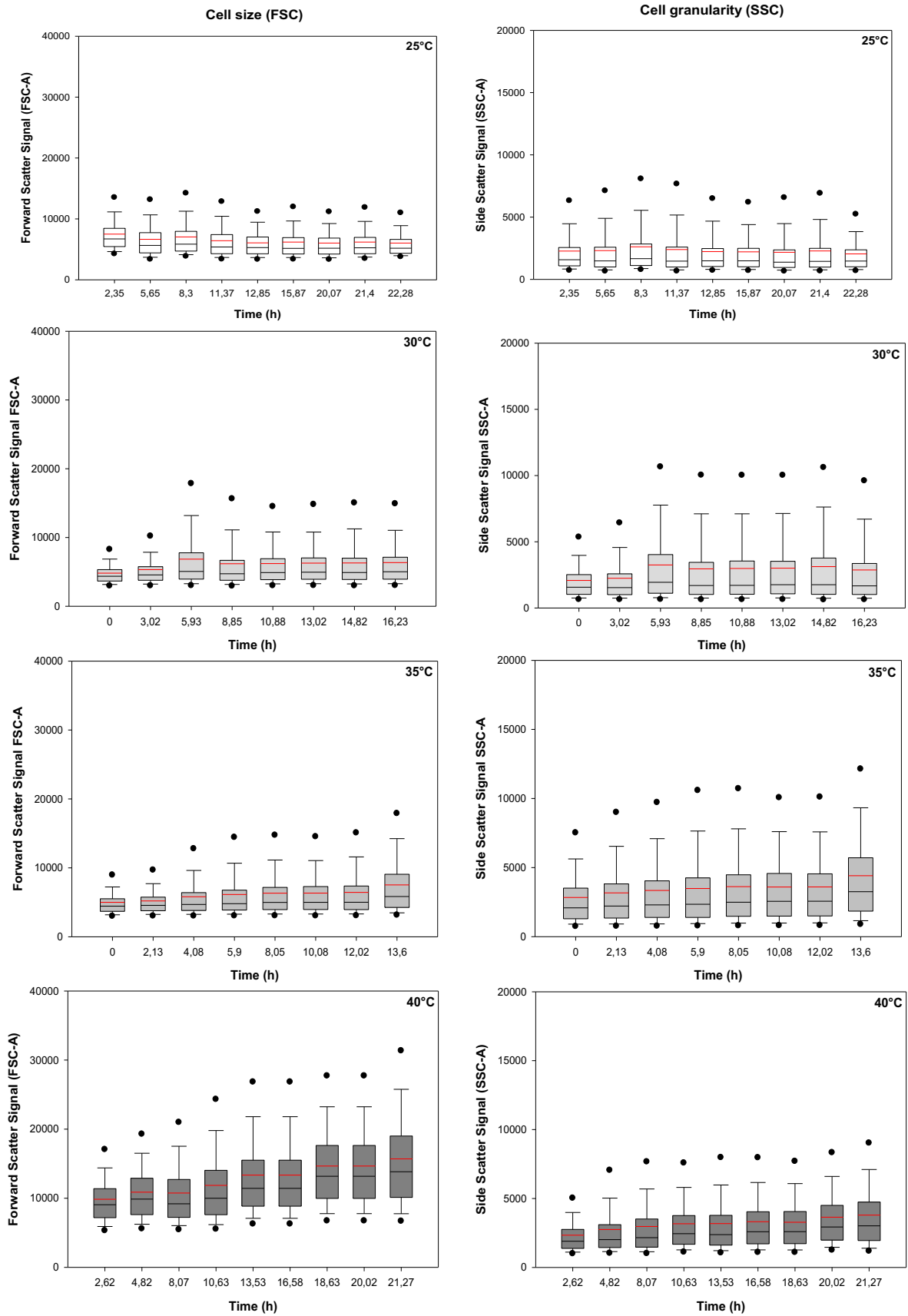


Figure 31 : Evolution of cell size and granularity measured by FSC and SSC signals distributions over time using different temperatures

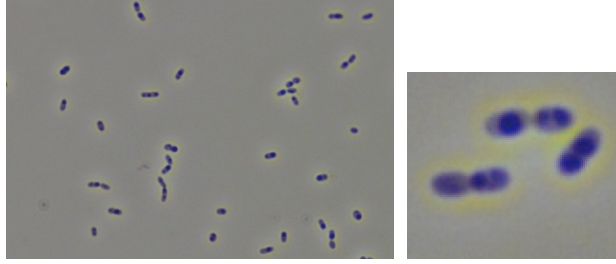


Figure 32 : Poly- β -hydroxybutyrate (PHB) granules at 40°C

2.2. SCP production and composition

Regarding protein production

The amounts of protein in the biomass were quantified and compared between the four temperature conditions. Two quantification methods were used and the results are presented in Figure 33 and Figure 34.

Figure 33 shows the kinetics of soluble protein content measured with the Bicinchoninic (BCA) protein assay after NaOH treatment at 100°C during 5 min.

Comparing the NaOH-solubilized proteins (Figure 33), protein content at 25 and 30°C presented similar dynamics. At 40°C at the end of the culture, and to a less extent at 35°C, the protein percentages decreased. The final protein content (in %_{DW}) presents values of 37.4±2.0; 41.3±4.5; 41.3±1.5 and 26.3±0.7 %_{DW} at 25, 30, 35 and 40 °C, respectively. The results suggest that at 25, 30 and 35 °C soluble proteins contents were similar. However, a lower protein content was observed at 40°C.

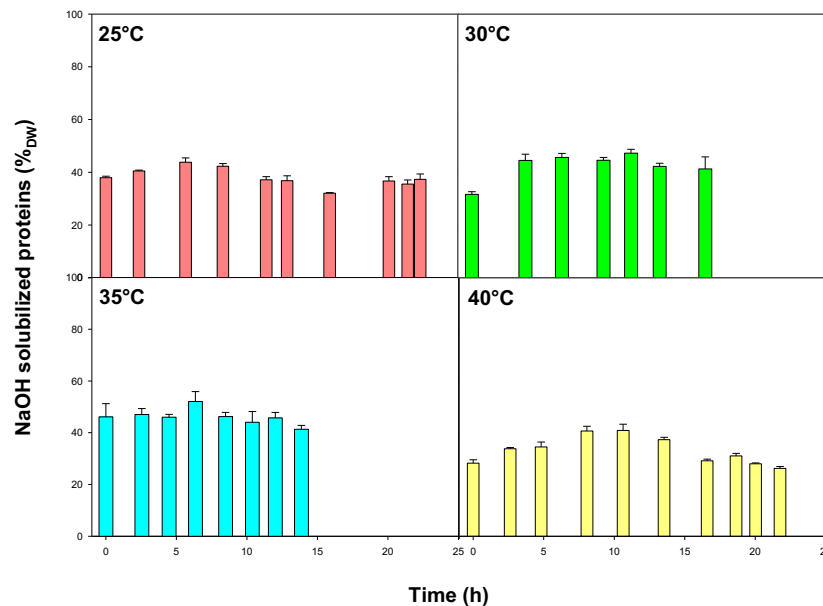


Figure 33 : NaOH-solubilized protein evolution quantified by BCA method over time at different temperatures

Figure 34 presents the evolution of total protein content calculated from the total nitrogen content measured by the Dumas method. Based on measured total nitrogen, a multiplication factor of 6.25 was applied to convert into protein content as in Kjeldahl method.

Figure 34 shows higher amounts compared to BCA assay results (about 30% higher whatever the moment of the cultivation) (Figure 33). This difference is explained by the principle of the two methods used. Indeed, the BCA method allows to quantify proteins which are soluble in NaOH after extraction. However, the Dumas method measures the total nitrogen, which includes soluble, insoluble proteins and other N-containing compounds such as nucleic acids. Figure 34 also confirms that, at 40°C, the total protein amount was lower (58%) compared to the other conditions where the protein contents were quite higher (79% at 25 °C and 30 °C and 67% at 35 °C).

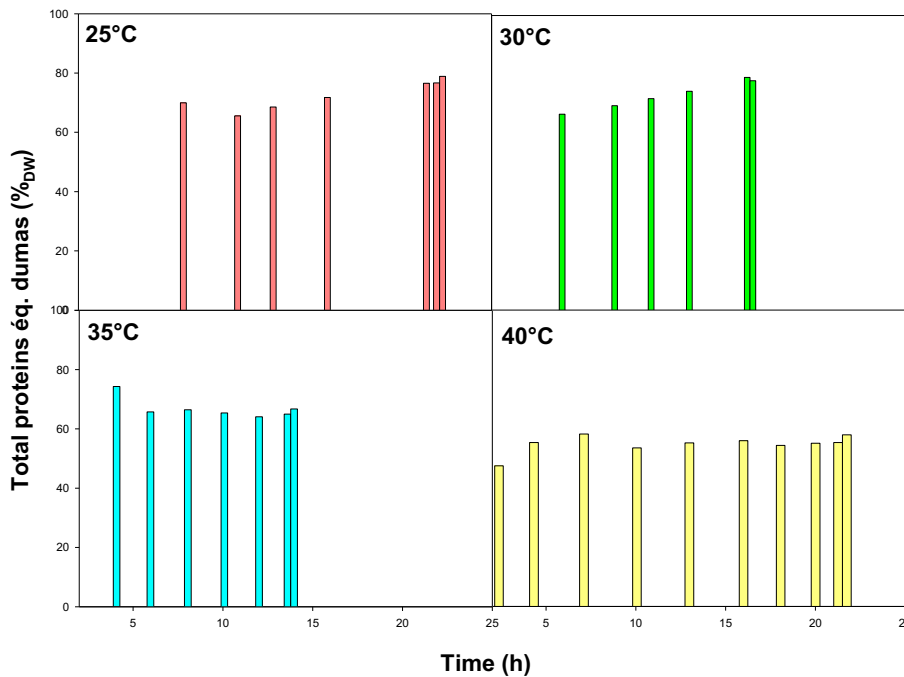


Figure 34 : Total protein content evolution (calculated from total nitrogen quantification by Dumas Method) over time at different temperatures

These results were also confirmed using the reference Kjeldahl method (analysis done in external lab) (Figure 35). Indeed, total protein amounts were 82.0; 81.9; 69.9 and 59.3 %_{DW}, respectively, at 25, 30, 35 and 40°C which is very close to those obtained by Dumas's method.

As a conclusion, the higher temperatures used (40°C) and at less extent (35°C) showed to have a negative impact on both NaOH-solubilized and total proteins production. Protein amounts were, therefore, dependent on the temperature value in *Cupriavidus necator*.

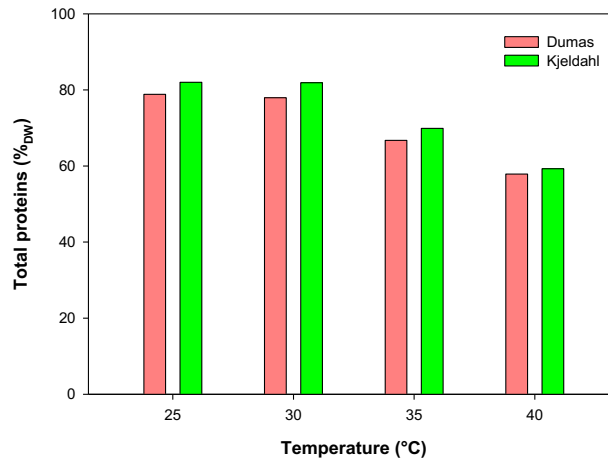


Figure 35 : Comparison between the final total protein amounts using two quantification methods Kjeldahl and Dumas

In the literature, for other microorganisms, the protein content had the same tendency as the growth in response to temperature stress. The protein content was optimal between 30 and 40°C for *Aspergillus terreus* [173], lower when the temperature was above 28°C for *Cryptococcus aureus* [104], and decreased when the value was above 34°C for *Schanniomyces castellii* [146]. In the case of *C. necator*, the amount of total protein (%_{DW}) obtained in the literature at a temperature of 30°C were quite similar (75%_{DW} in the literature against 79%_{DW} in this work) even if the strains were different (*H16=ATCC17699*, *Z1* and *B5786*) [158, 226, 249]. However, there is no article evaluating the protein production in response to temperature stress.

Regarding nucleic acid production

Nucleic acid quantifications were performed after lysis of cell pellets by the means of the FastPrep system. Figure 36 presents both RNA and DNA evolution profiles over time for the four batch cultures.

Figure 36-A shows that, at 30 and 35°C, the RNA profiles were quite similar. However, lower profiles were observed at 25°C and 40°C. However, the final points at 25, 30, 35 and 40°C were at 6.03 ± 0.15 , 5.76 ± 0.18 and 7.96 ± 0.06 and 4.35 ± 0.17 %_{DW}, respectively. The highest final amount was measured at 30°C and the lowest at 40°C.

Figure 36-B shows DNA percentages ranged between 1.5 and 3 %_{DW}. DNA profiles at 25, 30 and 35 °C were quite similar with comparable final amounts of 2.86 ± 0.05 , 2.27 ± 0.03 , 2.76 ± 0.05 %_{DW}, respectively. However, at 40°C, DNA production was lower compared to other temperatures, with a final amount of 1.63 ± 0.07 %_{DW}.

In conclusion, a high temperature value (40°C) gave lower nucleic acid production percentages. The temperature had an effect on RNA and DNA production in *C. necator*.

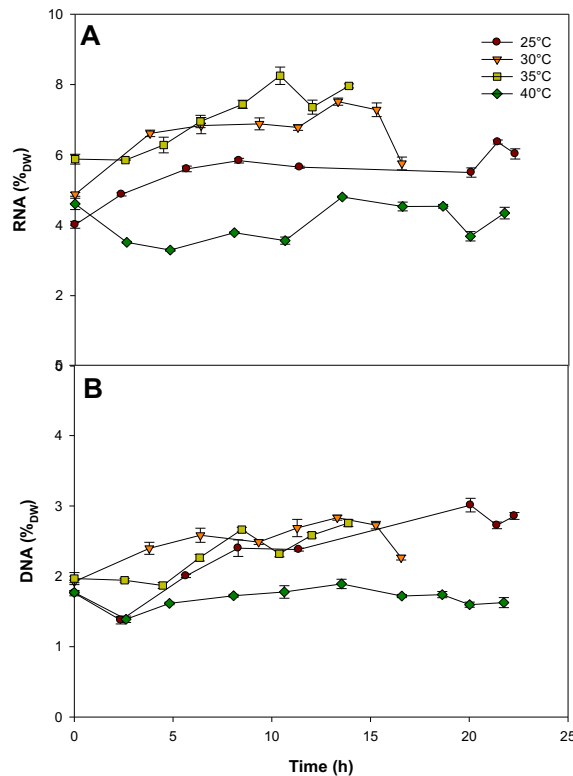


Figure 36 : Evolution of nucleic acid percentages over time using different temperatures

Regarding biomass composition

Figure 37 presents the biomass compositions considering the proteins recalculated from the Dumas analysis (by subtraction of the part of nitrogen coming from nucleic acids), the nucleic acids and the estimated values carbohydrates (cell wall) and fats (cell membrane). In the literature, values for *C. necator* were 5 and 6 % for carbohydrates and fats, respectively [158]. The results suggested that the calculated percentages of proteins decreased with the increasing temperature.

At 25 and 30°C, the macromolecular composition was close to 100% (89.85 at 25°C and 88.94 at 30°C) but less at 35°C (75.73 %) and especially for 40°C, an increasing deviation is observed (68.86 %). That can be due either to a lower estimation of carbohydrate and fats under high temperature of growth or to a production of other compounds during this culture.

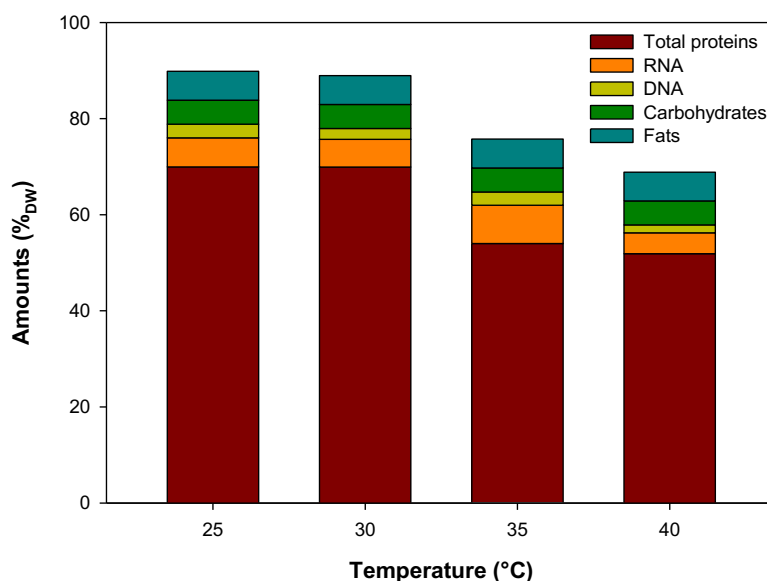


Figure 37 : Estimated biomass composition in percentages at different temperatures

- **Flow cytometry for PHB detection**

In order to verify if other compounds were produced during cultures, Poly- β -hydroxybutyrate (PHB) was investigated by flow cytometry in combination with the fluorescent probe (Nile Red). Indeed, the strain used can produce PHB since its production pathway is not deleted. Figure 38 presents the distribution of labelled sub-populations at three points of the cultures (initial point, point in exponential phase and final point) compared to 100% cells which have accumulated PHB (PHB⁺_(control) on Figure 38).

The initial points of the four cultures were slightly different despite the fact that they came from precultures prepared under the same conditions, but the Nile Red⁺ percentage was quite similar, ranged between 33 and 40%. During the exponential phases, Nile Red⁺ populations were around 35-37 at 25 and 30°C, but higher at 40°C (54%) and more at 35°C (83%). At the end of the culture (lines in blue), the Nile Red⁺ population was also observed, its percentage increased with the value of the temperature with a higher percentage at 40°C. On the other hand, the Nile Red is not a specific label for PHB granules. Indeed, it can be used to detect lipids and proteins' mixtures [250]. Thus, Figure 38 could not present the real PHB⁺ populations, only at 40°C according to the microscopic results showed previously.

Regarding the literature, only few studies evaluated the PHB production in response to temperature stress. For instance, Kerketta and Vasanth [222] studied the influence of culture parameters including temperature on the production of PHB from *Madhuca indica* flower extract as cheaper carbon source. The highest production was given at 35°C after 48 hours of culture [222]. Added to that, the study of Nowroth *et al.*, [221] compared PHB accumulation

using two different temperatures (5°C and 30°C). 5°C was found as the best value to produce the biopolymer on nutrient broth medium [221].

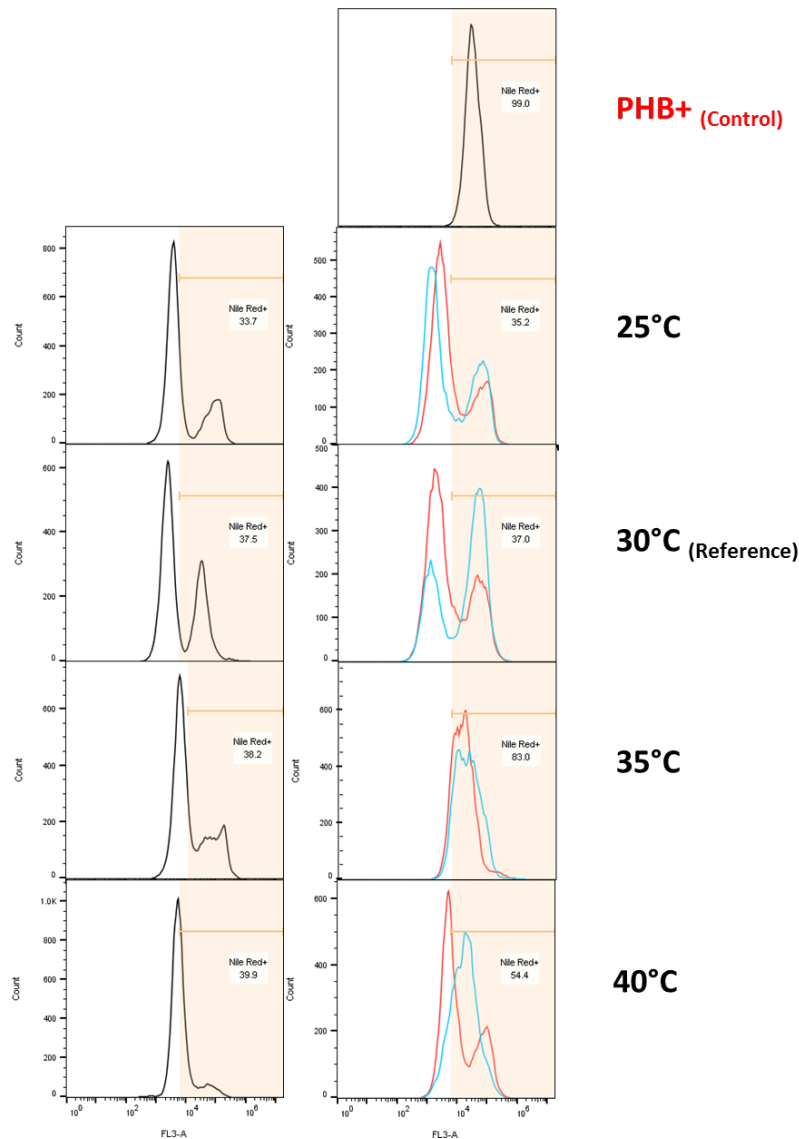


Figure 38 : Poly- β -hydroxybutyrate (PHB) detection with flow cytometer at different temperatures (**Orange rectangle**: Nile Red⁺, the indicated percentages were obtained for black profile on the left and red profile on the right); **black**: initial point; **red**: point in the middle of the exponential phase; **blue**: final point)

- **CHONS composition**

In order to verify biomass macromolecular composition over time in response to temperature change, a CHONS analysis were carried out at the end of each culture. Figure 39 compares the percentages for each element (C, H, O, N, and S) for the different culture conditions used. No variation in carbon (46-48 %) and hydrogen (7%) amounts were detected. Oxygen value was

slightly different (24% at 25 and 35°C and 27 % at 30 and 40°C). However, the nitrogen percentages decrease when the temperature increased and values varied from 12.01 ± 0.06 to 8.33 ± 0.33 %_{DW} at 25 and 40°C respectively. The same result was obtained with sulfur, but the values always ranged between 0.4 and 0.6 %.

Elemental compositions were obtained from the previous percentages (Figure 39), as presented in Table 42. The table presents the biomass formula at the end of the culture for the different culture temperatures. Oxygen values were similar (ranged between 0.39-0.43). Hydrogen, sulfur and nitrogen decreased with higher temperatures however to a higher extent for nitrogen. For instance, comparing the cultures at 25°C and 40°C, the hydrogen value passed from 1.85 to 1.75 and the nitrogen passed from 0.22 to 0.15.

The N percentage evolution agrees with the total proteins results (Figure 34 and Figure 35). Indeed, the total protein contents obtained with both Dumas and Kjeldahl methods decreased by increasing the temperature, passing from 25 to 40°C. These total protein percentages were calculated from the total nitrogen in biomass [243, 251]. In addition, the production of PHB at 40°C can cause the decrease in N percentage since it is not a nitrogen-based product [208].

The sulfur values can be influenced by the amino acids' composition, notably by the amount of methionine and cysteine in the proteins. The ratio S/N were relatively constant at 0,020 at temperature 30 and above, however at 25°C the ratio was 13% higher at 0,023. This would need to be confirmed by amino acid composition quantification in the proteins. Indeed, bacterial biomass showed high levels of essential and sulfur amino acids [124] and methionine contents are comparable with recommended values from FAO [75]. However, using different culture conditions the amino acid composition of *C. necator* reported in Foster and Litchfield [226] and Calloway and Kumar [99] studies showed higher methionine values compared to some other bacterial proteins and changes in sulfur amino acids compositions comparing the two studies [99, 226].

Considering all these compounds, molecular weights of culture biomasses were calculated (without considering the ashes). Table 42 presents their values depending on the temperatures of cultures. At 25, 30 and 35°C, the values were comparable (around 23 g/Cmol). However, the value is slightly lower (22.72 g/Cmol ± 0.16) at 40°C, where the nitrogen impact was significant compared to the other conditions.

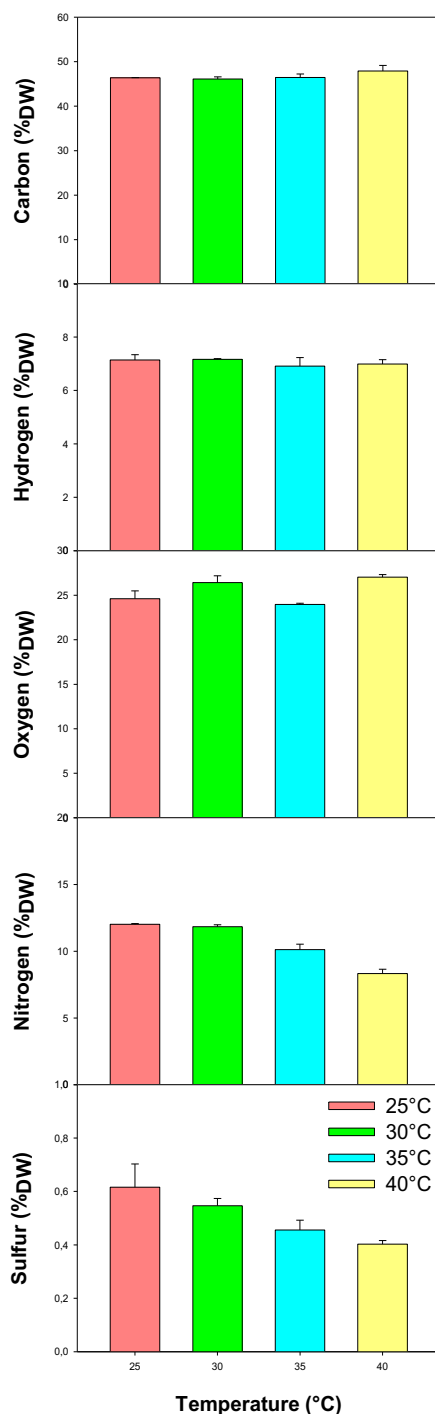


Figure 39 : CHONS composition for different temperatures

Table 42 : Elemental composition of the final biomass at different temperatures

Temperature (°C)	Elemental composition					Molar weight (g/Cmol)
	C	H	O	N	S	
25	1	1.85	0.40	0.22	0.0050	23.48±0.09
30	1	1.86	0.43	0.22	0.0044	23.96±0.26
35	1	1.79	0.39	0.19	0.0037	22.71±0.01
40	1	1.75	0.42	0.15	0.0031	22.72±0.16

3. Conclusion

This chapter had as an objective to evaluate SCP production and biomass composition using different temperature (25, 30, 35 and 40°C). To do that, growth dynamics, cell morphology and biomass composition were compared.

The maximal growth rate reached a higher value at 35 °C and lower at 25°C and 40 °C with no significant difference was detected in biomass yields between cultures. On the morphological level, no changes in size and granularity at 25°C, 30°C and 35°C were obtained. However, at 40°C, both cell size and granularity increased slightly over time, possibly caused by PHB granules production seen through a macroscopic analysis. Protein production presented the same dynamic, the protein amount increased overtime. The protein amounts were slightly similar at 25, 30 and 35°C, but lower at 40°C. Nucleic acids profiles were quite similar at 25, 30 and 35°C, but lower at 40°C. For the PHB detection, the results of flow cytometry in combination with the fluorescent probe (Nile Red) show that the Nile Red⁺ population were observed during all the culture. This percentage was higher at the 40°C culture. At this condition, the labelled population is probably PHB⁺ population according to macroscopic images. For the biomass CHONS composition, the nitrogen and the sulfur percentages were relatively stable at 30°C and above, but slightly higher at 25°C.

Considering the growth and the biomass composition, the chapter results showed that temperature value has an impact on Single Cell Protein production. At high temperature, the growth is slower and the production of RNA, DNA and proteins was impacted. Indeed, low percentages were obtained at 40°C which was also confirmed with low percentage of nitrogen. Nile Red + population, changes in cell size and granularity and also the low N percentage reinforced PHB production, especially at 40°C.

**Chapter III-3: Impact of the pH on
Single Cell Protein production and
composition in *Cupriavidus necator***

Chapter III-3: Impact of the pH on Single Cell Protein production and composition in *Cupriavidus necator*

1. Introduction

The aim of this part was to characterize cell growth and single cell protein production and composition by *C. necator* in response to different pH. In literature, some works have been reported concerning microbial growth and/or protein production using different pH values using fungi (*Aspergillus terreus* [173]), bacteria (*Raoutella ornithinolytica* [124], *Acetobacter diazotrophicus* [96]) and yeasts (*Schawanniomyces castellii* [146], *Rhodotorula glutinis* [140], *Cryptococcus aureus* [104] and *Saccharomyces cerevisiae* [175]). On the other hand, no evaluation of protein production under different pH conditions was done using *C. necator* in the literature. Only the study of Sakarika *et al.*, [167] gave information about growth and protein production in *C. necator* in response to two pH values, pH 6 and pH 7.

In our work, the bacteria *C. necator* was cultivated in well controlled bioreactors at different pH (5, 6, 7 and 8) to quantify pH impact on growth behavior and both SCP production and composition. All the bioreactor inocula were prepared under the same conditions at pH7 which is the optimal pH for growth reported in the literature for this strain.

The different pH values were maintained constant during the cultivations by the means of a pH regulation by addition of ammoniac solution.

In this chapter, growth parameters, SCP production and also the elemental composition were presented. Biomass growth and substrate concentrations were described by presenting the evolution of these state variables during the different cultures and through both growth rates and biomass yield values. For SCP production, evolutions of both soluble and total protein and nucleic acid quantifications were presented. Added to that, Poly- β -hydroxybutyrate production was evaluated. Finally, the evolution of the elemental composition of the biomass was compared through a CHONS analysis.

2. Results and discussion

2.1. Growth dynamics and viability

Cell growth

For all the different pH values, biomass growth and substrate consumption evolutions were quantified and compared, as shown in Figure 40. The kinetic profiles at pH6, pH7 and pH8 followed a similar exponential growth. However, at pH5, a very low growth was observed and this experiment was stopped after 124 hours. With an initial glucose concentration of 15-17 g/l,

a biomass concentration of 8 g/l was obtained after complete exhaustion of the C-substrate at pH6, pH7 and pH8 compared to only 1 g/l at pH5. In addition, no production of other metabolites was detected in any culture supernatants.

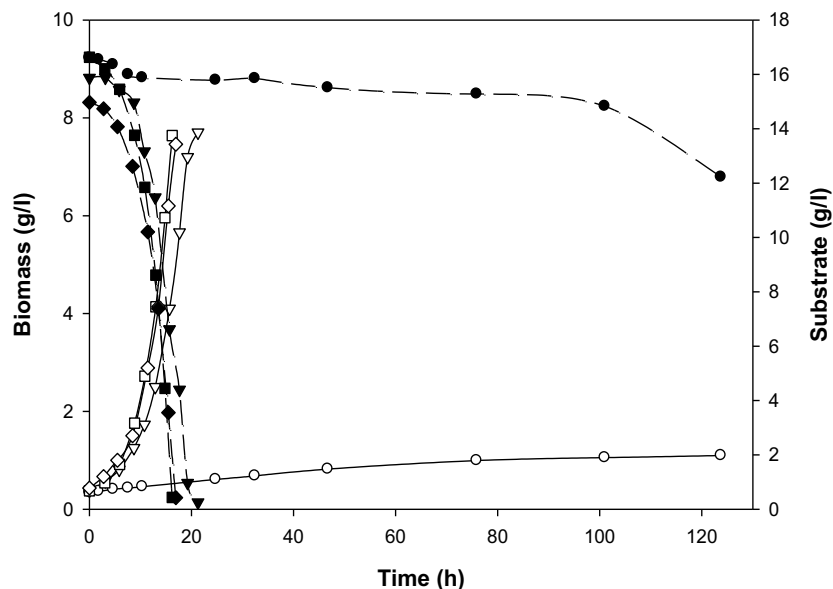


Figure 40 : Evolution of the concentration of biomass (solid line) and glucose (dotted line) over time for each tested pH: pH5 (●○), pH6 (▲△), pH7 (■□) and pH8 (◆◇).

Using the evolutions of the state variables, growth rates and biomass yields were calculated, as shown in Table 43. At the reference growth conditions (pH7), the maximum growth rate value was $0.21 \pm 0.01 \text{ h}^{-1}$, corresponding to a unique phase of growth along the culture until carbon depletion. For the other pH conditions, different growth phases were observed during the culture. Table 41 showed the growth rate determination for each condition. Two growth phases were obtained at pH6 (0.11 ± 0.01 and $0.16 \pm 0.01 \text{ h}^{-1}$) and pH8 (0.12 ± 0.01 and $0.18 \pm 0.01 \text{ h}^{-1}$), and the obtained values are lower than those obtained for pH 7. At pH5, three growth rates values were obtained 0.027 ± 0.004 ; 0.014 ± 0.001 and $0.0021 \pm 0.0002 \text{ h}^{-1}$. In addition, no significant difference in biomass yields was detected between pH6, 7 and 8. Indeed, the values were at 0.45 ± 0.03 ; 0.46 ± 0.01 and $0.49 \pm 0.02 \text{ g}_x/\text{g}_s$, respectively. However, much lower yield was obtained at pH5 ($0.22 \pm 0.04 \text{ g}_x/\text{g}_s$). That could be a result of the conversion of a higher part of glucose into cellular energy facing pH stress or the production of co-products such as PHB although considered in the biomass dry weight as an intracellular compound. Thus, the maximal growth was obtained at pH7 as reported in the literature [169, 199, 201, 209, 215, 247, 252, 253] and the lower was at pH5.

Table 43 : Growth rate and biomass yield values depending on pH

pH	μ (h ⁻¹)	R _{x,s} (g _x /g _s)
5	0.027±0.004	0.22±0.04
	0.014±0.001	
	0.0021±0.0002	
6	0.11±0.01	0.45±0.03
	0.16±0.01	
7	0.21±0.01	0.46±0.01
8	0.12±0.01	0.49±0.02
	0.18±0.01	

According to the literature, other strains were used to evaluate SCP production for the studies under pH stress. Applying an acidic stress (pH5) gave the highest growth to some microorganisms : *Gluconacetobacter diazotrophicus* grown on waste [96], *Aspergillus terreus* using cheap energy sources [173], *Rhodotorula glutinis* grown on potato wastewater and glycerol [140], *Schanniomyces castellii* using cassava starch [146] and *Saccharomyces cerevisiae* grown on date extracts [175]. However, alkaline stress (pH8.5) was found as the best for others such as *Raoutella ornithinolytica* cultivated in potato residue value [124].

In the case of *C. necator*, the optimal pH was normally pH7 (or 6.8 sometimes) considering the literature [169, 199, 201, 209, 215, 247, 252, 253] which agrees with the results obtained in this work. However, there is no articles evaluating SCP growth in response to pH stress yet. Only one information was given from the study of Sakarika *et al.*, [167] comparing growth under two pH values. Indeed, pH 7 gave the highest growth rates compared to pH 6 [167].

Cell morphology and viability

Figure 41 shows cells morphology during the cultures at pH5, 6, 7 and 8. At pH 6, 7 and 8, cell size and granularity were slightly the same during all the culture, with more homogeneous profiles at pH6. However, at pH5, both cell size and granularity increased over time. Indeed, for pH5, the big changes on cell size were started in the second phase of growth when the growth rate decreased from 0.027 to 0.014 h⁻¹. Elongation and cell size enlargement could be explained by PHB production [217, 248].

Cell viability and membrane permeability measured by PI dye were quantified by flow cytometry. Lower percentages of dead populations were observed for three different cultures (pH5, 6 and 8). Indeed, percentages did not exceed 9.48, 7.64 and 2.49%, respectively. Microbial cells were mainly intact and a weak loss in cell viability was detected.

Part III - Results and discussions

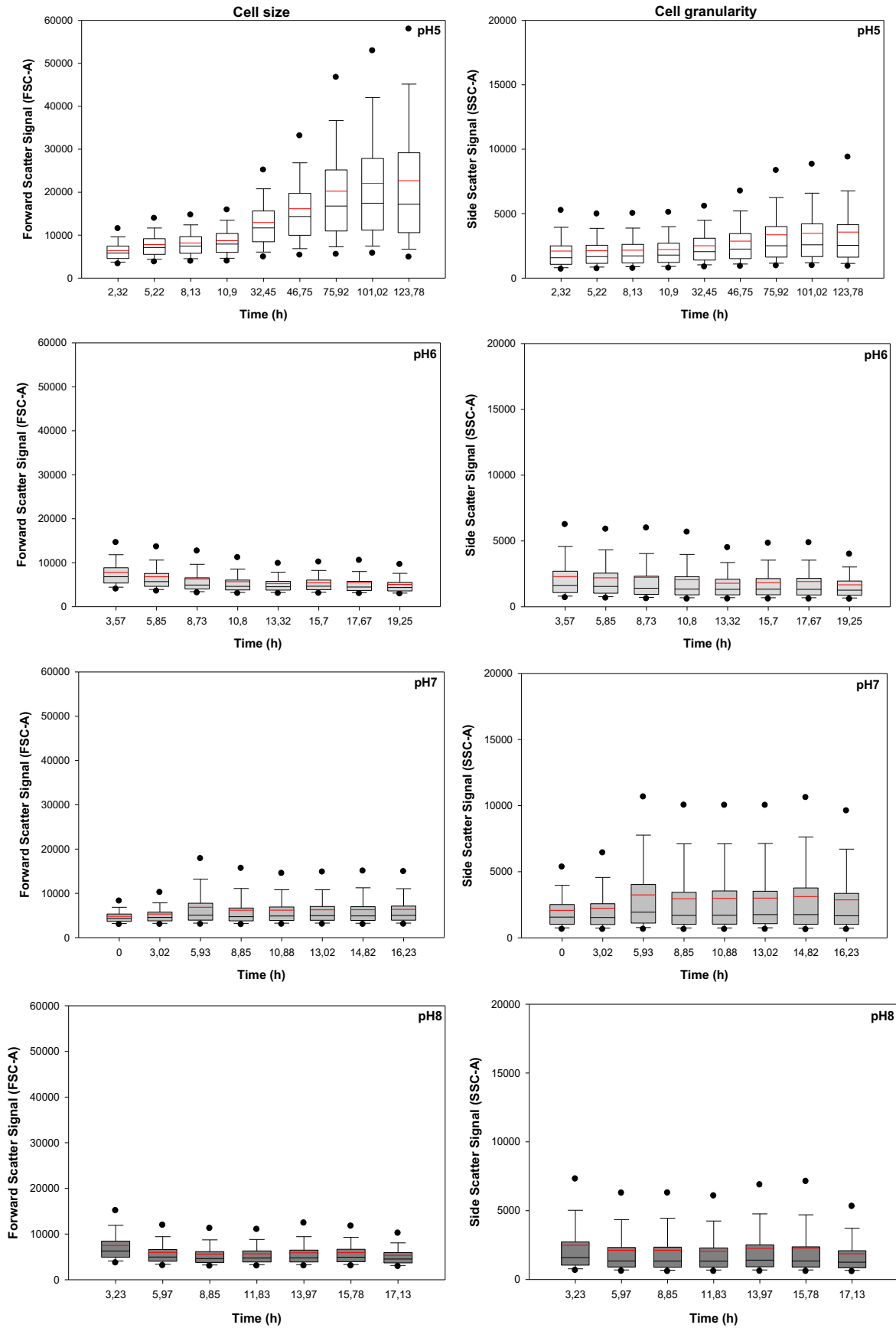


Figure 41 : Evolution of cell size and granularity measured by FSC and SSC signals distributions over time using different temperatures

2.2. SCP production and composition

Regarding protein production

Using two different quantification methods, protein production evolutions were compared between four pH conditions, as show Figure 42 and Figure 43.

Figure 42 showed the NaOH-solubilized protein amounts quantified by the BCA assay. Protein accumulation was faster at pH7 and pH6 but slower at pH8 and especially at pH5. The profiles were stable during the cultures but at pH5 a degradation of protein (proteolysis) could be understood considering Figure 42. Comparing the final protein percentages, the different pH conditions led to similar values: 35.26 ± 2.07 , 32.69 ± 1.08 , 41.3 ± 4.56 and 33.62 ± 1.14 %_{DW} at pH5, 6, 7 and 8, respectively.

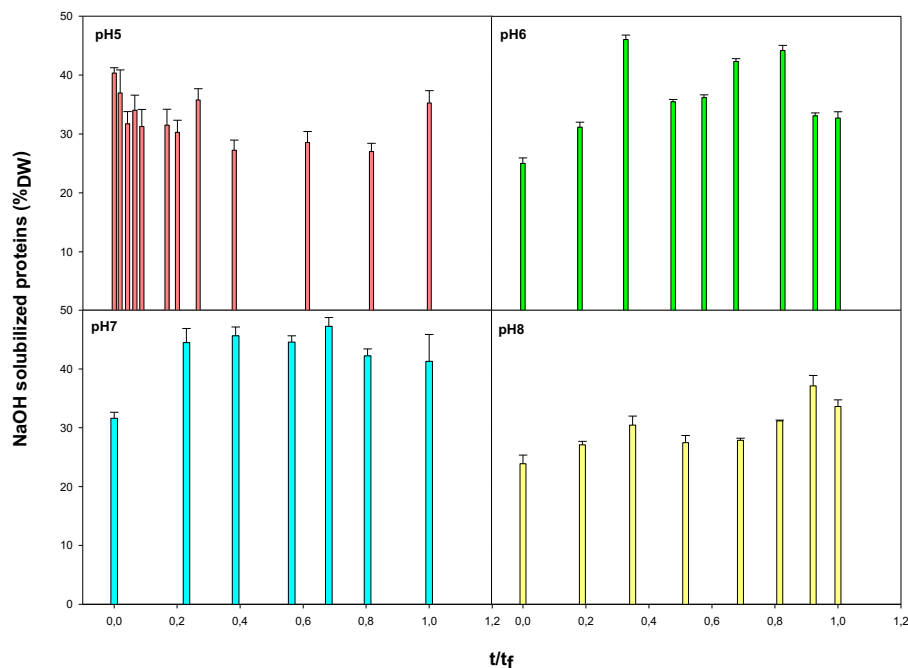


Figure 42 : NaOH-solubilized protein evolution quantified by BCA method over time at different pH

Nitrogen amount converted into total protein percentages observed by the Dumas method (Figure 43) were different depending on the pH. The profiles showed that the protein production increased overtime in every condition. Similar final amounts were obtained at the end of cultures at pH6, pH7 and pH8 (75.67, 77.95 and 78.05%_{DW}) but a much lower percentages was measured at pH5 (50.41%_{DW}). So, at pH5, lower values were obtained not only for the growth, but also the protein production.

Therefore, protein production is dependent on the pH value choice. The acidic pH gave lower amounts compared to the other conditions.

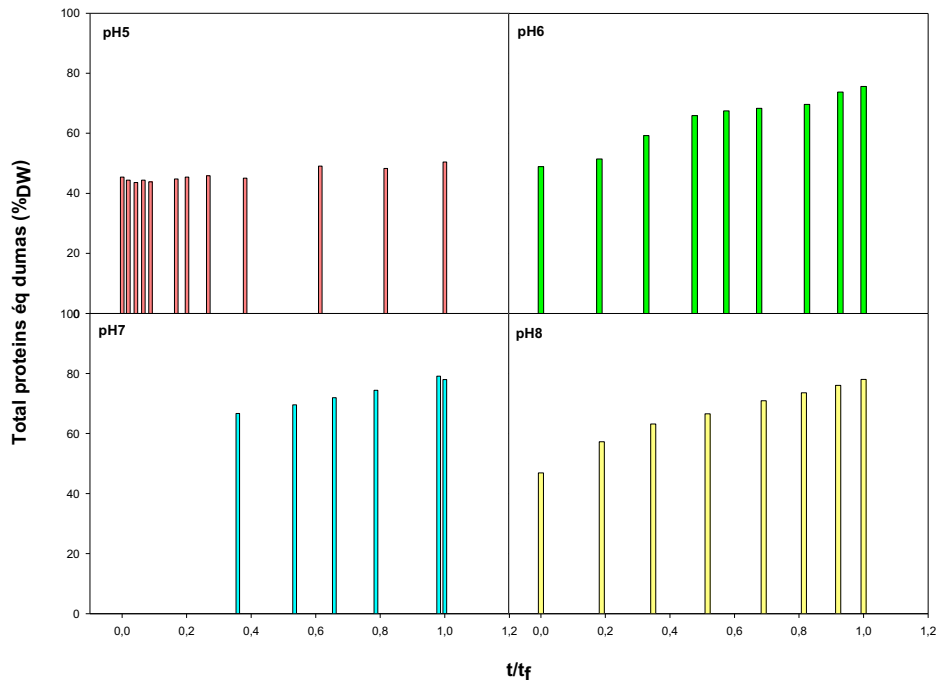


Figure 43 : Total protein content evolution (calculated from total nitrogen quantified by Dumas's method) over time at different pH

According to the SCP papers, no studies have investigated the pH effect on *C. necator* growth. However, some erlen flask cultures investigated this effect using other microorganisms.

An acidic pH stress gave the highest protein amount in the case of *Acetobacter diazotrophicus* [96] and *Aspergillus terreus* [173]. However, for *Cryptococcus aureus*, the protein amount was lower at acidic medium pH3 and pH4 (< 2.5 g_{DW}/l) and also at alkaline medium pH8 (< 3 g_{DW}/l), the highest protein content was estimated between pH5 and pH6 (3.5 g_{DW}/l), which were the optimal condition of growth [104].

In other studies, pH is not a main factor affecting protein amounts. For example, the amounts were around 40%_{DW} using pH between pH3 and pH8, in the case of *Rhodotorula glutinis* [140].

Regarding nucleic acid production

Figure 44 presented both RNA and DNA evolution over time, comparing the different cultures. For RNA, the curves are similar at pH6 and pH8, higher at pH7 and lower at pH5. Final values were comparable at pH6 and pH7 (5.8%_{DW}). However, a lower percentage was measured at pH5 (3.31±0.07%_{DW}) and higher amount was obtained at pH 8 (6.36±0.06%_{DW}) (Figure 44-A). Regarding DNA profiles, the amounts were ranged between 1.5 and 3 %_{DW} during the cultures. The profiles were similar at pH6 and pH7 but lower at pH8 and especially at pH5. The final values were quite similar at pH 6, 7 and 8 (2.60±0.03 and 2.27±0.03 and 2.41±0.08%_{DW}) but lower at pH5 (1.32±0.01%_{DW}) (Figure 44-B).

Therefore, the nucleic acid amounts were dependent on pH value of the culture. The acidic pH (pH5) gave lower amounts for both RNA and DNA.

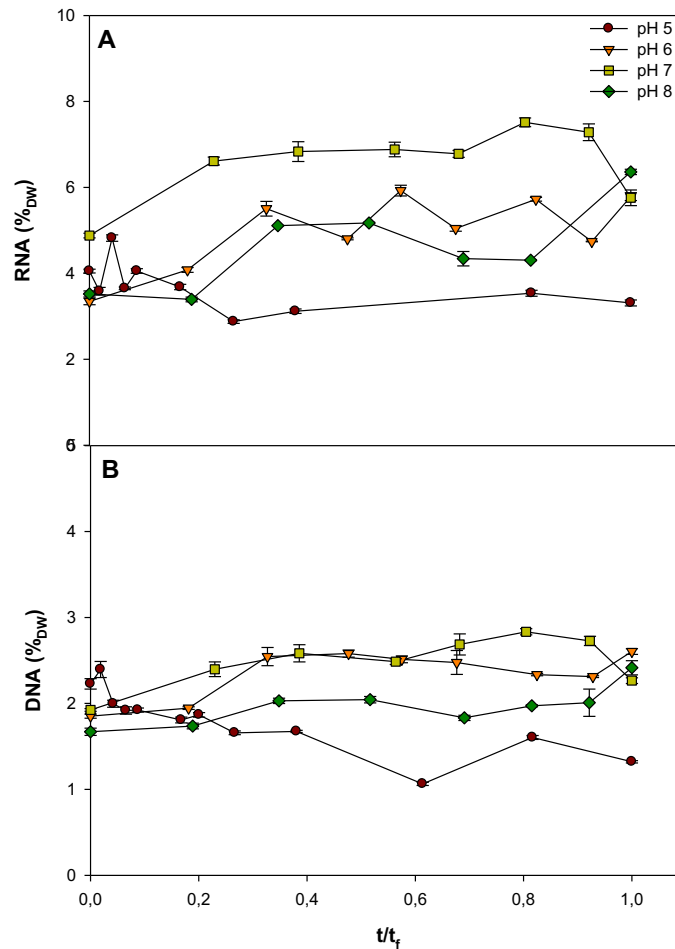


Figure 44 : Evolution of nucleic acid production over time using different pH

Regarding biomass composition

Figure 45 presents the biomass composition considering the quantified compounds (proteins recalculated considering nucleic acid nitrogen and nucleic acids) and also the compounds not quantified (carbohydrates and fats) from the literature [216]. The results suggest that the percentage increased with the pH value. They are close to 100% for pH6, 7 and 8 (87%, 89% and 89%, respectively), but far for the pH5 (60%). That can be due to variation in fats and carbohydrate amounts or to a production of other intracellular compounds during this culture.

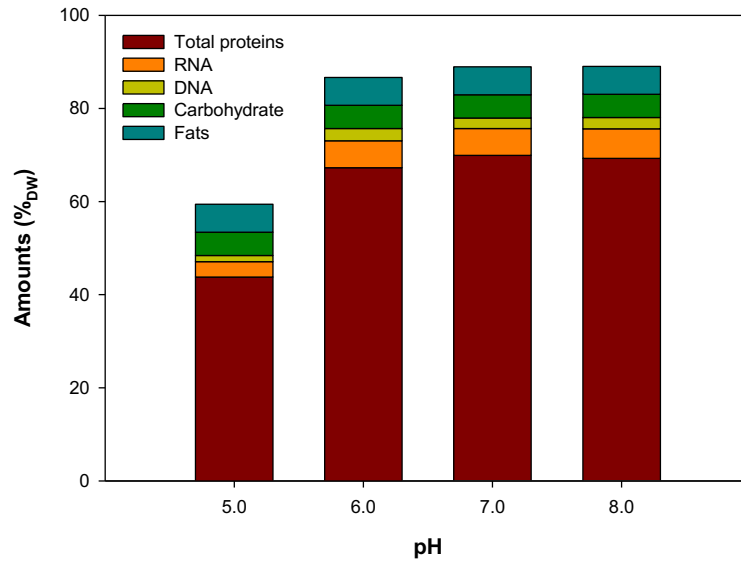


Figure 45 : Estimated biomass composition in percentages at different pH.

- **Flow cytometry for PHB detection**

C. necator naturally produces poly- β -hydroxybutyrate under nutrient limiting conditions. In our case, the strain 4623 can produce PHB since its synthesis pathway is not deleted. In order to verify the presence of this product during the cultures, Nile Red (NR) fluorescent probe was used for flow cytometry measurements. Figure 46 shows the distribution of the population during cultures compared to 100% cells that have accumulated PHB (reference). At pH 6, 7 and 8, the culture presented significant percentages of Nile Red⁺ cells. However, higher percentage of the Nile Red⁺ population was observed at pH5. These high percentages started at the second phase of growth when growth rate passed from 0.027 to 0.014 h⁻¹. These results can explain the lower amount of protein produced using this pH.

In some studies, PHB content was evaluated during *C. necator* cultures with non-regulated pH. In Aramvach et al., study [219], the PHB content decreases when initial pH increases from pH6 to pH8 [219]. Also, in the study of Khanna et al., [220], the decrease of pH from 7 to 5.26 increased the PHB yield [220]. However, other studies showed that pH value around 8 stimulates considerably PHB production [254, 255].

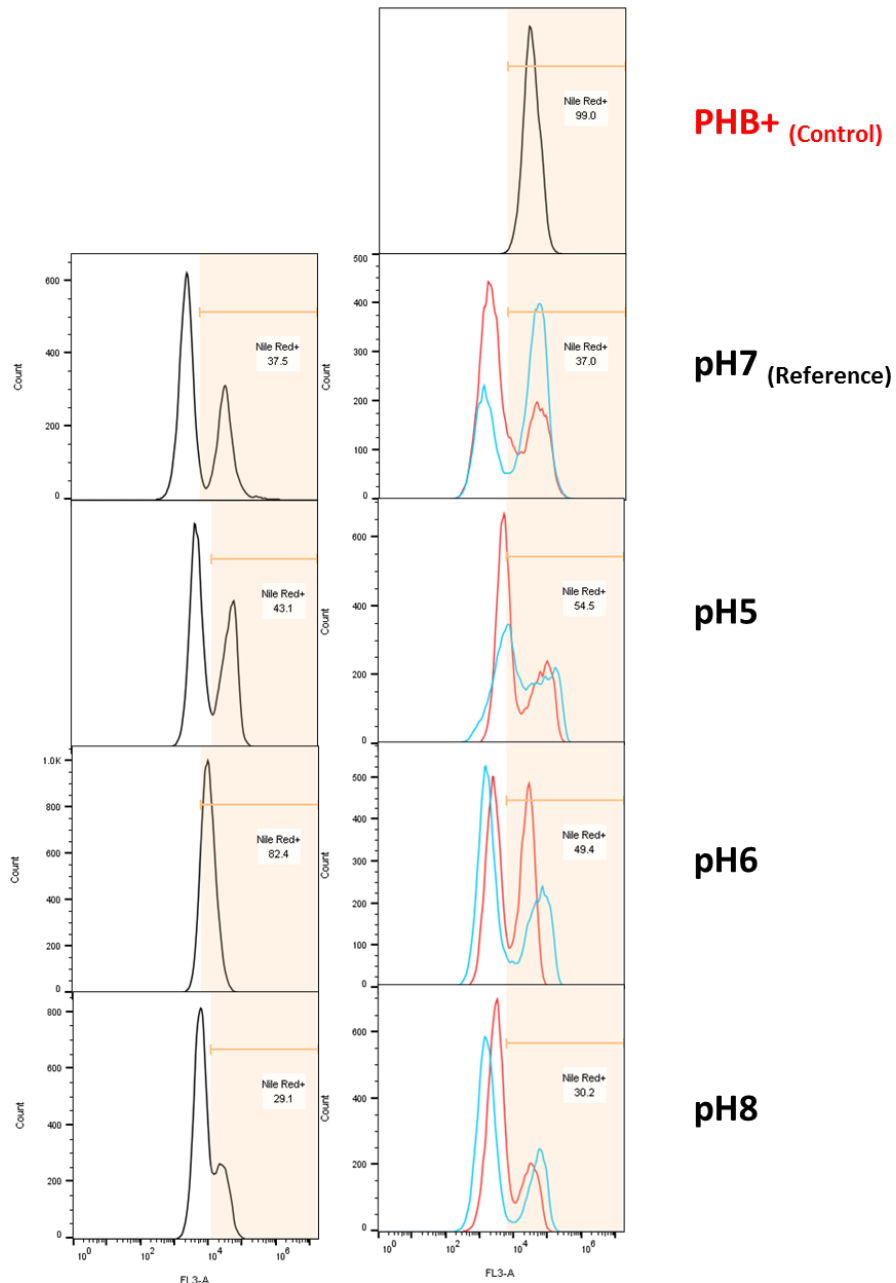


Figure 46 : Poly- β -hydroxybutyrate (PHB) detection with flow cytometer at different pH (**Orange rectangle**: Nile Red⁺, the indicated percentages were obtained for black profile on the left and red profile on the right); **black**: initial point; **red**: point in the middle of the exponential phase; **blue**: final point)

- **CHONS composition**

In response to pH stress, the CHONS composition variation was measured at the end of each culture, as shown in Figure 47. The figure compares carbon, hydrogen, oxygen, nitrogen and sulfur percentages in the biomass. The percentage of carbon increased when the pH value is decreased from 8 to 5, from 42.86 ± 4.7 to $48.87 \pm 1.38\%_{DW}$. No high variation in hydrogen (7%) amounts was detected. Oxygen value was slightly different (26 % at both 5 and 7, 25.48 ± 1.96

at pH8 and $23.55 \pm 0.09\%_{\text{DW}}$). Nitrogen and sulfur percentages have the same profile. Indeed, the values increased when the pH value increased. From pH8 to pH5, the values vary from $10,84 \pm 1,11$ to $7,01 \pm 0,15\%_{\text{DW}}$ for nitrogen and $0,56 \pm 0,09$ to $0,36 \pm 0,01\%_{\text{DW}}$ for sulfur.

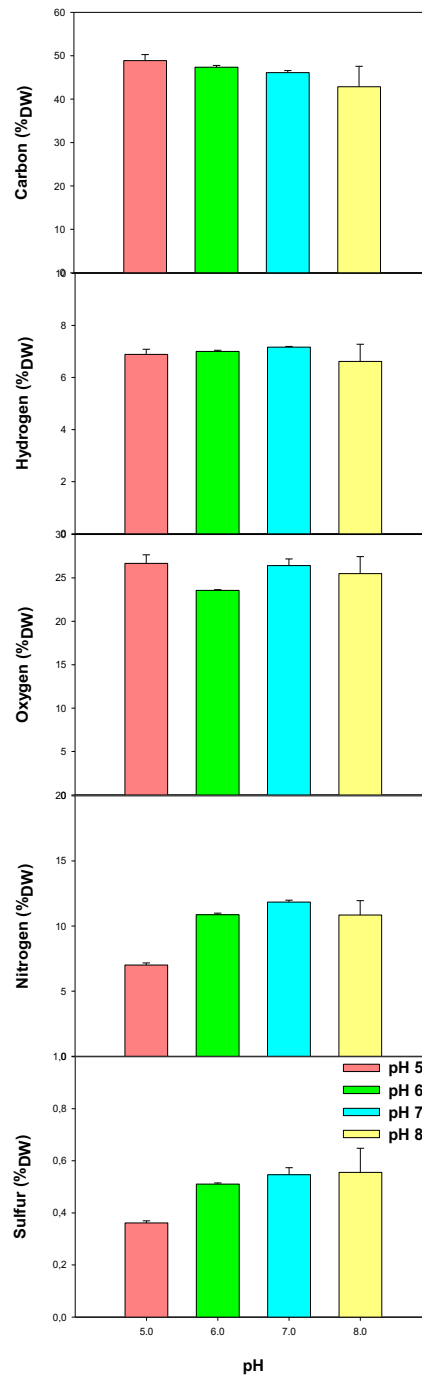


Figure 47 : CHONS composition for different pH

Elemental composition was obtained with the previous percentages (Figure 47). Table 44 presents the biomass formula at the end of the culture for the four pH values. Oxygen and

hydrogen values were slightly similar at different pH. However, nitrogen and sulfur values decrease slightly with lower pH. For example, comparing the cultures pH7 and pH5, the nitrogen passed from 0.22 to 0.12 and the sulfur passed from 0.0048 to 0.0028. S/N ratio were found identical at pH 6 and 7 (0.02) and slightly higher at pH8 (0.022) and specially pH5 (0.023). These results could suggest different amino acid composition, in particular sulfur-containing amino acids (methionine and cysteine), during cultures with different pH. However, this should be confirmed by quantifying amino acid composition in the proteins.

The molar weight of the biomass was thus very impacted by this composition changes from 26 g/Cmol at pH 7 to 22 g/Cmol at pH5. Indeed, the biomass value passed from 24.25±0.97 to 22.05±0.03 Cmol from pH8 to pH5.

Table 44 : Elemental composition of final biomass at different pH

pH	Elemental composition					Molar weight (g/Cmol)
	C	H	O	N	S	
5	1	1.69	0.41	0.12	0.0028	22.05±0.03
6	1	1.77	0.37	0.20	0.0040	22.62±0.04
7	1	1.86	0.43	0.22	0.0044	23.96±0.26
8	1	1.85	0.45	0.22	0.0048	24.25±0.97

3. Conclusion

This chapter investigated SCP production and biomass composition using different pH values (5, 6, 7 and 8). Indeed, growth dynamics, morphology of cells and SCP production and composition were compared.

The maximal growth rate was obtained only with pH7, however, for the other conditions many growth phases were obtained especially for the pH 5 where the culture was very slow. On the morphological level, changes in both size and granularity were observed during the pH5 culture. Protein profiles were almost the same at pH 6, 7 and 8 but lower amounts at pH5. The same results were obtained for nucleic acid production profiles. Confirming these results, the use of the fluorescent probe (Nile Red) shows that the Nile Red+ population were observed during all the culture, but with higher percentages at pH5. All these results agree with the total proteins results and also, the production of PHB at this condition.

Considering the growth and the biomass composition, the chapter results showed that pH has an impact on Single Cell Protein production by *C. necator*. At low pH value, the growth is slower and the production of both nucleic acids and proteins was lower compared to the other conditions.

Chapter III-4: Impact of the nitrogen source on Single Cell Protein production and composition in *Cupriavidus necator*

Chapter III-4: Impact of the nitrogen source on Single Cell Protein production and composition in *Cupriavidus necator*

1. Introduction

For Single Cell Protein evaluation, few studies investigated the use of organic (peptone, polypeptone, tryptone, yeast extract, soybean meal, corn steep powder, urea) and/or inorganic (ammonium sulfate, ammonium chloride, nitrate, nitrite) sources of nitrogen [24, 60, 103, 168, 169]. Among the microorganisms used for this aim, we can report a halophilic bacterial strain [24], *Bacillus brevis* [103]), the yeast *Candida utilis* [60] and the fungus *Trichoderma spp.* [168]. A study with *C. necator* was also done, using different forms of nitrogen sources (ammonium sulfate, nitrate, nitrite and urea) under autotrophic conditions [169]. However, no study was done using well-controlled heterotrophic conditions.

One of the objectives of SCP studies was the use of industrial or agricultural wastes or other by-products in order to valorize them as cheaper nutrients [38, 39]. Wastewater plants could be a good candidate also [256]. Urea is found as the most significant nitrogen compound in urine with around 90% [257, 258]. This source is found as a good candidate for microbial protein production. Indeed, for *C. necator*, protein amount can reach 65%_{DW} under autotrophic conditions using urea [169]. In addition, it presents not only a nitrogen source but also a carbon source. Indeed, metabolization of urea releases carbon dioxide and ammonia according to the following hydrolysis reaction : $(\text{NH}_2)_2\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2 \text{NH}_3$ [169]. This reaction is catalyzed by the urease enzyme, which seems to be more likely cytoplasmic according to KEGG and UniProt data basis considering H16 *Cupriavidus necator* strain. Indeed, search in genome databases of *C. necator* H16 and N1 showed annotations of urease subunits and urease accessory proteins located in the cytosol.

In this context, the aim of this chapter was to characterize cell growth and protein production by *C. necator* using two different forms of nitrogen under well controlled heterotrophic conditions using glucose as sole carbon source.

Two different sources of nitrogen were evaluated and compared, ammonium sulfate (usually used for *C. necator* [259]) and urea. Batch cultures were performed under controlled conditions (pH7, 30°C). Only the nitrogen source was changed in the preculture and bioreactor media.

In this chapter, growth parameters, cell morphology, protein production and the elementary composition of the biomass will be investigated and discussed.

2. Results and discussion

2.1. Growth dynamics and viability

Cell growth

During the batch cultures, both growth and glucose consumption of *C. necator* were measured in order to evaluate the growth dynamics.

Figure 48 presented their evolutions overtime for urea. An exponential growth was observed. With 14 g/l of glucose, 7 g/l of biomass were obtained with no organic acids and alcohols has been detected in the supernatants. Thus, visibly, all the glucose was converted into carbon dioxide and biomass.

Since urea releases both carbon dioxide (CO₂) and ammonium (NH₄⁺) in the medium after hydrolysis, both urea and ammonium concentrations were quantified (Figure 48) to evaluate the accumulation of each in the medium depending on the microbial growth phase.

Urea concentration passed from 3.5 to 1.4 g/l, indicating that no nitrogen limitation occurred during the course of the culture. Low residual ammonium concentration was detected along the culture, but its concentration reached 95.3 mg/l at the end of the culture when the glucose concentration was close to zero.

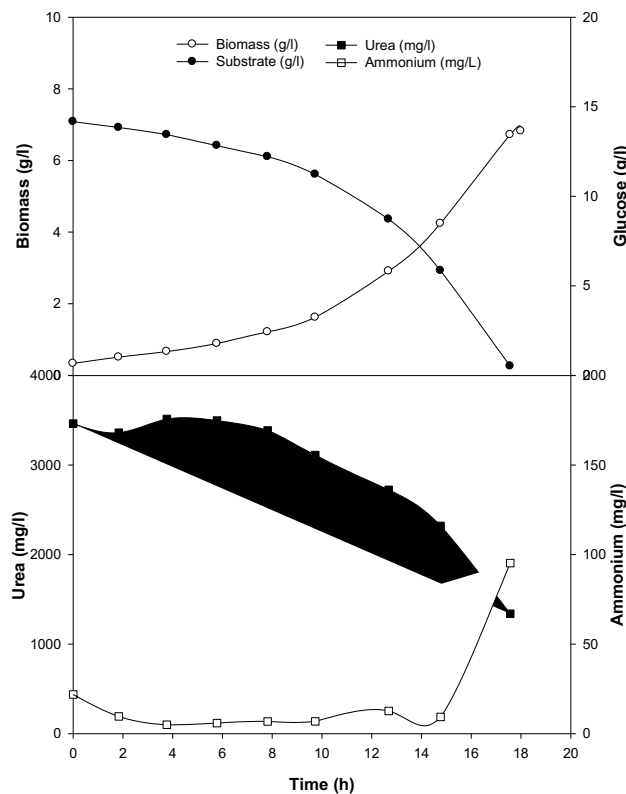


Figure 48 : Evolution of the concentration of biomass (○), glucose (●), urea (■) and ammonium (□) over time for the urea culture.

For ammonium sulfate used as N-source, the state variable evolutions were presented in Figure 49. Indeed, 8g/l of biomass were obtained at the end of the culture from 17 g/l of consumed glucose with no detection of organic acids or other compounds occurred in the samples' supernatants.

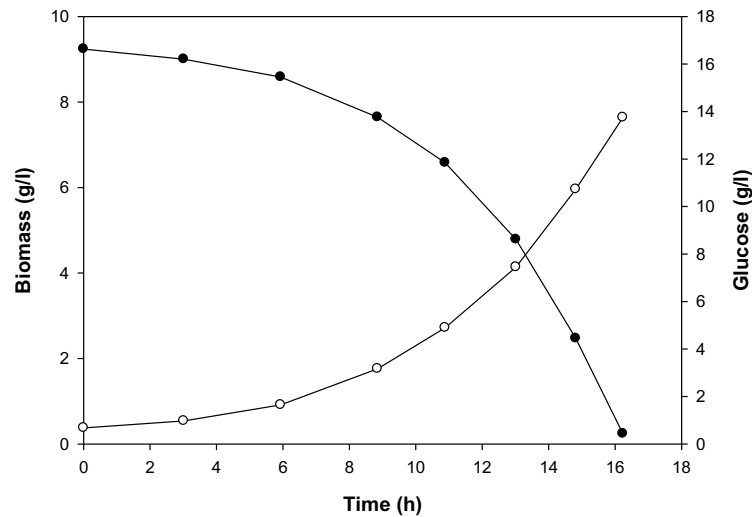


Figure 49 : Evolution of the concentrations of biomass (○) and glucose (●) over time for the ammonium sulfate culture.

In order to compare the growth using two nitrogen forms, growth rates and biomass yields were calculated, as presented in Table 45.

The growth was faster using ammonium than urea, as nitrogen source, with $0.21 \pm 0.01 \text{ h}^{-1}$ against $0.17 \pm 0.01 \text{ h}^{-1}$.

However, no significant difference was detected between the biomass yields. The values were comparable, $0.46 \pm 0.01 \text{ g}_x/\text{g}_s$ for ammonium sulfate and $0.47 \pm 0.01 \text{ g}_x/\text{g}_s$ for urea.

In conclusion, the use of urea slows down the cell growth of *Cupriavidus necator*.

Table 45 : Growth rate and biomass yield values depending on nitrogen source

Nitrogen form	$\mu \text{ (h}^{-1}\text{)}$	$Y_{x,s} \text{ (g}_x/\text{g}_s\text{)}$
Ammonium sulfate	0.21 ± 0.01	0.46 ± 0.01
Urea	0.17 ± 0.01	0.47 ± 0.01

These results are in agreement with the results of Yang *et al.*, [169] who investigated the effect of the nitrogen sources (ammonium sulfate, nitrate, nitrite and urea) on *C. necator* (strain DSM531) growth under autotrophic conditions. The results of this study showed that with

ammonium sulfate, the bacterium grew faster than on urea. Indeed, the maximal growth rates values were 0.26 h^{-1} and $0.18 \pm 0.01 \text{ h}^{-1}$, respectively [169]. In this study, the obtained values are slightly lower probably due to the difference of the strain, or the culture conditions. Indeed, Yang *et al.*, [169] study used the *DSM531* strain grown in autotrophic conditions using glass serum bottles with no controlled parameters.

With the strain *ATCC 17699* of *C. necator*, Aramvach *et al.*, [219] studied also the effect of different nitrogen sources on growth and ammonium sulfate presented lower growth compared to other sources (peptone, tryptone, yeast extract, urea and ammonium chloride) which is not in agreement with our results [219]. The same results were obtained with Khanna and Srivastava [220] study, where urea provided higher growth than ammonium chloride, ammonium phosphate and ammonium nitrate in Erlenmeyer flask culture using the strain NRRL B14690 [220]. However, these studies aimed to optimize polyhydroxybutyrate (PHB) production by *Cupriavidus necator* not SCP production, thus, it could be the reason why the results were not the same.

The results concerning studies about N-source effect using other microorganisms, such as *Trichoderma reesei* (grown in synthetic media) [168] and *Candida utilis* (grown in capsium powder waste medium) [60] were comparable to our results regarding SCP growth. The result of the study of Zhao *et al.*, [60] agrees also with Choi and Park [139] study for *Candida utilis* growth, where the urea an adverse effect on the cell growth compared to others nitrogen sources.

Cell morphology and viability

Figure 50 shows cells morphology evolution during the cultures using urea and ammonium sulfate. No drastic change was detected in both cell size and granularity. Cell viability was also assessed by flow cytometry using Propidium Iodide (PI). PI-permeability was measured along the culture, reaching around 2.4% at the end of the culture. Thus, there was no loss in membrane permeability and microbial cells were kept intact during the batch culture using urea as a nitrogen source.

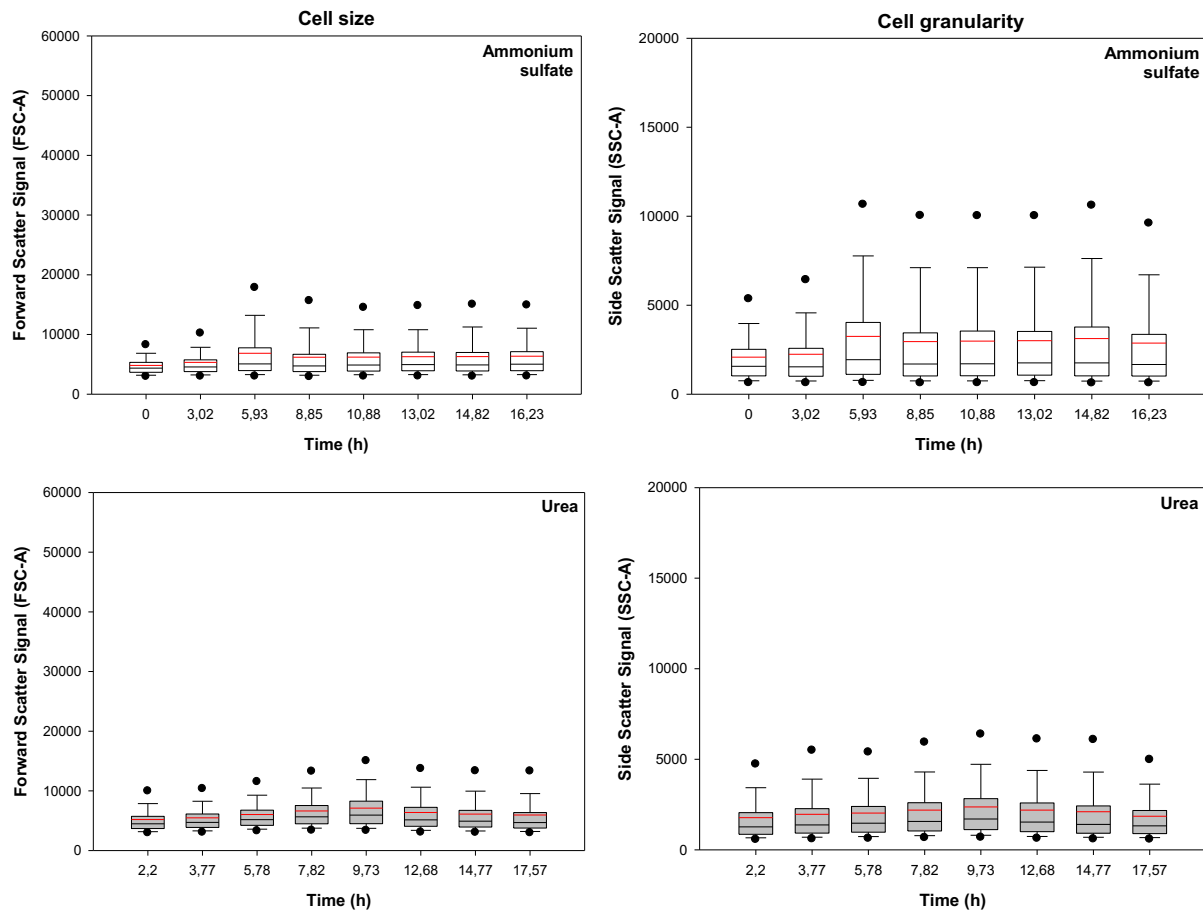


Figure 50 : Evolution of cell size and granularity measured by FSC and SSC signals distributions over time using ammonium sulfate (white) and urea (gray) as nitrogen sources

2.2. SCP production and composition

Regarding protein production

Two methods were used along the two batch cultures in order to quantify proteins and to compare their productions' dynamics. Thus, both NaOH-solubilized proteins (measured by BCA method to get soluble proteins) and total proteins (eq. Dumas) evolutions are presented in Figure 51.

The results showed different dynamics in protein production. For ammonium sulfate (Figure 51-A-C), the protein contents increased overtime, between 32-47%_{DW} and 67-78%_{DW} for soluble and total proteins, respectively. However, for urea (Figure 51-B-D), the protein percentage didn't have the same dynamic as ammonium sulfate profiles. Fluctuations were observed between the beginning and the end of that culture and the proteins amounts fluctuated between 26 and 45%_{DW} for soluble proteins and between 47 and 61%_{DW} for total proteins.

Regarding the final amounts of proteins, the use of ammonium sulfate as nitrogen source gave the highest protein amounts using the two different methods, with $41.30 \pm 4.56\%_{DW}$ (against

35.65 ± 1.28 %_{DW}) for NaOH-solubilized proteins and 78%_{DW} (against 55.3%_{DW} for urea) for total proteins measured with the Dumas's method.

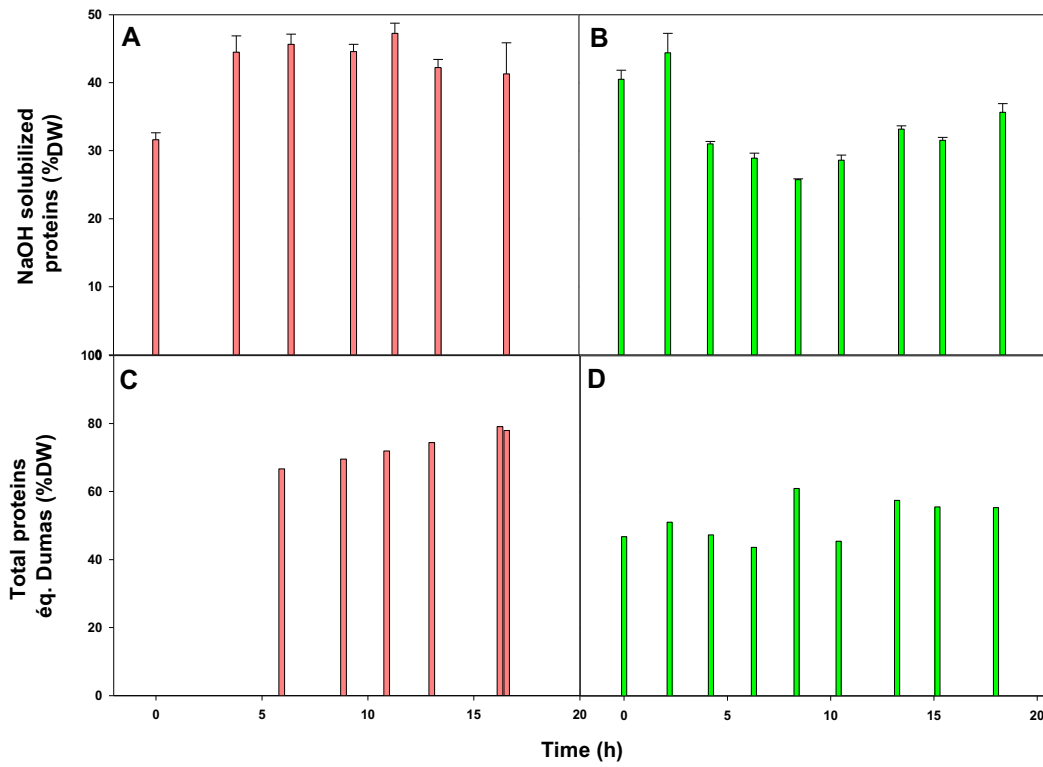


Figure 51 : NaOH-solubilized protein and total protein content evolutions overtime quantified by BCA method and Dumas Method using ammonium sulfate (pink: A, C) and urea (green: B, D) as nitrogen sources

According to the SCP literature, in the study of Yang et al., [169], *C. necator* (DSM531) was used for SCP production using ammonium sulfate, nitrate, nitrite and urea. The ammonium sulfate was found to permit higher production 83%_{DW} against 65%_{DW} using urea [169] measured by the BCA protein assay. According to the article, this could be caused by the higher C/N ratio during the urea hydrolysis compared to the ammonium assimilation leading to a low percentage of proteins [169].

These values are in agreements with our results, but if we compare the amounts measured with the same method, which is BCA, our results were lower. That could be caused by the difference of the strain or the culture conditions (autotrophic conditions in the study of Yang *et al.* [169]) or the difference in protein extraction method (treatment with HCl in the study of Yang *et al.* [169]).

On the other hand, the total protein amount obtained, using ammonium sulfate (Figure 51-C), was comparable to the amounts obtained in two other studies with the strain *C. necator* DSM

531 cultivated in autotrophic studies [260-262]. Indeed, a SCP content of 74 % was measured using the Kjeldahl method (as total nitrogen quantification method) [260-262].

The difference between ammonium sulfate and urea utilization in our work agrees with the studies using other microorganisms' types. For instance, higher protein contents were measured using ammonium sulfate for *Trichoderma reesei*, with 0.94 ± 0.02 g/100ml against 0.81 ± 0.02 for urea [168].

As a conclusion, with both BCA measurements and Dumas's method, ammonium was found as the best nitrogen source for protein production.

Regarding nucleic acid production

Along the culture, the nucleic acids amounts were quantified (Figure 52).

The DNA evolution profile (Figure 52-A) was higher using ammonium as nitrogen source along the culture. Indeed, the values were ranged between 1.92 ± 0.02 and 2.83 ± 0.04 %_{DW} for ammonium, against 1.40 ± 0.02 and 2.06 ± 0.03 %_{DW} for urea.

For the RNA evolutions (Figure 52-B), values were higher during the culture using ammonium as nitrogen source, presenting the same dynamics as the DNA profiles (Figure 52-A). The RNA amounts were ranged between 4.88 ± 0.05 and 7.51 ± 0.10 %_{DW} for ammonium against 3.63 ± 0.02 and 4.98 ± 0.03 %_{DW} for urea.

Both RNA and DNA amounts were slightly stable during the cultures. That could probably due to the nitrogen metabolism during the two cultures. Indeed, as mentioned previously, the urea hydrolysis releases high concentration of CO₂ causing a high C/N ratio in the media. Therefore, high percentages of C-based compounds (such as carbohydrates and fats) [169] against lower amounts of N-based compounds (including nucleic acids) are detected.

Therefore, the production profiles of both RNA and DNA were higher in the biomass cultivated with ammonium as nitrogen source.

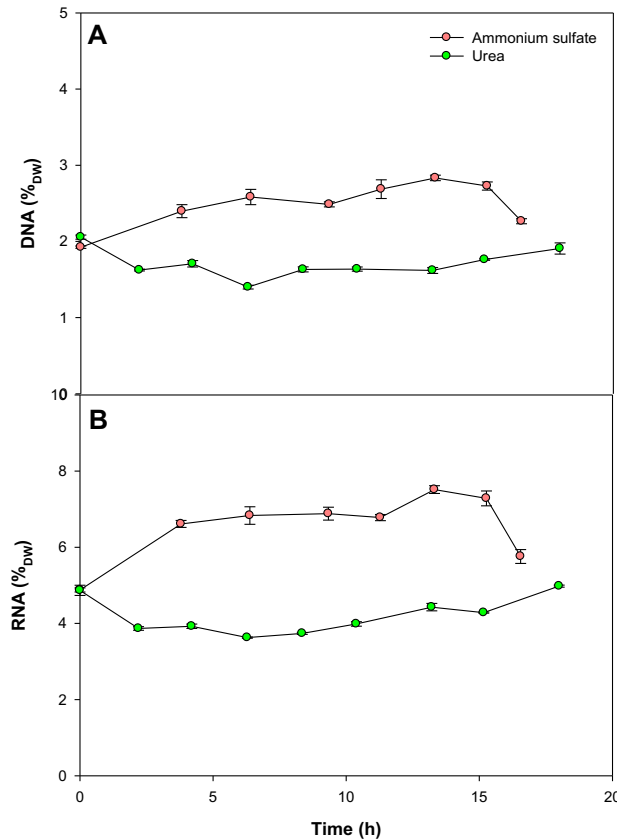


Figure 52 : Evolution of nucleic acid percentages over time using two different nitrogen sources: ammonium sulfate and urea.

Regarding biomass composition

Biomass composition in macromolecules were calculated for every culture condition, as presented in Figure 53. Recalculated protein content from the Dumas analysis (by subtraction of the part of nitrogen coming from nucleic acids), RNA, DNA carbohydrates and fats values were used. The values of carbohydrates and fats for *C. necator* were estimated to 5 and 6 %DW, respectively [158]. Comparing the N-source, the percentage of recovery regarding the total biomass dry weight is much lower in the case of urea 66% than with ammonium (89%). That could be explained by the variability of the values used from the literature. Indeed, carbohydrates and fats values could be low estimated for urea since the release of carbon dioxide in the media during urea hydrolysis brings a higher C/N ratio as suggested by Yang *et al.*, [169]. On the other hand, that could be caused by the production of other intracellular compounds during this culture. To verify this latter hypothesis two analyses were done: flow cytometry combined to the Nile Red labelling for PHB detection and CHONS analysis for biomass composition comparison.

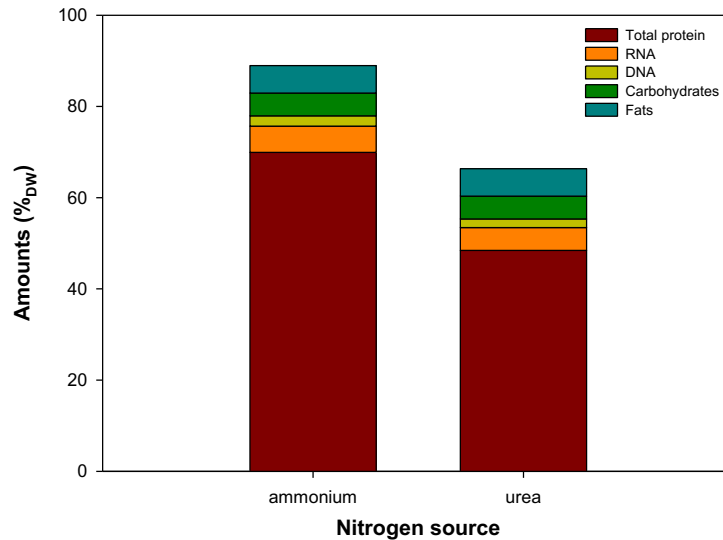


Figure 53 : Estimated biomass composition using two different nitrogen sources

- **Flow cytometry for PHB detection**

The results of the distribution of sub-populations in response to NR during cultures are presented in Figure 54. We chose to present three points for each culture: the initial point (in black), a point in the middle of the exponential phase (in red) and the final point (in blue) in comparison to 100% cells that have accumulated PHB (PHB⁺_{control}). (Figure 54).

The two initial points presented different results since they are not prepared with the same protocol. Indeed, the environmental parameters were the same for the inoculum preparation, however, the nitrogen source was not the same. In the middle of the exponential phase (red profiles), the Nile Red⁺ population percentage was 37% for ammonium sulfate against 42.6% for urea. However, for the final point, ammonium sulfate presented a higher percentage of Nile Red⁺ population.

If we suppose that Nile Red⁺ labelled population is PHB granules, the results regarding final points did not agree with studies done under PHB production conditions. In the study of Aramvash *et al.*, [219], ammonium and urea nitrogen sources gave the same PHB concentrations and in the study of Khanna *et al.*, [220] study, urea addition as nitrogen led to higher PHB production 6.75 g/l compared to 1.4 g/l with ammonium sulfate [220].

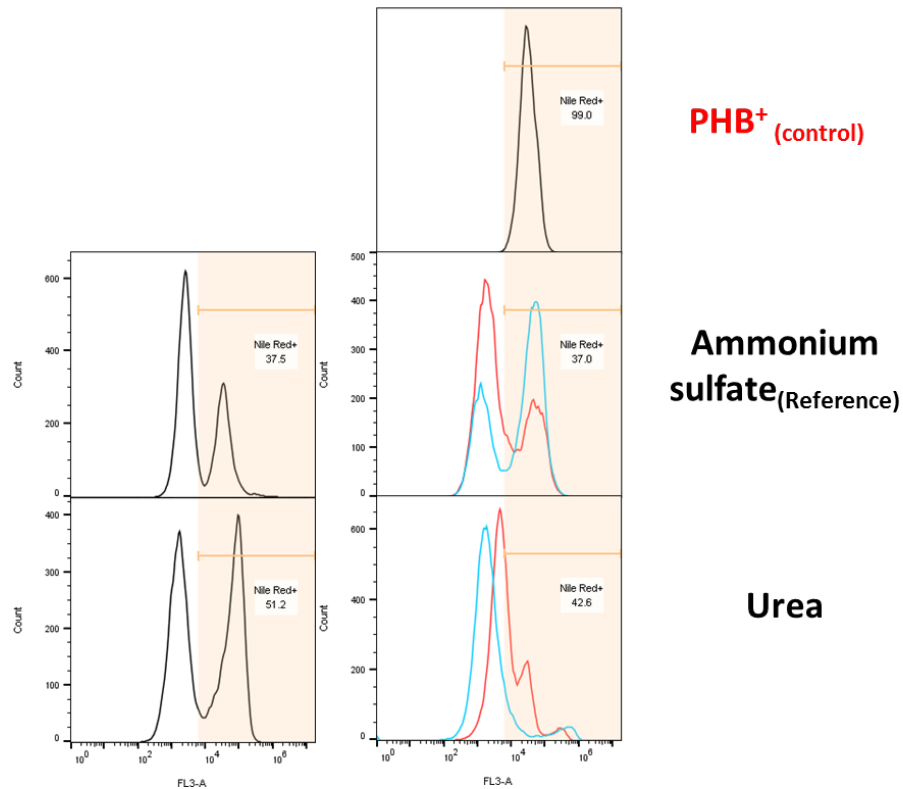


Figure 54 : Poly- β -hydroxybutyrate (PHB) detection with flow cytometer using two different nitrogen sources (**Orange rectangle**: Nile Red⁺, the indicated percentages were obtained for black profile on the left and red profile on the right); **black**: initial point; **red**: point in the middle of the exponential phase; **blue**: final point)

- **CHONS composition**

The biomass elemental compositions (C, H, O, N, and S) for the two cultures are presented in Figure 55. The percentages for each element were higher using ammonium sulfate, especially for carbon and oxygen, with a difference of 3%_{DW} and 2 %_{DW}, respectively.

The elemental compositions were then calculated using these percentages, the results are detailed in Table 46. No high differences were obtained for both ammonium and urea with comparable values of the molar weight (around 23.5 g/Cmol), which was calculated without considering the ashes percentages.

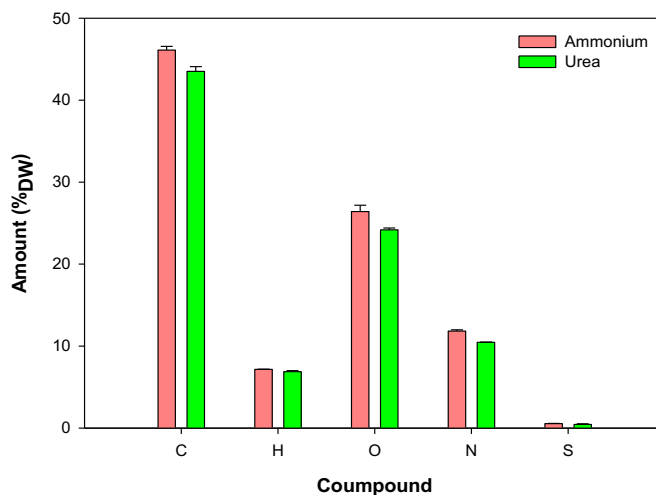


Figure 55 : CHONS composition using two different nitrogen sources

Table 46 : Elemental composition of the final biomass using two different nitrogen sources

Nitrogen source	Elemental composition					Molar weight (Cmol)
	C	H	O	N	S	
Ammonium sulfate	1,00	1,86	0,43	0,22	0,0044	23.96±0.26
Urea	1,00	1,90	0,42	0,21	0,004	23.57±0.03

3. Conclusion

The objective of this chapter was to characterize growth behavior and biomass composition comparing two batch cultures using ammonium sulfate and urea as nitrogen sources.

The growth was faster using ammonium than urea with no changes in cell size, granularity and permeability during the two cultures.

Both NaOH-solubilized protein, total protein and nucleic acid profiles were higher using ammonium which could be caused by the different nitrogen metabolism in each condition. Indeed, the urea release carbon dioxide in the media which caused high C/N ratio in the media and, thus, high percentages in C-based intracellular compounds. Therefore, lower percentages in N-based compounds were produced.

The Nile Red + population was lower at the end of the urea culture compared to the other nitrogen source and the elemental composition did not present high differences between ammonium and urea and the molar weight was comparable.

Therefore, the macroscopic behavior and SCP production for *C. necator* depends on the nitrogen source. Indeed, a faster growth and high SCP compounds were obtained with ammonium sulfate. However, cell morphology and CHONS composition appeared to be independent on the nitrogen source used.

Chapter III-5: Impact of the carbon source on Single Cell Protein production and composition in *Cupriavidus necator*

Chapter III-5: Impact of the carbon source on Single Cell Protein production and composition in *Cupriavidus necator*

1. Introduction

This chapter presents the impact of the use of different carbon sources on growth and protein production by *C. necator*. In the literature, papers presented mainly the use of different complex substrates, such as, agricultural wastes (for *Rhodococcus opacus*, *Streptomyces* strains, *Penicillium expansum* [54, 117, 121, 165]) and food industrial wastes (for *Rhodococcus opacus* and *Saccharomyces cerevisiae* [21, 54]) for Single Cell Protein production. Such complex substrates, provide mixture of different nutrients, C sources but also N-sources, salts, vitamins, leading to difficulties to interpret the specific impact of one of the compounds on the performance of SCP production

However, few studies have evaluated the effect of the carbon source alone. Khan *et al.*, [165] evaluated different pure sugars in order to optimize the protein contents by *Penicillium expansum*. Another study of Sakarika *et al.*, [167] evaluated growth and SCP production from pure microorganisms and mixed cultures, including *Cupriavidus necator*, using two organic acids, formate and acetate [167].

Since the bacterium *Cupriavidus necator* has the ability to grow on various carbon sources [194, 199-202], in this work, carbon sources of different nature were used to evaluate SCP production. The choice was made considering their different chemical nature (sugar, organic acid and fatty acid), their different number of carbon (from C₁ to C₁₈), the different metabolic pathways involved for their catabolism and their availability in terms of industrial streams. The strain used here (CECT 4623) has the ability to grow on glucose (C₆H₁₂O₆). Thus, glucose was the sugar tested. The formic acid (CH₂O₂) was used, as an organic acid, offering the opportunity to valorize industrial wastes [69, 82, 263, 264] and also, to use, at the same time, a carbon and energy source [265], and to mimic the growth under autotrophic condition [206, 265, 266]. In *C. necator*, the formic acid is converted in one NADH and one CO₂ by a formate dehydrogenase, the latter product could be assimilated by the Calvin Benson Bassham cycle which is a very energy demanding cycle [267]. The key enzyme of CO₂ fixation in the CBB cycle is RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) which has nutritional and functional promising values such as a good protein digestibility [268]. The oleic acid (C₁₈H₃₄O₂) was also tested as being the most abundant fatty acid in nature. The use of this fatty acid could be also an opportunity to valorize the wastes from the oil industries. In the bacterium, oleate is converted to acetyl-CoA via the fatty acid degradation pathway and then inserted into

the central carbon metabolism. Oleic acid was also used by *C. necator* for other biotechnological applications, but not yet for SCP production. Indeed, in the literature, many studies used fatty acids, as carbon source, to improve PHA production by *C. necator*. Fatty acids used, as carbon source, were generally in the form of pure plant oils [201, 269-272] or wastes from oils such as used cooking oil [273-276]. Fatty acids were also used in addition to another carbon source such as sugars in PHB works in order to optimize its production [277-281]. For oleic acid, there is no articles studying protein production.

Herein, for all experiments, inocula were prepared in 2-L Erlenmeyer flask cultures under the same environmental conditions (30°C, pH7, 140 rpm) but using the different carbon sources. Bioreactors were performed in batch mode under the same environmental conditions (30°C, pH7) except for formic acid cultivation where fed batch mode was preferred. Indeed, for formic acid culture, a pH-controlled fed-batch was carried out to maintain low concentrations of formic acid. As formic acid is toxic for cells [281-283], batch mode could not be a suitable strategy to evaluate this carbon source. pH-controlled fed batch culture was inspired from the study of Grunwald *et al.*, [206], which had as objective to compare pH-regulation fed batch cultures using different initial concentrations of formic acid in the medium and to evaluate toxicity towards cells.

In this context, this chapter presented and discussed results between these cultures using three different carbon sources considering their growth evolutions, cell morphology, biomass composition (in terms of SCP, nucleic acids, and elemental composition).

2. Results and discussion

2.1. Growth dynamics and viability

Cell growth

Cell growth and substrate consumption curves of *C. necator* grown on glucose, formic acid and oleic acid are presented in Figure 56. However, quantification of the oleate consumption was not possible for this work, due to unavailability of the required equipment. According to the HPLC results, no other metabolite was detected in supernatants during these cultures.

- For the glucose culture, with 17g/l of glucose, 7.5 g/l of biomass was produced and a maximal growth rate was obtained, $0.21 \pm 0.01 \text{ h}^{-1}$.
- For the oleic acid culture, a maximal growth rate was obtained during all the culture and its value was $0.19 \pm 0.01 \text{ h}^{-1}$.

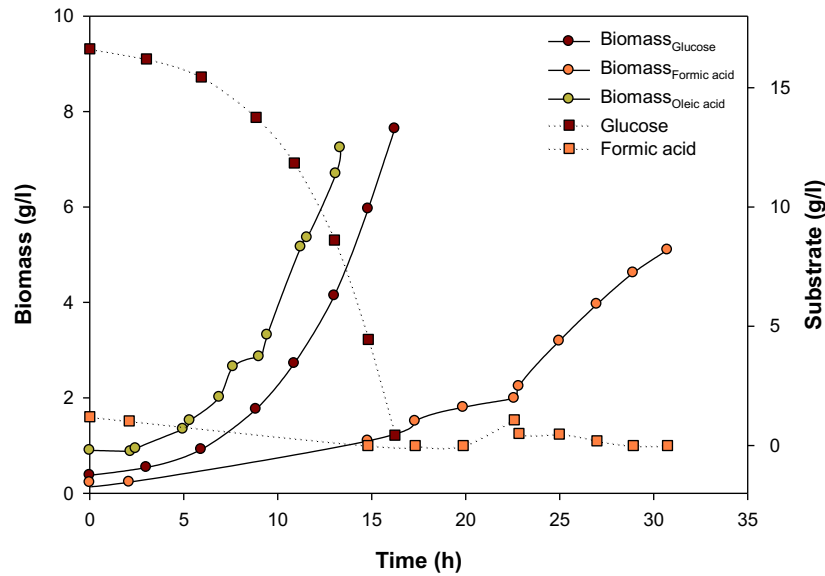


Figure 56 : Evolution of biomass (○) and substrate (□) concentrations over time during cultures on different carbon sources

Surprisingly, in the literature and to our knowledge, no article detailing growth rates using oleic acid only as a carbon source for *C. necator* could be found. Indeed, oleic acid was always used as a supplement to another carbon source in PHA improvement studies [284-286].

- For formic acid, a pH-controlled fed-batch culture was carried out in order to avoid growth inhibition by the acid. The kinetics could be divided on four phases, as presented in Figure 57:

At the beginning, a concentration of 2 g/l (3.7 g) of formic acid was added to the medium and the pH-regulation system (solution containing 50% (w/v) formic acid solution with 4.3 g/l (252 mM) of $\text{NH}_{3(\text{aq})}$) was activated upon inoculation.

Phase A: It presents a growth on formic acid, as the biomass passed from 0.5 to 4.5 g and the residual formic acid decreased from 3.5 to 0 g. After 15 hours of cultivation, the residual formic acid concentration was null indicating that the growth was limited by the feeding of formic acid via the pH-regulation system. The growth rate during this phase was $0.117 \pm 0.003 \text{ h}^{-1}$.

Phase B: As growth at the end of phase A was limited by the feeding of formic acid, a pulse of 1.2g/l of formic acid solution was added in the reactor. As expected, the growth rate increased from $\mu = 0.117 \pm 0.003 \text{ h}^{-1}$ to $0.17 \pm 0.04 \text{ h}^{-1}$. However again the residual formic acid concentration dropped quickly, indicating that the feeding of formic acid by the pH-regulation system was not sufficient.

Phase C: In order to feed higher amount of formic acid by the pH-regulation system, the feed formic acid solution was changed with a solution of pure formic acid. The growth rate during this phase was at $0.10 \pm 0.01 \text{ h}^{-1}$

Phase D: It concerns the final phase of the culture where the growth rate decreased to $0.05 \pm 0.01 \text{ h}^{-1}$. Indeed, even the pure solution of formic acid was not sufficient as feeding system, the residual formic acid concentration decreased until reaching zero. The experiment was stopped when biomass concentration reached 5 g/l.

Maximal growth rates were obtained in similar strategy of culture in Friedrich *et al.*, [287] and Grunwald *et al.*, [206] studies, with 0.17 h^{-1} and 0.18 h^{-1} values, respectively. This growth rate value was reached during phase B of the fed batch, just after the formic acid pulse. In the study of Sakarika *et al.*, [167], μ_{max} reached 0.29 h^{-1} which was much higher than the other studies. However the medium used in this study contained 5g/l succinate, which could be most probably used a substrate; thus its impact on the growth in presence of formate was not clear [167].

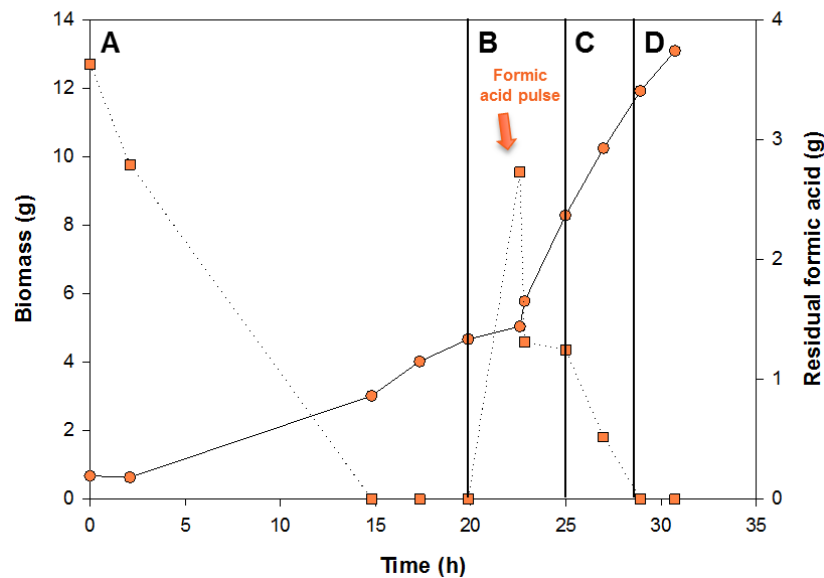


Figure 57 : Evolution of the biomass (○) and the substrate (□) over time during the pH-regulation formic acid fed batch

Biomass yields were also calculated for the formic acid culture (Table 47). The results are presented considering the residual formic acid concentrations. Compared to the theoretical biomass yield (0.180 Cmol/Cmol), the biomass yields calculated during the culture were lower, ranged between 0.110 and 0.145 Cmol/Cmol . The best yield was obtained at the beginning of the culture (phase A) where formic acid was not limiting and reached 80% of the theoretical yield. Using identical pH-regulated fed-batch strategy, Grunwald *et al.* [206] reached yields

from 0.09 to 0.17 Cmol/Cmol with *C. necator* H16. In their study, the highest yield (0.17 Cmol/Cmol) was obtained when the residual formic acid concentration was kept below 0.5 g/L. The lowest yield (0.09 Cmol/Cmol) was obtained when the residual formic acid was between 1.5 and 2 g/l. They found an intermediate yield (0.14 Cmol/Cmol) for formic acid concentrations in the range of 0.5 and 1 g/l. In our study, the residual formic acid concentrations fluctuated between 0 and 1.2 g/l which could approximate the intermediate conditions of Grunwald et al [206]. Therefore, both yields (0.126-0.145 in our study; 0.14-0.15 in Grunwald *et al.*, (2014)) were not very far even though the strains were different (H16 strain in Grunwald *et al.*, (2014)).

Table 47 : Biomass yield values during the formic acid culture

Culture phase	Residual formic acid (g/l)	$Y_{x,s}$ (Cmol/Cmol)	$Y_{x,s}^{theo}$ (Cmol/Cmol)	$Y_{x,s} / Y_{x,s}^{theo}$ (%)
A	0-1.2	0.145	0.180	80.5
	0	0.120		66.7
B	0-1.1	0.126		70.0
C+D	0-0.5	0.110		61.1

Cell morphology and viability

During glucose and formic acid cultures, flow cytometry measurements were done overtime to characterize the cell morphology. It was not possible to evaluate cell morphology from the oleate culture because of the oily mixture. The cell size and granularity distribution results were shown in Figure 58. No drastic variations in size (FSC) and granularity (SSC) were observed using these two carbon sources. However, formic acid profiles were more homogeneous compared to glucose. Then, in order to evaluate toxicity of the formic acid, cell viability was analyzed by flow cytometry after staining by Propidium Iodide (PI) during the formic acid culture. Low percentages were obtained (between 1.6 and 6.1 %). Cells remained then intact during the fed batch culture with no loss in cell permeability.

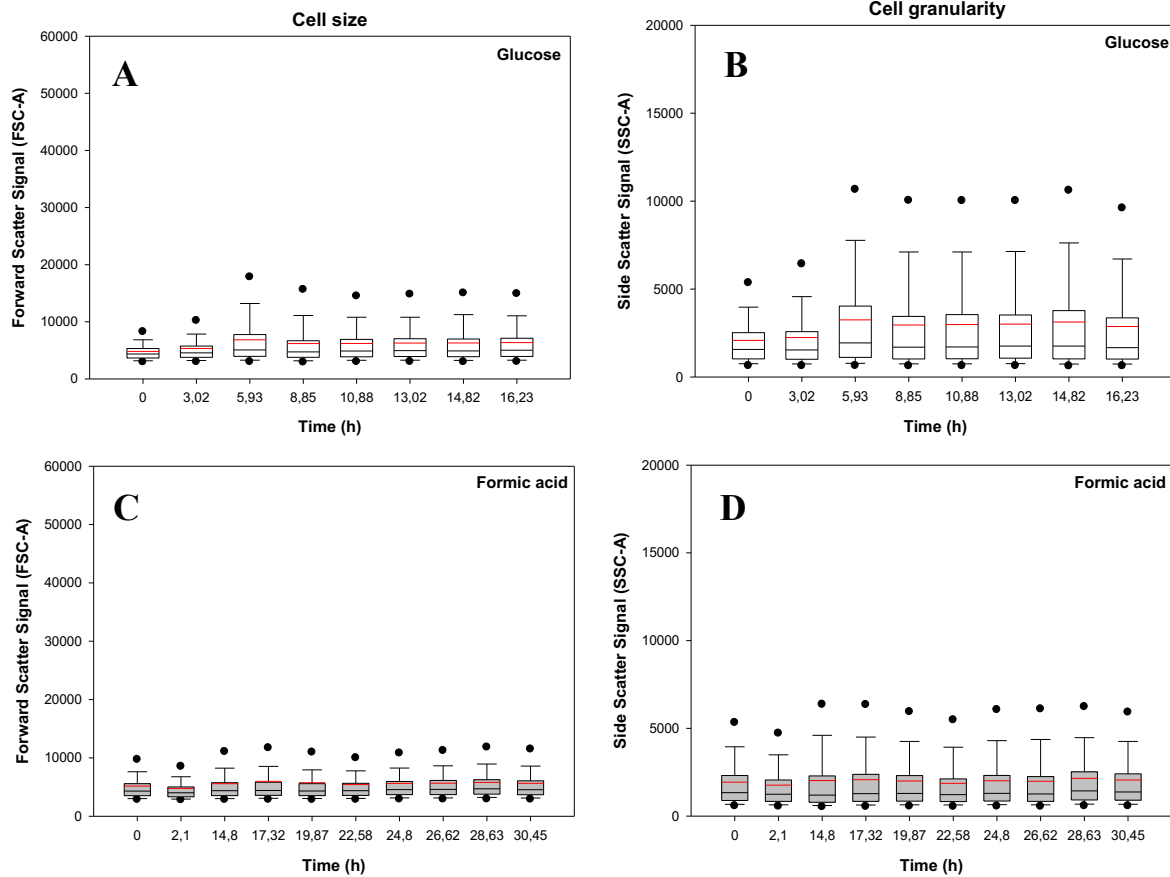


Figure 58 : Evolution of cell size and granularity measured by FSC and SSC signals distributions over time for two different carbon sources: glucose in white (A, B), formic acid in gray (C, D)

2.2. Biomass composition

Regarding protein production

NaOH-solubilized proteins and total proteins percentages in the biomass were measured. The results are presented in Figure 59 : A and D for glucose, B and E for formic acid and C and F for oleic acid. Figure 59-ABC presents the soluble protein amounts quantified after NaOH treatment (5min, 100°C) with the BCA method. For the final points, the values were comparable for glucose and formic acid cultures (around 42%_{DW}). However, the percentage was lower for the oleic acid culture with 31.38±1.30%_{DW}.

On the other hand, Figure 59-DEF showed the total protein production during the cultures quantified with the Dumas method. The final amounts of proteins were different comparing the three cultures. Indeed, the formic acid culture presented the highest protein content (91%_{DW}), the glucose culture come after with 78%_{DW} and, then, the oleic acid with just 62%_{DW}.

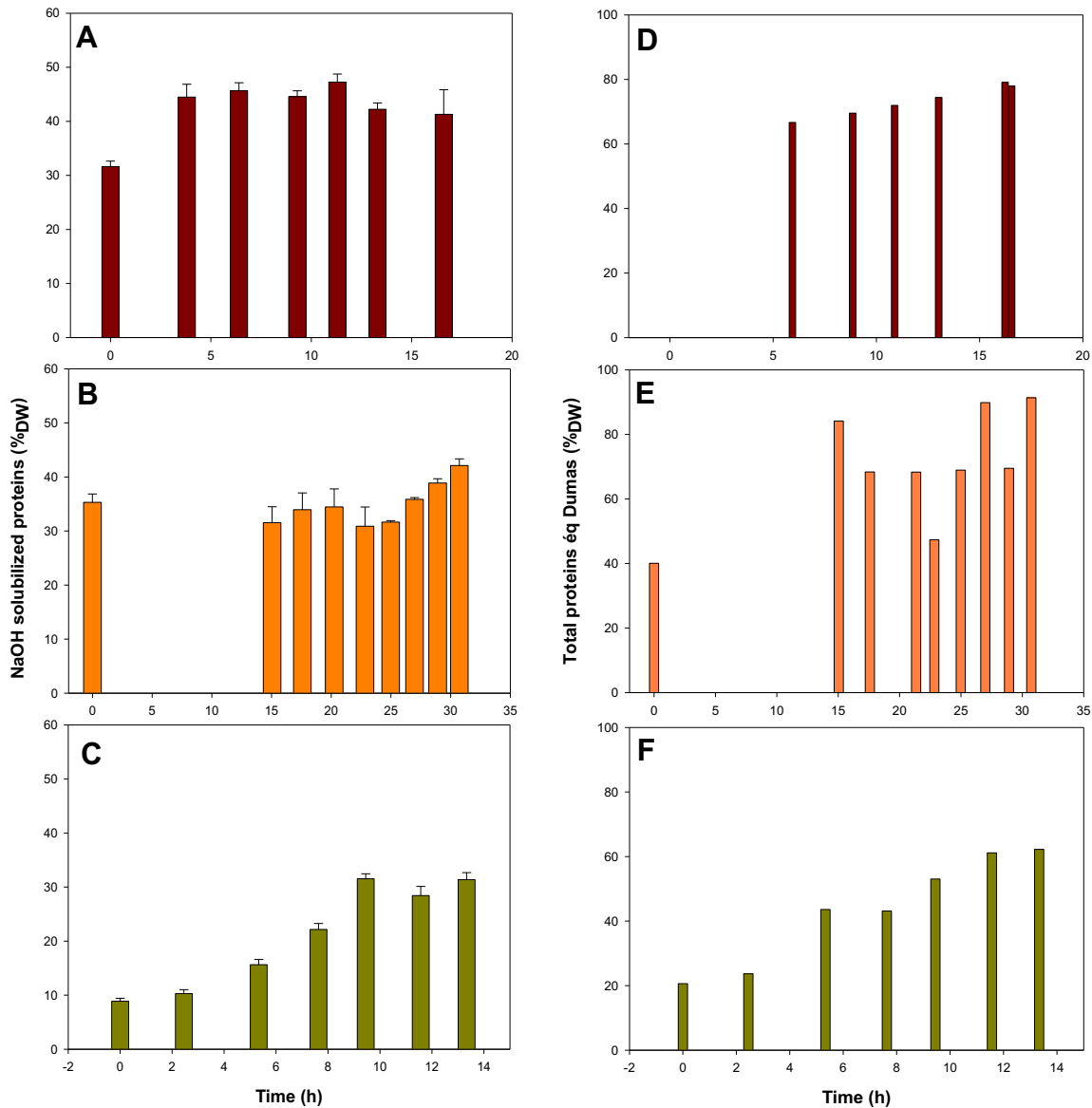


Figure 59 : NaOH-solubilized protein evolution quantified by BCA method (A, B, C) and total protein content evolution calculated from total nitrogen quantification by Dumas Method (D, E, F) over time for different carbon sources (glucose: A-D; formic acid: B-E; oleic acid: C-F)

Total protein content obtained were higher than Sakarika *et al.* [167] study (45-60%_{DW}) which is the only study evaluating SCP production using formic acid as carbon source. This difference compared to the Sakarika *et al.* [167] study could be explained by the different culture mode (Erlenmeyer flask), the strain (LMG 1199), the protein extraction method (not mentioned in Sakarika *et al.*, study) and quantification method (a modified Lowry method called Markwell method [288]) and probably the presence of succinate in the medium [167]. Another study showed that latex wastewaters rich in formic acid could be a good alternative substrate for SCP production by *Rhodospseudomonas palustris* reaching high amounts of total protein up to 65%_{DW} [67].

Regarding the oleic acid culture, the low amounts of proteins are probably caused by the quantification protocol. Indeed, the elimination of residual oleic acid from the cell samples may not have been efficient or reproducible enough. In further analysis, we observed that only one treatment with IPA was not sufficient to eliminate oily texture from the samples.

The difference in protein production profile could also be explained by the different carbon source metabolism in *Cupriavidus necator*. Indeed, as showed the Figure 9 of the literature part, the three carbon sources follow different pathways. The glucose is consumed directly and follows the central carbon pathway [204, 205]. However, the formic acid enter to the Calvin-Besson-Bassham cycle allowing the fixation of CO₂ and the oleic acid follows the fatty acid degradation pathway [204, 207]. Thus, the carbon source assimilation pathway by the strains is not the same, requiring a complete rearrangement of the metabolic machinery.

In conclusion, comparing the three carbon sources, the formic acid gave the best protein percentage which was ranged between 70 and 90%_{DW} (Dumas) and the oleic acid presented the lowest amounts, not exceeding 60%_{DW} (Dumas).

Regarding nucleic acid production

Figure 60 presents the evolution of both RNA and DNA during the cultures.

RNA evolutions were quite different. Indeed, the evolution curve was higher on glucose than on formic acid, and oleic acid profile was different. The final amounts were comparable using glucose and formic acid (5.76 ± 0.18 and 5.51 ± 0.40 %_{DW}), but higher on oleic acid (7.97 ± 0.01 %_{DW}). However, it has to be noticed that RNA content was fluctuated a lot between 1.8 to 7.97 %_{DW} with an increasing trend. Whereas the contents were relatively stable on glucose (4.88-7.51 %_{DW}) and formic acid (4.66-6.22%_{DW}) (Figure 60-A).

DNA profiles were also different. Evolution curve was slightly higher on glucose (2-2.8%_{DW}) than on formic acid (1.7-2.5%_{DW}), and oleic acid DNA amounts were lower than 2%. The final values were comparable for formic acid and oleic acid (1.7%_{DW}) but higher for glucose (2.27 ± 0.03 %_{DW}) (Figure 60-B). As for RNA, DNA content on oleic acid showed an increasing trend and a high variation (0.5-1.7 %_{DW}) compared to a more stable evolution on glucose and formic acid. Figure 60-B show also difference in oleic acid ratio values, which is higher than the other conditions.

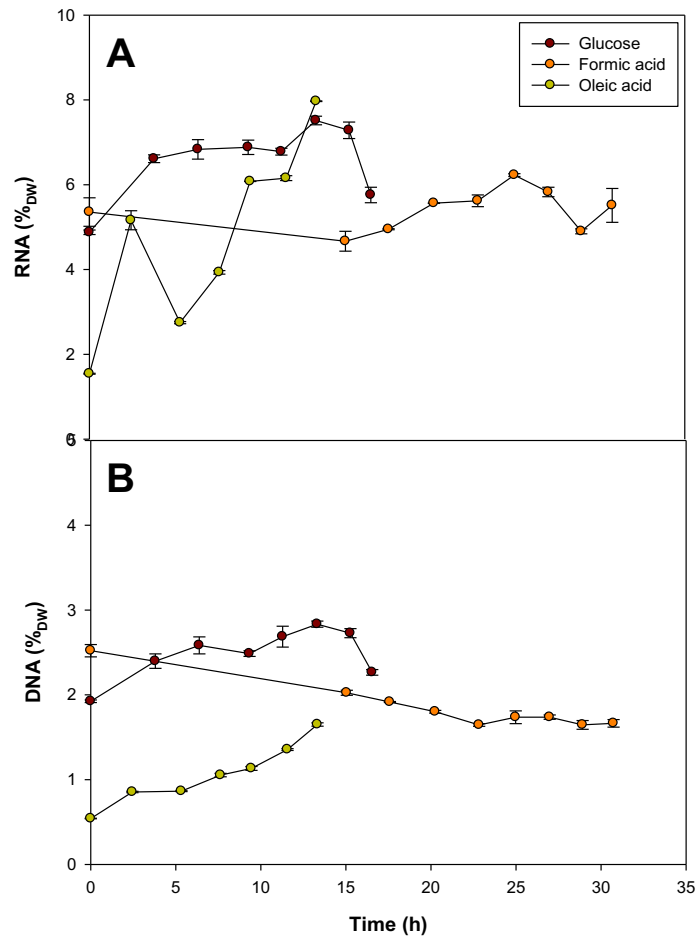


Figure 60 : Evolution of nucleic acid percentages and their ratio in biomass over time for different carbon sources.

Regarding the oleic acid, the difference in the dynamic profiles is probably caused by the IPA treatment efficiency and reproducibility, which is already mentioned below, in the protein discussion paragraph.

If we compare all the different profiles regarding the different carbon source cultures' profiles, we can also explain this difference by the carbon assimilation and pathway in *Cupriavidus necator* (central carbon pathway for glucose [204, 205], Calvin-Besson-Bassham cycle for formic acid and the fatty acid degradation pathway for oleic acid [204, 207]).

As a conclusion, the nucleic acid profiles were higher using the glucose but lower amounts were given by the formic acid. However, different dynamics were observed during the oleic acid culture.

Regarding biomass composition

Biomass composition in macromolecules were calculated for each culture condition, as presented in Figure 61. Recalculated protein content from the Dumas analysis (by subtraction

of the part of nitrogen coming from nucleic acids), RNA, DNA carbohydrates and fats values were used. The values of carbohydrates and fats for *C. necator* were estimated to 5 and 6 %_{DW}, respectively [158]. The calculated percentages were 89, 102 and 73 %_{DW} for glucose, formic acid and oleic acid cultures, respectively. For formic acid culture, this percentage could be due to a over estimation of some biomass compounds such as protein content value considered (90%) that could be overestimated because the protein content estimated from the Dumas method fluctuated between 70 and 90%_{DW} (Figure 59). For percentages less than 100%, a production of other compounds like PHB (not detected by the used HPLC method) during the culture could occur. To investigate these hypotheses, both PHB detection and CHONS composition analysis will be presented in the following paragraphs.

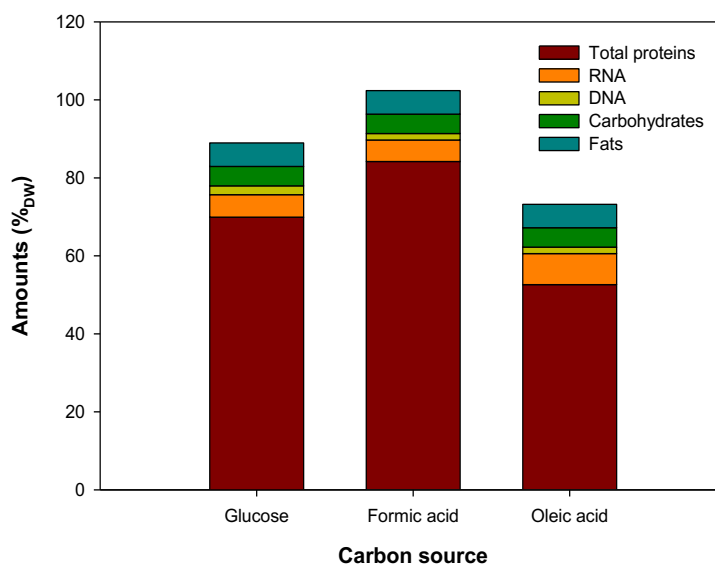


Figure 61 : Estimated biomass composition (in percentages) using different carbon sources

- **Flow Cytometry for PHB detection**

Three points from the cultures were presented after Nile Red utilization (the initial point, a point from the middle of the exponential phase and the final point) and compared to a PHB⁺ control (PHB accumulated cells) in Figure 62.

Comparing the glucose and the formic acid cultures, the initial and the final points were different. Indeed, the use of formic acid as carbon source presented lower Nile Red⁺ population, 9% against 37% out of the total population for glucose in the middle of the exponential phase of growth (red profile in Figure 62). This result clearly showed the absence of PHB in the cells grown on formic acid agreeing with the higher protein percentages (Figure 59-C-E) whereas the glucose culture generated lower percentages of protein. For oleic acid cultures, cells were

always been labelled with Nile Red⁺ from the beginning to the end of the cultures. No information for PHB could be obtained for this culture, since the Nile Red is also used to label cholesteryl esters, triacylglycerol and mixture of lipids [250]. Thus, the oleic acid localized in the cell membrane must be labelled.

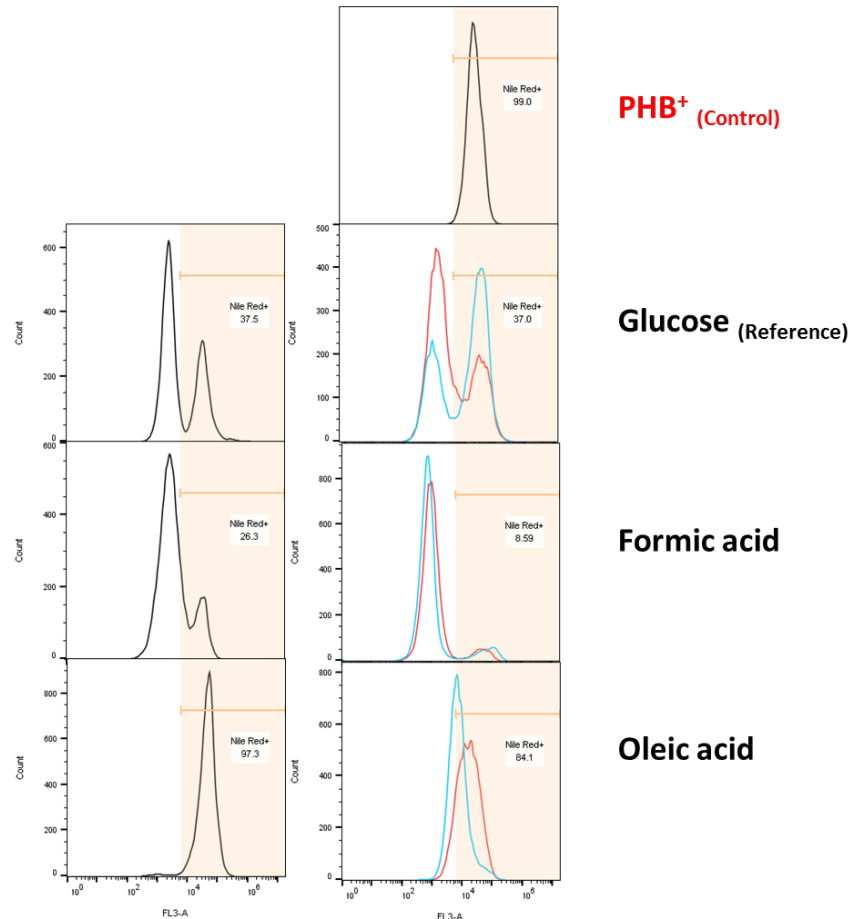


Figure 62 : Poly-β-hydroxybutyrate (PHB) detection with flow cytometer using three different carbon sources (**Orange rectangle**: Nile Red⁺, the indicated percentages were obtained for black profile on the left and red profile on the right); **black**: initial point; **red**: point in the middle of the exponential phase; **blue**: final point)

- **CHONS composition**

Biomass macromolecular composition was also verified at the end of each culture via CHONS analysis. Figure 63 presents the percentages C, H, O, N, and S for the different carbon source used. Oleic acid culture biomass showed lower percentages for all the compounds. Comparing biomasses obtained using glucose and formic acid, higher percentages were found in carbon (46%), hydrogen (7%) and oxygen (26%) for glucose, but higher percentages in nitrogen (13%) and sulfur (0.65%) for formic acid were measured. High percentages in nitrogen and sulfur

could validate the higher protein contents obtained in formic acid culture. However the S/N ratio were comparable on glucose and formic acid (0.02) suggesting identical sulfur amino acid contents in the proteins when cultivated on those C-sources. On the contrary the S/N was lower (0.012) on oleic acid.

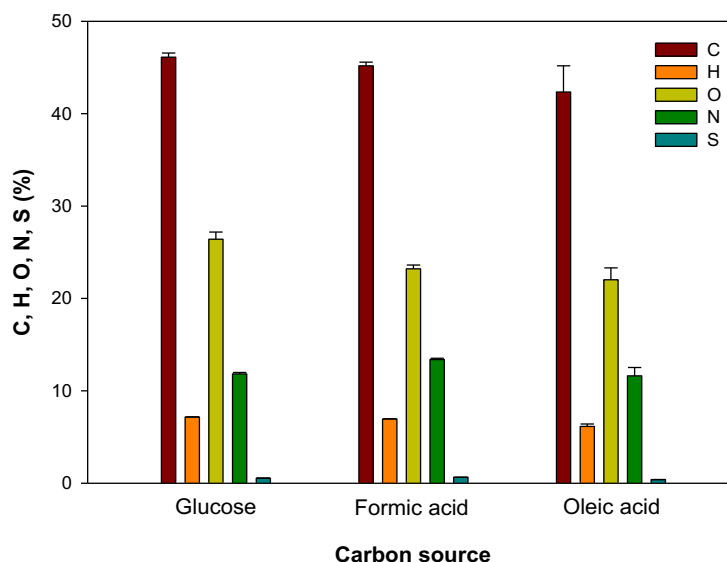


Figure 63 : CHONS composition using different carbon source

Considering these percentages, elemental composition and biomass molecular weights were calculated (Table 48). Despite the differences in elemental composition, the molecular weights were comparable using glucose, formic acid or oleic acid as carbon sources (23-24 g/Cmol).

Table 48 : Elemental composition of the final biomass using different carbon sources

C source	Elemental composition					Molar weight (g/Cmol)
	C	H	O	N	S	
Glucose	1.00	1.86	0.43	0.22	0.0044	23.96±0.26
Formic acid	1.00	1.84	0.39	0.25	0.005	23.74±0.17
Oleic acid	1.00	1.74	0.39	0.24	0.003	23.41±0.56

3. Conclusion

The aim of this chapter was to evaluate growth and SCP production using three different carbon sources, glucose, formic acid and oleic acid.

Maximal growth rates were obtained for glucose and oleic acid, but the growth was faster with glucose. For the formic acid because of the culture strategy, the growth was divided into four phases and the best yield was obtained at the beginning of the culture reaching 80% of the

theoretical yield. No variation in cells morphology (cell size and granularity) and low permeable cell percentages were obtained. A higher protein content was obtained for the biomass produced from formic acid compared to glucose. The RNA and DNA evolution curves were higher using glucose than on formic acid. Therefore, the formic acid culture gave an interesting biomass composition for human consumption with high protein content and low nucleic acid content. The oleic acid culture showed lower protein amounts with high variations in RNA and DNA contents that can be due to the residual oleic acid in the cells. The intracellular composition could be also explained by *C. necator* metabolism. Indeed, different pathways were followed using the three different carbon sources (the central carbon pathway for glucose, the Calvin-Besson-Bassham for formic acid and the degradation pathway for the oleic acid).

The use of formic acid presented lower Nile Red⁺ population compared to glucose. This percentage agree with the high protein content at the formic acid culture. For oleic acid cultures, no information for PHB could be obtained here, since the Nile Red is also used to detect lipids [250]. Comparing CHONS compositions using glucose and formic acid, higher percentages were found in carbon, hydrogen and oxygen for glucose with higher percentages in nitrogen and sulfur for formic acid. Higher percentages in N and S during the formic acid culture could agree the higher protein content. Oleic acid culture biomass shows lower percentages for all the compounds. The molecular weights calculated were slightly comparable.

As a result, the macroscopic behavior and the biomass composition of *C. necator* depends on the carbon source. Formic acid was found as the best source to produce proteins. However, protein content on oleic acid results were very low compared to the other condition what can be a problem of sample treatment.

Conclusions and perspectives

Conclusions and perspectives

Single Cell Proteins production come as alternative proteins from microorganisms for human consumption and animal feed to solve the problem of deficiency of protein. They present attractive composition and high protein amounts presenting a complete amino acid profile with presence of all the essential amino acids [4, 9, 27, 74]. However, some drawbacks could be presented such as toxins, indigestible cell wall or high nucleic acid content [4, 156, 178, 182]. Many studies have the objective to evaluate and to optimize SCP production and/or growth using different factors which could influence the biomass composition. Most of the studies were performed using mono-factorial approaches in this aim. Substrate composition, strain types and environmental parameters were included in these factors.

Many studies had as an objective to evaluate the use of different agricultural or industrial residuals or different substrates compositions using different carbon sources, nitrogen sources or C/N ratio, etc. A generic conclusion cannot be drawn because it is very dependent on the microorganisms chosen. Therefore, the choice of strain is important. Indeed, for the same type of microorganism, the choice of the strain had an impact on growth and protein production. Regarding the impact of environmental parameters on SCP, temperature and pH were the most affecting parameters independently of the microorganism type.

However, many limits could be mentioned considering these investigations such the use of Erlenmeyer-flask cultures and the absence of dynamics details on Single Cell Protein production along the cultures. Indeed, the cultures were mainly performed under non-controlled parameters and only the final protein amounts were given, so the production phases could not be understood. On the other hand, it was not possible to compare these articles' results because of the difference of extraction and quantification methods used for proteins and/or nucleic acids. Among these microorganisms, *C. necator* was found as an efficient Single Cell Protein's source, providing more than 70%_{DW} of proteins [210, 225, 226]. However, these studies were, in the most of the cases, performed under autotrophic conditions [99, 158, 212, 224, 227-229]. Now, regarding the papers studying the SCP impacting factors, *Cupriavidus necator* was rarely used. For instance, no article was found studying the SCP growth and composition in response to environmental stress (including temperature and pH) or using different media composition (including carbon and nitrogen sources) except the study of Sakarika *et al.*, [167] which had as aim to compare different carbon sources utilization.

Thus, in response to these literature limits, the objective of the thesis was to characterize *Cupriavidus necator* biomass including growth behavior, protein production and nucleic acid

content (which are one of the drawbacks of SCP) along bioreactor culture in response of some factors, environmental parameters and substrate composition in bioreactors.

Considering the literature studies in this context, the scientific questions of my thesis were:

- What are the optimized methods for protein and nucleic acids extraction and quantification in *Cupriavidus necator*?
- What are the impacts of environmental parameters on the dynamics of growth and protein production using *Cupriavidus necator*?
- What are the impacts of substrate composition on the dynamics of growth and protein production using *Cupriavidus necator*?

Methodologies for extraction and quantification SCP and nucleic acid content in *Cupriavidus necator*

The first step in my thesis objectives was to develop optimized extraction methods and quantification methods to measure proteins and nucleic acids within the biomass in *C. necator*. The literature review chapter detailed some lysis methods used to extract protein and/or nucleic acids from cells with the objective to quantify SCP composition. These methods can be divided into two types, chemical (cell autolysis using endogenous or exogenous enzymes [100], acid cell hydrolysis (using trichloroacetic acid [22], chlorhydric acid [31, 99]) or heating in alkaline solution [103] (sodium hydroxide (NaOH) treatment [101, 102])) or physical (enzymatic digestion at high temperature [104], sonication [95-98], destruction using mortar and sea sand [105] and breaking with glass beads [106, 107]). However, no information was really found for extractions regarding *C. necator*.

For the extraction methods, chemical and/or physical treatments were investigated using the different samples of the same dry weight: FastPrep-24™ beating grinder, sonication and sodium hydroxide treatment. Trying to find the optimized protocol, parameter values were fixed considering their impact for cell extraction and according to some preliminary assays.

For the quantification methods, sample dilution and microplate reader sensitivity were evaluated. The quantity of the biomass was also studied in order to identify the efficient extraction cell weight regarding the established protocols.

As conclusion, chemical protocols presented a better protein extraction yield (NaOH (10%w/v, 5min)) and the physical treatments were better for nucleic acid extraction (FastPrep system (3 cycles of lysis)). These methods were found to be more efficient for their extraction. For the quantification methods, the dilution 1/10 is found as the optimal dilution for protein

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quantification by BCA protein assay method after NaOH extraction protocol. The choice was done regarding the stability of the assay considering the interferences with the BCA reagent. Sensitivity of 50 for the microplate reader and 2 min for the incubation time were found as the optimal conditions for nucleic acid quantification via RiboGreen and PicoGreen assays after beads treatment protocol (FastPrep system).

This step was a very important phase in our work. It was crucial for us to, not only, optimize both soluble proteins and nucleic acids extractions but also to establish efficient quantification protocols for *Cupriavidus necator*.

Impacts of the environmental parameters on the dynamics of growth and protein production in *Cupriavidus necator*

This part had as an objective to evaluate Single Cell Protein production and biomass composition under environmental stress. Two parameters were evaluated, temperature (25, 30, 35 and 40°C) and pH (5, 6, 7 and 8) using a mono-factorial approach which was interesting to understand the effect of every parameter. Batch cultures were performed.

The microbial dynamics of *C. necator* at different temperatures in batch cultures confirmed the temperature effect on *C. necator* growth. A maximal growth was obtained at 35°C ($0.25 \pm 0.01 \text{ h}^{-1}$) and the lower was at 25°C and 40 °C (0.14 h^{-1}). However, in the literature, the temperature of *C. necator* used was always 30°C [158, 167, 169, 199, 201, 209, 215, 228, 246, 247]. This result could be explained by the different strains used in the literature (ATCC17699=H16, LMG1199, Z1, ATCC17697, DSM 531, DSM 541 and DSM 545).

pH had also an effect on *C. necator* growth. A very low biomass production was obtained at pH5 which was stopped after 124 hours with three growth phases (0.027 ± 0.004 ; 0.014 ± 0.001 and $0.0021 \pm 0.0002 \text{ h}^{-1}$) and a low biomass yield ($0.22 \pm 0.04 \text{ g}_x/\text{g}_s$) which could be a result of glucose conversion into cellular energy facing pH stress. Two growth phases were obtained at pH6 (0.11 ± 0.01 and $0.16 \pm 0.01 \text{ h}^{-1}$) and pH8 (0.12 ± 0.01 and $0.18 \pm 0.01 \text{ h}^{-1}$) and the best growth was obtained at pH7 ($0.21 \pm 0.01 \text{ h}^{-1}$) which is the optimal pH value for *C. necator* growth considering the literature [169, 199, 201, 209, 215, 247, 252, 253]. In case of SCP production, the study of Sakarika *et al.*, [167] showed also that pH 7 gave the highest growth rates compared to pH 6 [167].

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At the morphological level, flow cytometry measurements were done during the cultures. Evolutions of both cell size and granularity were obtained and the results showed that:

Cell morphology has changed in response to temperature stress. Cells tend to elongate (FSC) and to change in granularity (SSC) at 40°C. That could be due most probably to PHB production [217, 248] which was proved with microscopy observation. However, no significant changes in size and granularity distributions at 25°C, 30°C and 35°C were observed.

pH had also an effect on cells morphology especially at pH5 where both cell size and granularity increased over time. The changes started in the second growth phase (0.014 h⁻¹).

Regarding the membrane permeability to Propidium Iodide, *C. necator* cells seemed to keep intact in response to the different environmental conditions tested.

In this work, SCP production was not only evaluated via biomass production. Indeed, two different methods were used in order to measure more specifically protein production during the culture. The difference was the type of protein quantification assay. The first one is a colorimetric assay after NaOH treatment (NaOH-solubilized proteins) and the second one is a total nitrogen measurement which is then converted to total protein content. The results revealed that:

Protein amounts were dependent on the temperature value in *Cupriavidus necator*. The high temperature used (40°C) seems to have a negative impact on proteins accumulation. Comparing the NaOH-solubilized proteins, the four profiles had almost the same dynamics at 25, 30 and 35°C (around 40%_{DW} at the end of the culture). However, a lower profile was obtained at 40°C (26.3±0.7 %_{DW} at the end of the culture). Regarding the Dumas and also Kjeldahl measurements, the results confirmed a lower protein production at 40°C (around 59%_{DW}) compared to the other conditions (above 75%_{DW}). The results obtained at 30°C agree with the literature results at the same temperature condition (75%_{DW} in the literature against 79%_{DW} in this work) [158, 226, 249].

Protein amounts were also dependent on the pH value in *Cupriavidus necator*. Similar final amounts were obtained at the end of cultures at pH6, 7 and 8 (75.67, 77.95 and 78.05%_{DW} at the end of the culture) but lower percentages were obtained at pH5 (50.41%_{DW} finally). The pH effect on SCP in *C. necator* is not yet evaluated in the literature. However, for other microorganisms, the pH effect was very dependent on the strain. An acidic pH stress gave higher protein content sometimes (for *Acetobacter diazotrophicus* [96] and *Aspergillus terreus* [173]), but lower amounts in other cases (*Cryptococcus aureus* [104]). However, pH (4-7) is found not as a main factor affecting protein amounts for *Rhodotorula glutinis* [140].

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The nucleic acid presents a drawback in SCP production. However, it was rarely presented in SCP investigations. It was essential to quantify it to verify its amount in the biomass. After lysis with the FastPrep method, RNA and DNA were quantified using fluorescence RiboGreen and PicoGreen assays, respectively. The results showed that:

The temperature had an effect on RNA and DNA production in *C. necator*. Indeed, a high temperature value (40°C) gave lower nucleic acid production profile and percentages (4.35±0.17 %_{DW} at the end of the culture) with higher RNA and DNA contents at 30 and 35°C (around 6 %_{DW} at the end of the culture).

On the other hand, the nucleic acid amounts were dependent on the pH value. The RNA and DNA profiles were similar at pH 6, 7 and 8. However, the acidic pH (pH5) gave lower amounts for both RNA (3.31±0.07%_{DW}) and DNA (1.32±0.01%_{DW}).

These profiles could be caused by the growth rates which are different for each culture, for both temperature and pH.

Poly-β-hydroxybutyrate (PHB) is normally produced by wild strains of *C. necator* under nutrient limitations such as nitrogen and/or phosphate. The used strain in this study was not deleted in dedicated metabolic pathways. In order to verify its accumulation during the cultures, flow cytometry analysis was done using Nile Red as the fluorescent probe. The analysis profiles showed that:

For the temperature impact, Nile Red⁺ populations were observed during all the cultures with a higher percentage at 40°C. However, these profiles could not present the absolute PHB⁺ populations, because the probe is not specific to PHB since it can detect lipids [250]. But as previously indicated, at 40°C, PHB granules were observed under microscope which explained the lower protein production at this condition. In the literature, the relation between temperature stress and PHB production was investigated in order to optimize its production [221, 222]. Temperature stress had a positive impact on PHB production.

Regarding the pH effect, significant Nile Red⁺ percentages were obtained for all the conditions especially at pH5. If it refers to PHB percentages, these results could explain the lower percentage of protein produced at this pH. PHB content was evaluated during *C. necator* cultures with pH stress. The PHB amounts decreased when the pH increased (from 6 to 8) [219, 220]. However, in other cases, pH value around 8 stimulates PHB production [254, 255].

To characterize the biomass macromolecular composition, a CHONS analysis was carried out using the final biomasses. Its results showed that:

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In response to temperatures' values, no variation in carbon (46-48 %), hydrogen (7%) and oxygen (24-27%) amounts were detected. However, the nitrogen, the sulfur and their ratio decrease in response to the temperature increase. That could be explained by a difference in sulfur amino acids (methionine, cysteine) amounts. In addition, the nitrogen amount agrees with the total protein percentages measured accordingly also with the increased production of PHB (not a nitrogen-based product) in the case of 40°C. The synthesis of PHB can indeed explained the decrease the N% in the total biomass [208]. The molecular weights calculated were also dependent on the temperature with a lower value at 40°C (22.72 g/Cmol \pm 0.16).

In response to pH, the percentage of carbon increased when the pH value is decreased from 8 to 5, from 42.86 \pm 4.7 to 48.87 \pm 1.38%_{DW}. No high variation in hydrogen and oxygen amounts was detected. However, Nitrogen and sulfur percentages increased when the pH value increased and also their ratio. That could suggest different amino acids composition especially sulfur-containing amino acids (Methionine, Cysteine). The molar weight calculated was very impacted. Its value passed from 24.25 \pm 0.97 to 22.05 \pm 0.03 Cmol from pH8 to pH5.

The study results show that both temperature and pH have an impact on SCP production and composition. Indeed, this impact was especially observed at the highest temperature (40°C) or the lowest pH value (pH5) providing lower growth rates, lower amounts of NaOH-solubilized proteins, total proteins, RNA and DNA. These low percentages could probably be due to a production of other compounds which could be PHB according to the Nile Red⁺ populations and also the CHONS compositions. On the other hand, nucleic acids and PHB granules are both drawbacks for SCP production for human consumption from *C. necator*. Producing low percentages of nucleic acids is favorable but producing PHB is not satisfactory. However, this polymer could be eliminated from the biomass with separation methods.

Impacts of the substrate composition on the dynamics of growth and protein production in *Cupriavidus necator*

This thesis part evaluated SCP production and composition using different substrate composition. We chose to vary the nitrogen source (ammonium sulfate and urea) and the carbon source (glucose, formic acid and oleic acid). The aim was to compare different C- or N-sources having different nature with different microbial metabolisms, in order to modulate SCP production and composition. Batch cultures were used in this aim using a mono-factorial

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approach, but for the formic acid a fed batch mode was used due to its toxicity for microbial cells. The same analyses of the previous part were performed in this part.

The growth dynamics showed that:

Regarding the nitrogen sources, the growth was faster using ammonium ($0.21 \pm 0.01 \text{ h}^{-1}$) than urea ($0.17 \pm 0.01 \text{ h}^{-1}$), as nitrogen source. But no significant difference was detected between the biomass yields. These results agree with Yang *et al.*, [169] where the maximal growth rates values were 0.26 h^{-1} and $0.18 \pm 0.01 \text{ h}^{-1}$, for ammonium and urea, respectively. The values were however lower probably due to the difference of the strain, or the culture conditions. Aramvach *et al.*, [219] and Khanna and Srivastava [220] studies were not in agreement with the results obtained in this work, the urea gave a highest growth rate than ammonium but under different culture conditions (unregulated pH, shake flasks, ..) [220].

For carbon source cultures, the growth kinetics revealed that the maximal growth rate was, $0.21 \pm 0.01 \text{ h}^{-1}$ for glucose and $0.19 \pm 0.01 \text{ h}^{-1}$ for oleic acid. However, the growth profile was divided into different phases for the formic acid due to the fed batch control passing from $0.117 \pm 0.003 \text{ h}^{-1}$, $0.17 \pm 0.04 \text{ h}^{-1}$, $0.10 \pm 0.01 \text{ h}^{-1}$ and $0.05 \pm 0.01 \text{ h}^{-1}$ at the end of the culture.

Regarding cell morphology, the results showed that:

Using different nitrogen sources, no drastic changes were detected in both size and granularity. For carbon sources, it was not possible to evaluate cell morphology from the oleate culture because of its texture. No variation in cells morphology was obtained for glucose and formic acid profiles. However, the population distribution (SSC and FSC signals) seemed to be more homogenous compared during the formic acid fed batch culture.

Cell viability was also assessed by flow cytometry using Propidium Iodide (PI). Permeability to PI was measured along the culture. No loss in membrane permeability was detected and microbial cells were kept intact during these cultures.

Protein profiles detected via two different methods showed that:

Regarding the N-sources, ammonium sulfate was found as the best for protein production with both BCA measurements and Dumas's method. The protein production increased overtime and was ranged between $32\text{-}47\%_{\text{DW-BCA}}$ and $67\text{-}78\%_{\text{DW-DUMAS}}$. For urea, the protein profile showed fluctuations between the beginning and the end of the culture; proteins amounts fluctuated between 26 and $45\%_{\text{DW-BCA}}$ and between 47 and $61\%_{\text{DW-DUMAS}}$. These results agree with the study of Yang *et al.*, [169] evaluating SCP production using ammonium sulfate, nitrate, nitrite

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and urea. The ammonium sulfate gave higher production 83%_{DW} against 65%_{DW} for urea [169]. According to their study, urea caused higher C/N ratio in the media (since urea degradation released CO₂) leading to a low percentage of N-products, including proteins [169].

Comparing the final NaOH-solubilized proteins, the percentages were comparable for both glucose and formic acid cultures (around 42%_{DW}) but low percentage was obtained for the oleic acid culture with 31.38±1.30%_{DW}. For the final total amounts, the formic acid culture presented also the highest protein content (91%_{DW-DUMAS}) against 78%_{DW-DUMAS} for glucose and 62%_{DW-DUMAS} for the oleic acid with. This difference is probably caused by the difference of carbon metabolism for each carbon source (central carbon pathway for glucose, Calvin-Benson-Bassham cycle for formic acid and fatty acid degradation pathway for oleic acid).

After a bead lysis protocol, RNA and DNA amounts were measured along the cultures and the results showed that:

The nitrogen source has an impact on RNA and DNA production profiles. Profiles were higher in the biomass cultivated with ammonium as nitrogen source which could be caused by the nitrogen metabolism explained previously in the protein part [169]. This result could be also explained by the growth rates during these cultures (0.21±0.01 h⁻¹ for ammonium sulfate and 0.17±0.01 h⁻¹ for urea). The percentages were ranged between 1.92±0.02 and 2.83±0.04 %_{DW} for ammonium and between 1.40±0.02 and 2.06±0.03 %_{DW} for urea. The production profiles were quite stable during the culture.

For carbon sources, the nucleic acid profiles were higher using the glucose but lower amounts were given by the formic acid. However, different dynamics were observed during the oleic acid culture. The final RNA amounts were comparable using glucose and formic acid (5.76±0.18 and 5.51±0.40 %_{DW}), but higher on oleic acid (7.97±0.01 %_{DW}). DNA evolution profiles was slightly higher on glucose (2-2.8%_{DW}) than on formic acid (1.7-2.5%_{DW}), and lower than 2% for oleic acid.

To evaluate PHB production, flow cytometry analysis profiles using the Nile Red label showed that:

Comparing cellular biomasses produced using urea and ammonium sulfate, in the middle of the exponential phases, the Nile Red⁺ population percentage was 37% for ammonium sulfate against 42.6% for urea. However, the final percentage was higher for ammonium sulfate. If we suppose that Nile Red⁺ in a PHB⁺ population, the results of the final points did not agree with other studies regarding PHB where urea lead to higher concentration [219, 220]. These

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differences could be due to the culture conditions. Indeed, the studies of Aramvash *et al.*, [219] and Khanna *et al.*, [220], the cultures were done under PHB production conditions.

Comparing the carbon source profiles, the formic acid as carbon source presented lower Nile Red⁺ population, 9% against 37% out of the total population for glucose in the middle of the exponential phase of growth. This result showed clearly the absence of PHB in the formic acid biomass agreeing with high protein amount.

To characterize the biomass elemental composition, the CHONS compositions results at the end of the cultures showed that:

Regarding the N source effect, urea biomass presented lower amounts for CHONS composition compared to ammonium sulfate. However, the molar weights values calculated were comparable (around 23.5 g/Cmol).

For the C source effect, Oleic acid culture biomass showed lower percentages for all the compounds. Comparing biomasses obtained using glucose and formic acid, higher percentages were found in carbon, hydrogen and oxygen in glucose biomass, but higher percentages in nitrogen and sulfur for formic acid biomass were measured. High percentages in nitrogen and sulfur could validate the higher protein contents obtained in formic acid culture. The molar weights were comparable around 23-24 g/Cmol.

In conclusion, the macroscopic behavior (growth, morphology and viability) of *C. necator* depends on the nitrogen source and also the carbon source.

Regarding N-sources, the growth was faster using the sulfate ammonium as nitrogen source. Single Cell Protein production appeared to be dependent on the nitrogen source used too. Ammonium sulfate culture produces more proteins and higher amounts of nucleic acids than urea which is probably due to the N metabolism. However, there is no impact on cell morphology and CHONS composition either.

Regarding C-sources, the growth was faster using glucose as carbon source. The formic acid gave the best protein percentage which was ranged between 70 and 90%_{DW} (Dumas) (with low nucleic acid amounts) and the oleic acid presented the lowest amounts, not exceeding 60%_{DW} (Dumas). That could be caused by *C. necator* different metabolism considering the different carbon nature. Indeed, the glucose is consumed following the central carbon pathway [204, 205], the formic acid enter through the Calvin-Besson-Bassham cycle allowing the fixation of CO₂ and the oleic acid enter in the fatty acid degradation pathway [204, 207]. The low amounts in oleic acid biomass are due to the

residual oleic acid contained in the samples where the elimination protocol may not have been efficient enough. Therefore, formic acid was found as promoting carbon source for SCP production and human consumption due to the high protein percentage, low nucleic acid amounts and low Nile Red+ labelled population. Formic acid culture results could be due to the carbon source, the mode of culture (fed batch) or both of them.

Perspectives

Some experiment suggestions are presented below, regarding the previous results obtained during my thesis:

- To complete this study, an amino acid profile quantification could be a good way to characterize the nutritional composition of the proteins produced along the cultures, especially the essential amino acids composition (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), which cannot be made by the human body and must come from food. Then, a comparison with the FAO recommendations amounts and the other protein sources (animal and vegetal) could be investigated. Added to that, we could compare the amino acid profiles for different temperatures, different pH, different carbon sources and different nitrogen sources using *Cupriavidus necator* grown on defined chemical media. This quantification will allow us to see if these parameters impacted or not the nature of the produced proteins. Added to that, it could validate or not the conclusions made from the analyses of the C/N ratio and then provide new information as to the modulation of the amino acid composition in SCP in relation to the culture environment. The quantification of the amounts in amino acids could be done using chromatographic analysis.
- A PHB quantification is necessary to verify its production during all my bioreactor cultures. Indeed, it is crucial to calculate the balances percentages with PHB concentrations measurement. Indeed, with the presented results, we could not close neither the Carbon balances nor the macromolecular balances. The Nile Red + populations percentage were obtained in almost all my cultures. Thus, it is essential to verify the composition of the obtained biomasses and to confirm the PHB presence. The quantification of the PHB could be done using gas chromatography analysis.

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- The amounts of lipopolysaccharides (LPS) present in the biomass is interesting to quantify since it is inevitable produced by bacteria, including *Cupriavidus necator*. Their evaluation is important because of the health diseases caused by these endotoxins' presence in food and feed. This quantification will investigate also the effect of the environmental parameters and the media composition on LPS production.

In addition to the complementary analyses:

- Other factors could be also tested, such as the culture mode. Indeed, a fedbatch culture could be performed using glucose and/or oleic acid, as substrate in order to compare to the results obtained in the case of the formic acid-fedbatch culture. That could help to decipher the formic acid culture results: performance due to the nature of the substrate or the mode of culture.
- A multi factorial approach could be tested for both environmental parameters and media composition. Indeed, it's interesting to evaluate not only the impact of one parameter but the two or more parameters combined taking into account the interaction effects between parameters.

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