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Optimization of fermentative biohydrogen production by *Thermotoga neapolitana*

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— PARIS-EST

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Spécialité : Science et Technique de l'Environnement



Dottore di Ricerca in Tecnologie Ambientali



Degree of Doctor in Environmental Technology



TAMPERE UNIVERSITY OF TECHNOLOGY

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Gilbert Dreschke

Optimization of fermentative biohydrogen production by *Thermotoga neapolitana*

05/12/2018, Cassino

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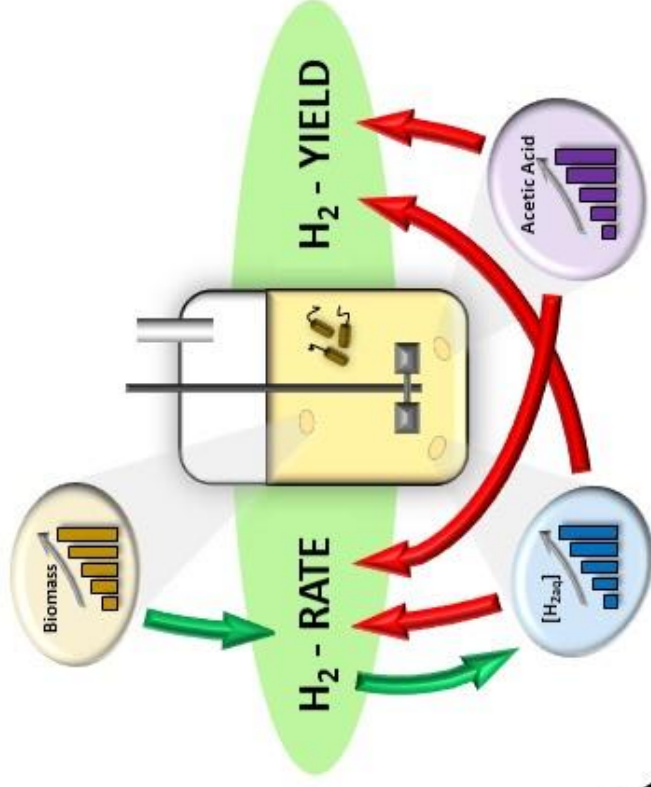
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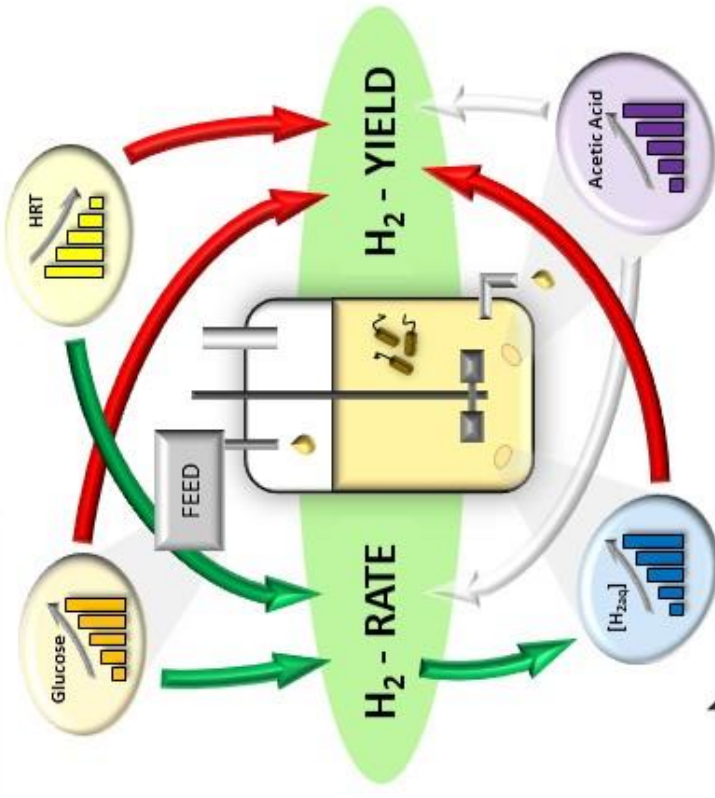
80 °C
T. neapolitana

Positive correlation
Negative correlation
No correlation

Batch



Continuous



OPTIMIZATION

Abstract

Hydrogen has revealed a great potential as a versatile and non-polluting energy carrier of the future providing a high energy density and an efficiently conversion to usable power. Dark fermentation is one of the most promising biological production processes, but still has to overcome major challenges, most importantly low hydrogen production rates (HPRs) and hydrogen yields (HYs), before its industrial application becomes cost- and energy-efficient.

In this work, we aimed to optimize the hydrogen production via dark fermentation by *Thermotoga neapolitana*. The main objectives were to enhance the HPR and maintain a high HY using different approaches to counteract process limitations and prevent the most relevant inhibitions. Furthermore, a development of the industrially preferred continuous-flow process was projected.

An increase of the initial biomass concentration from 0.46 to 1.74 g cell dry weight (CDW)/L in batch bioassays resulted in a more than 2-fold enhancement of the HPR up to 654 (± 30) mL/L/h (mL of hydrogen produced per L of volume of reactor per hour of reaction or per hour of liquid retention) without negatively affecting the HY. However, while the volumetric productivity increased the specific HPR (per unit of biomass) was negatively correlated with the HPR and the biomass concentration.

Subsequently, we investigated the supersaturation of hydrogen in the liquid phase (H_{2aq}) in batch bioassays. At 100 rpm agitation H_{2aq} supersaturated up to 3 times the equilibrium concentration. Increasing the agitation speed diminished the accumulation of H_{2aq} until an equilibrium between the gas and liquid phase hydrogen was reached with 500 rpm agitation at low cell concentrations. A raise from 200 to 600 rpm gradually reduced H_{2aq} from 21.9 (± 2.2) to 8.5 (± 0.1) mL/L and approximately doubled the HPR, revealing a direct correlation between the two parameters. Similarly, the addition of K1 carrier and H_2 -rich biogas recirculation (GaR) successfully counteracted the accumulation of H_{2aq} . Accelerating the process by increasing the reactors biomass concentration up to 0.79 g CDW/L, GaR revealed to be more efficient in removing H_{2aq} than 500 rpm agitation. The application of GaR at 300 and 500 rpm enhanced the HPR by approximately 260% up to 850 (± 71) mL/L/h, compared to a sole 300 rpm agitation, reaching a HY of 3.5 mol H_2 /mol glucose. We demonstrated that an insufficient gas-liquid mass transfer leads to the accumulation of H_{2aq} which inhibits the yield but even more so the rate of dark fermentation.

In the final phase of this project we successfully maintained continuous-flow hydrogen production. Increasing the feed glucose concentration from 11.1 to 41.6 mM diminished the HY from 3.6 (± 0.1) to 1.4 (± 0.1) mol H_2 /mol glucose. The HPR increased concomitantly up to approximately 55 mL/L/h at 27.8 mM of glucose, whereas a further increase of feed glucose to 41.6 mM did not enhance the HPR and the acetic acid (AA) concentration. To investigate whether high levels of AA limited the process, the feed AA concentration was gradually increased. However, this revealed no negative effect on continuous dark fermentation up to 240 mM of feed AA and, throughout the 110 days of continuous fermentation, the HY increased by 47%. Decreasing the hydraulic retention time (HRT) from 24 to 7 h also led to a HPR enhancement from 82 (± 1) to 192 (± 4) mL/L/h, while decreasing the HY.

Concomitantly, the H_{2aq} accumulated, directly correlated to the HPR reaching 15.6 mL/L at an HRT of 7 h and 500 rpm agitation. The application of GaR efficiently counteracted the supersaturation of H_{2aq} and allowed the highest HPR of 277 mL/L/h at a HRT of 5 h.

Sommario

L'idrogeno è uno dei vettori di energia più versatili, essendo caratterizzato da un'elevata densità energetica ed essendo facilmente convertibile in ogni forma di energia. Inoltre, la sua combustione non comporta alcuna produzione di gas clima alteranti. L'idrogeno può essere prodotto in maniera sostenibile attraverso il processo biologico di "dark fermentation", o "fermentazione al buio", che, tuttavia, presenta ancora delle limitazioni, tra cui i bassi tassi e rendimenti di produzione di idrogeno, che non ne permettono al momento la sua applicazione su scala industriale.

In questo lavoro di tesi, l'obiettivo è stato quello di massimizzare la produzione di idrogeno attraverso il processo di dark fermentation condotto a mezzo di una coltura pura di *Thermotoga neapolitana*. Nello specifico, sono stati utilizzati differenti approcci sperimentali per garantire elevati valori di tassi di produzione di idrogeno (HPR) e rendimenti di produzione (HY) e, allo stesso tempo, contenere gli effetti inibenti dei principali parametri. Inoltre, è stato implementato e studiato un bioreattore con flusso in continuo, che rappresenta sicuramente un'alternativa più valida per un'eventuale applicazione del processo su scala reale.

I risultati hanno dimostrato che, in esperimenti batch, un incremento della concentrazione di biomassa di *T. neapolitana* da 0,46 a 1,74 g CDW/L ha comportato più che un raddoppio dell'HPR, che ha raggiunto un valore finale di 654 (± 30) mL/L/h senza che venisse alterato il valore di HY. Incrementando, invece, la velocità di agitazione della miscela liquida, si è osservato un minor accumulo dell'idrogeno in fase liquida (H_{2aq}) con il valore più basso ottenuto ad una velocità di 500 rpm a basse concentrazioni di biomassa. Ad esempio, un aumento della velocità da 200 a 600 rpm ha comportato una riduzione di H_{2aq} da 21,9 ($\pm 2,2$) a 8,5 ($\pm 0,1$) mL/ e quasi un raddoppio del valore di HPR, rivelando una correlazione diretta tra i due parametri. Sotto ogni condizione di esercizio, un equilibrio termodinamico tra la fase liquida e quella gassosa è stato raggiunto con differenti valori di H_{2aq} che hanno indotto differenti valori di HPR.

In maniera del tutto simile all'aumento della velocità di agitazione, anche l'utilizzo di una corrente di ricircolo del biogas (GaR) prodotto e l'introduzione di elementi solidi nella miscela (quali i supporti K1, utilizzati comunemente per la crescita dei biofilm) ha permesso una riduzione dei valori di H_{2aq} . In particolare, la GaR è risultata più efficiente di una velocità di agitazione pari a 500 rpm nell'abbattere il valore di H_{2aq} , nelle condizioni di biomassa presente pari a 0,79 g CDW/L. L'HPR è aumentato di circa del 260%, ovvero fino a 850 (± 71) mL/L/h, e l'HY ha raggiunto un valore di 3,5 mol H_2 /mol di glucosio quando la GaR è stata utilizzata a 300 e 500 rpm, rispetto alla condizione di assenza di GaR. Pertanto, un limitato trasferimento di idrogeno dalla fase liquida a quella gassosa favorisce l'accumulo di H_{2aq} , che comporta l'inibizione del processo in termini di riduzione delle rese di produzione e, ancor di più, dei tassi di produzione di idrogeno.

Nell'ultima parte della sperimentazione, il processo biologico è stato con successo implementato all'interno di un reattore esercito in continuo, valutando dapprima l'effetto di differenti concentrazioni di glucosio e acido acetico nella soluzione influente. Incrementando

la concentrazione in ingresso di glucosio da 11,1 a 41,6 mM, è stata osservata una netta riduzione dell'HY da 3,6 ($\pm 0,1$) a 1,4 ($\pm 0,1$) mol H₂/mol di glucosio. Allo stesso tempo, l'HPR ha subito un aumento fino a circa 55 mL/L/h per una concentrazione iniziale di glucosio di 27,8 mM, per poi rimanere costante, insieme alla produzione di acido acetico (AA), quando è stato effettuato l'ultimo step di incremento di glucosio a 41,6 mM. Al fine, appunto, di valutare se l'AA avesse avuto un effetto inibente sul processo, la sua concentrazione nella soluzione influente è stata aumentata con gradualità da 30 a 240 mM. Tuttavia, nessun effetto negativo dell'AA è stato osservato sulla produzione di idrogeno e, addirittura, al termine dei 110 giorni di sperimentazione, si è avuto un incremento dell'HY di circa il 47%. Successivamente, il reattore in continuo è stato esercitato abbassando gradualmente il tempo di detenzione idraulica (HRT) da 24 a 7 h, ottenendo un incremento dell'HPR da 82 (± 1) a 192 (± 4) mL/L/h, a discapito dell'HY che ha subito una diminuzione. In questa fase, si è osservato anche un aumento di H_{2aq}, in equilibrio con l'HPR, che ha raggiunto un valore massimo di 15,6 mL/L ad un HRT di 7 h. Ricircolando il biogas prodotto, è stata efficacemente limitata la supersaturazione dell'idrogeno in soluzione permettendo di raggiungere il valore più elevato di HPR, e pari a 277 mL/L, e di scendere ancor di più con l'HRT che, negli ultimi giorni di sperimentazione, è stato mantenuto pari a 5 h.

Résumé

L'hydrogène a un fort potentiel en tant que source polyvalente et non-polluante d'énergie du futur. La production de biohydrogène par voie fermentaire dite « sombre » est l'un des processus biologiques de production les plus prometteurs, mais son application est pour l'instant contrainte notamment par des cinétiques et des rendements de production d'hydrogène qui restent relativement faibles. Ce qui pour l'instant restreint toute application industrielle.

Dans ce travail de thèse, nous avons cherché à optimiser la production d'hydrogène par fermentation « sombre » avec *Thermotoga neapolitana*. Les principaux objectifs étaient d'améliorer la cinétique et de maintenir un rendement élevé de production en hydrogène en utilisant différentes approches pour contrecarrer les limites des processus et prévenir les inhibitions les plus significatives. En outre, le développement d'un procédé à mode continu potentiellement applicable à l'échelle industrielle a été étudié.

Une augmentation de la concentration initiale de la biomasse de 0,46 à 1,74 g masse sèche / L dans des bioréacteurs discontinus a entraîné un doublement de la cinétique de production d'hydrogène jusqu'à 654 (\pm 30) mL / L / h sans affecter négativement les rendements de production d'hydrogène. Cependant, alors que la productivité volumétrique augmente, la cinétique spécifique de production d'hydrogène (i.e. par unité de biomasse) est négativement corrélée aux cinétiques de production d'hydrogène et à la concentration de biomasse.

Par la suite, nous avons étudié la sursaturation en hydrogène de la phase liquide (H_{2aq}) dans des bioréacteurs discontinus. À une agitation de 100 tr / min, H_{2aq} est sursaturé jusqu'à 3 fois la concentration à l'équilibre. L'augmentation de la vitesse d'agitation diminue la concentration de H_{2aq} jusqu'à atteindre un équilibre entre l'hydrogène en phase gazeuse et phase liquide pour une agitation à 500 tr / min et de faibles concentrations en cellules microbiennes. Une augmentation de 200 à 600 tr / min a réduit progressivement l' H_{2aq} de 21,9 (\pm 2,2) à 8,5 (\pm 0,1) mL / L et a presque doublé cinétique de production d'hydrogène, révélant une corrélation directe entre les deux paramètres. De même, l'ajout de matériaux supports type AnoxKaldnes™ et la recirculation de biogaz riche en H_2 a permis de limiter l'accumulation de H_{2aq} . En augmentant la concentration de biomasse dans les réacteurs jusqu'à 0,79 g masse sèche / L, la recirculation de biogaz riche en H_2 s'est révélée plus efficace pour diminuer la concentration en H_{2aq} que l'agitation à 500 tr / min. L'application de la recirculation de biogaz riche en H_2 combinée à une agitation de 300 et 500 tr / min a augmenté la cinétique de production d'hydrogène d'environ 260% à 850 (\pm 71) mL H_2 / h / L, par rapport à une agitation à 300 tr / min, atteignant un rendement de 3,5 mol de H_2 / mol de glucose. Nous avons démontré qu'un transfert de masse gaz-liquide insuffisant conduit à une accumulation de H_{2aq} qui affecte le rendement de production d'hydrogène et la cinétique de la fermentation « sombre ».

Dans la phase finale de cette thèse, nous avons réussi à maintenir la production d'hydrogène à débit continu. L'augmentation de la concentration de glucose comme substrat entrant de 11,1 à 41,6 mM a conduit à une diminution des rendements de production en hydrogène de

3,6 ($\pm 0,1$) à 1,4 ($\pm 0,1$) mol H₂ / mol de glucose. La cinétique de production en hydrogène a augmenté simultanément jusqu'à environ 55 ml / L / h pour une concentration de 27,8 mM de glucose, tandis qu'une augmentation supplémentaire du glucose entrant, à 41,6 mM, n'a pas augmenté les cinétiques de production en hydrogène et les concentrations dissoutes en acides aminés. Pour déterminer si des concentrations élevées d'acides aminés sont susceptibles de limiter les processus biologiques, la concentration en acides aminés entrante a été progressivement augmentée. Cependant, cela n'a révélé aucun effet négatif sur la fermentation « sombre » en mode continu jusqu'à 240 mM d'acides aminés et, tout au long des 110 jours de fermentation « sombre » en mode continu alors que les rendements de production en hydrogène ont augmenté de 47%. La réduction du temps de séjour hydraulique (TSH) de 24 à 7 h a également entraîné une augmentation de la cinétique de production en hydrogène de 82 (± 1) à 192 (± 4) mL / L / h, tout en diminuant le rendement. De manière concomitante, le H₂aq accumulé est directement corrélé cinétique de production en hydrogène atteignant 15,6 mL / L pour un TSH de 7 h et une agitation de 500 tr / min. L'application de la recirculation de biogaz riche en H₂ a efficacement neutralisé la sursaturation en H₂aq et a permis d'obtenir une cinétique de production en hydrogène plus élevée de l'ordre de 277 mL / L pour un TSH de 5 h.

Samenvatting

Waterstof heeft een groot potentieel als een veelzijdige en niet-vervuilende energiedrager van de toekomst die een hoge energiedichtheid en een efficiënt conversie naar bruikbare energie. Donkere fermentatie is één van de meest veelbelovende biologische productieprocessen, maar nog steeds zijn grote barrières te overwinnen, de belangrijkste zijn de lage waterstofproductie snelheden (HPRs) en waterstof opbrengsten (HYs), voordat industriële toepassingen kosten- en energie-efficiënt worden.

In dit werk streefden we naar het optimaliseren van de waterstofproductie via donkere fermentatie door *Thermotoga napolitana*. De belangrijkste doelstellingen waren het verbeteren van de HPR en het handhaven van een hoge HY met verschillende benaderingen om proces beperkingen tegen te gaan en de meest relevante remmingen te voorkomen. Verder werd een ontwikkeling van het industriële geprefereerde continue-proces uitgewerkt.

Een toename van de initiële biomassaconcentratie van 0,46 tot 1,74 g CDW/L in batch bioassays resulteerde in een meer dan 2-voudige toename van de HPR tot 654 (\pm 30) mL/L/h zonder de HY negatief te beïnvloeden. Echter, terwijl de volumetrische productiviteit verhoogde, was de specifieke HPR (per eenheid van biomassa) negatief gecorreleerd met de HPR en de biomassaconcentratie.

Vervolgens onderzochten we de oververzadiging van waterstof in de vloeistoffase (H_{2aq}) in batch bioassays. Bij 100 RPM agitatie was de H_{2aq} oververzadigd, tot 3 keer de evenwichtsconcentratie. Het verhogen van de agitatie snelheid verminderde de accumulatie van H_{2aq} tot een evenwicht tussen de gas en vloeibare fase. Een verhoging van 200 tot 600 RPM geleidelijk verlaagde H_{2aq} van 21,9 (\pm 2,2) tot 8,5 (\pm 0,1) mL/L en verdubbelde de HPR, waaruit een directe correlatie tussen de twee parameters bleek. Ook de toevoeging van K1 carrier en recirculatie van het H_2 -rijke biogas (GaR) ging met succes de accumulatie van H_{2aq} tegen. Versnellen van het proces door het verhogen van de biomassaconcentratie tot 0,79 g CDW/L met GaR was efficiënter in het verwijderen van H_{2aq} dan 500 rpm agitatie. De toepassing van GaR bij 300 en 500 rpm verbeterde de HPR met ongeveer 260% tot 850 (\pm 71) mL H_2 /h/L, in vergelijking met alleen 300 rpm agitatie, en een HY van 3,5 mol/mol glucose werd bereikt. We hebben aangetoond dat een onvoldoende gas-vloeibare massaoverdracht leidt tot de accumulatie van H_{2aq} die de opbrengst remt, alsook de snelheid van de donkere fermentatie.

In de laatste fase van dit werk hebben we met succes continue waterstofproductie aangetoond. Het verhogen van de glucoseconcentratie in het influent van 11,1 tot 41,6 mM verkleinde de HY van 3,6 (\pm 0,1) tot 1,4 (\pm 0,1) Mol H_2 /mol glucose. De HPR steeg gelijktijdig tot ongeveer 55 mL/L/h bij 27,8 mM van glucose, terwijl een verdere verhoging van de influent glucose concentratie tot 41,6 mM de HPR en de AA concentratie niet verhoogde. Om te onderzoeken of de hoge AA concentraties het proces beperken, werd de influent AA concentratie geleidelijk verhoogd. Dit bleek echter geen negatief effect op de continue donkere fermentatie tot 240 mM feed AA te hebben, en gedurende de 110 dagen continue fermentatie verhoogde de HY met 47%. Het verminderen van de HST van 24 tot 7 h leidde

ook tot een HPR verhoging van $82 (\pm 1)$ tot $192 (\pm 4)$ mL/L/h, terwijl de HY verminderde. Tezelfde tijd, was de geaccumuleerde H_{2aq} direct gecorreleerd aan de HPR, die 15,6 mL/L bedroeg bij een HST van 7 h en 500 rpm agitatie. De toepassing van GaR compenseerde de oververzadiging van H_{2aq} en liet de hoogste HPR van 277 mL/L bij een HST van 5 uur toe.

Tiivistelmä

Vedyn uskotaan olevan merkittävä energiankantaja tulevaisuudessa, koska sillä on korkea energiatiheys ja ympäristöpäästöt ovat vähäiset. Vetyä pystytään tuottamaan biologisesti monella eri tavalla. Niistä pimeäfermentaatio on toistaiseksi yksi lupaavimmista vaihtoehdoista, mutta sen kaupallistamisen mahdollistaminen vaatii vielä useiden merkittävien haasteiden selvittämistä. Näistä tärkeimpiä ovat alhaiset vedyntuottonopeudet ja vetysaannot.

Tämän työn tavoitteena oli optimoida *Thermotoga neapolitana* puhdasviljelmän vedyntuottoa eri tavoin. Pyrkimyksenä oli selvittää vedyntuottoa rajoittavat tekijät ja löytää keinoja tyyppillisimpien inhibitioiden estämiseksi. Tavoitteena oli myös kehittää teolliseen soveltamiseen tähtääviä jatkuvatoimisia prosesseja.

Panoskokeissa *T. neapolitana* biomassan määrän nostaminen pitoisuudesta 0,46 solun kuivapainoa (CDS)/L pitoisuuteen 1,74 g CDS/L nosti vedyntuottonopeuden reaktoritilavuutta kohden yli kaksinkertaiseksi arvoon 654 (± 30) mL/L/h heikentämättä vetysaantoa. Vedyntuotto nopeus suhteutettuna biomassan määrään kuitenkin laski biomassapitoisuuden kasvaessa.

Seuraavissa panoskokeissa tutkittiin nestefaasin vetypitoisuuden (H_{2aq}) ja erityisesti vedyn ylikyllästymisen vaikutusta vedyntuottoon. Sekoitusnopeudella 100 rpm, H_{2aq} oli kolme kertaa korkeampi kuin teoreettinen vedyn tasapainopitoisuus. Sekoitusnopeuden lisääminen vähensi vedyn kertymistä nestefaasiin, ja kasvatuksissa, joissa solupitoisuus oli pieni, tasapaino kaasu- ja nestefaasin välillä saavutettiin sekoitusnopeudella 500 rpm. Sekoitusnopeuden nostaminen 200 rpm:stä 600 rpm:ään johti H_{2aq} :n pienenemiseen arvosta 21,9 ($\pm 2,2$) arvoon 8,5 ($\pm 0,1$) mL/L ja vedyntuottonopeuden kaksinkertaistumiseen osoittaen, että vedyntuottonopeuden ja H_{2aq} :n välillä on käänteinen korrelaatio. Myös K1-kantaja-aineen lisääminen ja tuotetun vetytöisen biokaasun kierrättäminen kasvatuksen läpi esti vedyn kertymistä nestefaasiin. Kun vedyntuottoprosessia oli tehostettu kasvattamalla reaktorin biomassapitoisuus 0,79 g/L:aan, kaasukierrätys reaktoriin pienensi H_{2aq} -pitoisuutta tehokkaammin kuin 500 rpm:n sekoitus. Kaasukierrätys nosti vedyntuottonopeutta 300 ja 500 rpm:n sekoituksella noin 260% verrattuna kasvatukseen, joissa käytettiin pelkästään 300 rpm:n sekoitusta. Korkeimmillaan vedyntuottonopeus oli 850 (± 71) mL/L/h ja vetysaanto 3,5 mol- H_2 /mol-glukoosia. Nämä tulokset osoittavat, että riittämätön massansiirto kaasu- ja nestefaasin välillä voi johtaa H_{2aq} -pitoisuuden nousuun, joka rajoittaa erityisesti vedyntuottonopeutta mutta jossain määrin myös vetysaantoa.

Jatkuvatoimisessa kokeessa syötteen glukoosipitoisuuden nostaminen laski vetysaantoa. Glukoosipitoisuudella 11,1 mM vetysaanto oli 3,6 ($\pm 0,1$) mol- H_2 /mol-glukoosia, kun taas pitoisuudella 41,6 mM saanto oli 1,4 ($\pm 0,1$) mol- H_2 /mol-glukoosia. Vedyntuottonopeus kasvoi syötteen glukoosipitoisuuden noustessa glukoosipitoisuuteen 27,8 mM asti ollen 55 mL/L/h, mutta syötteen glukoosipitoisuuden nostaminen 41,6 mM:iin ei enää kasvattanut vedyntuottonopeutta. Yhdeksi mahdolliseksi rajoittavaksi tekijäksi epäiltiin syötteen korkeilla glukoosipitoisuuksilla havaittua effluentin korkeaa asetaattipitoisuutta. Siksi seuraavaksi tutkittiin asetaatin lisäämistä reaktorin syötteeseen. Asteittainen syötteen

asettaattipitoisuuden nosto 240 mM:iin ei kuitenkaan heikentänyt vedyntuottoa. Sen sijaan 110 päivän pimeäfermentaation aikana vetysaanto kasvoi 47 %. Hydraulisen viipymän lyhentäminen kasvatti vedyntuottonopeutta, sillä 24 h viipymällä vedyntuottonopeus oli 82 (± 1) mL/L/h ja 7 h viipymällä 192 (± 4) mL/L/h. Vetysaanto oli kuitenkin sitä pienempi mitä lyhyempää hydraulista viipymää käytettiin. Vedyntuottonopeuden kasvaessa myös H_{2aq} kasvoi ollen 15,6 mL/L 7 h viipymällä ja 500 rpm:n sekoituksella. Tuotetun kaasun hyödyntäminen reaktorin sekoituksessa kuitenkin ehkäisi vedyn ylikyllästymistä nestefaasiin ja mahdollisti korkeimman vedyntuottonopeuden, 277 mL/L/h, 5 h viipymällä.

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List of Symbols and Abbreviations

Abbreviation	Definition
AA	Acetic acid
BM	Biomass
BMY	Biomass yield
CDW	Cell dry weight
CSTR	Continuous stirred tank reactor
EC	Electron carrier
GaR	Recirculation of H ₂ -rich biogas
H _{2aq}	Liquid phase hydrogen
HPR	Hydrogen production rate
HRT	Hydraulic retention time
HY	Hydrogen yield
LA	Lactic acid
NADH	Nicotinamide adenine dinucleotide
ORL	Organic loading rate
VFA	Volatile fatty acid

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Author's Contribution

Paper I, Chapter 3:

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Paper II, Chapter 4:

Gilbert Dreschke designed and performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Stefano Papirio, Antonio Panico, Giuliana d'Ippolito, Angelo Fontana, Piet N.L. Lens and Giovanni Esposito participated in the preparation of the manuscript.

Paper III, Chapter 5:

Gilbert Dreschke and Stefano Papirio planned the experiments, whereas their execution including the analysis was performed by Gilbert Dreschke, who wrote the manuscript and is the corresponding author. The manuscript was thoroughly revised by Stefano Papirio, Piet N.L. Lens and Giovanni Esposito.

Paper IV, Chapter 6:

Gilbert Dreschke planned and carried out the experiments including all the analysis, wrote the manuscript and is the corresponding author. Désirée M.G. Sisinni helped with the laboratory work, whereas Stefano Papirio participated in designing the experiments. Stefano Papirio, Piet N.L. Lens and Giovanni Esposito thoroughly revised and commented on the manuscript.

Chapter 7:

Gilbert Dreschke planned and carried out the experiments including all the analysis, wrote the manuscript and is the corresponding author. Alessio Scala helped with the laboratory work and Stefano Papirio participated in designing the experiments. Stefano Papirio, Piet N.L. Lens and Giovanni Esposito thoroughly revised and commented on the manuscript.

1 Introduction

The combined increase in global population and per capita energy consumption (Narbel and Hansen, 2014) has resulted in a 27-fold increase of global energy consumption since 1800 (Sorrell, 2015, p. 75). Currently, this energy demand is covered up to 80% by fossil resources (Mohr et al., 2015), a finite energy source whose natural deposits are rapidly declining and will inevitably cease someday (Shafiee and Topal, 2009, p. 188). Furthermore, the burning of fossil fuels jeopardizes human health by aggravating air pollution and greatly contributes to the effect of global warming (Elbeshbishy et al., 2017; Pfenninger and Keirstead, 2015, p. 83). To counteract this development, 195 countries declared to reduce their greenhouse gas emissions by 40% until 2030 in the Paris agreement of 2015. To realize this goal, renewable energy is seen as a key technology to provide low-carbon energy in the future (Pfenninger and Keirstead, 2015).

In the transition from a fossil to a renewable energy-based system, hydrogen gas is considered one of the most promising energy carriers. Its most important qualities include a high energy density, efficient conversion to usable power, the absence of greenhouse gas production upon combustion and its versatile applicability (Baykara, 2018; Marbán and Valdés-Solís, 2007). Hydrogen does not naturally occur in its molecular form as hydrogen gas and is consequently classified a carrier rather than a source of energy. Hence, hydrogen is only as environmentally friendly as the process used for its production (Marbán and Valdés-Solís, 2007). Currently, the vast majority of hydrogen is produced non-sustainably from fossil resources through energy intensive and ecologically harmful processes. Dark fermentation is, instead, an environmentally friendly biological alternative which allows the use of organic waste streams as substrates for the generation of hydrogen (Sivagurunathan et al., 2016).

While dark fermentation is widely seen as the most promising biological hydrogen production process (Arimi et al., 2015), further improvement of process performance is required for an economically viable application at industrial scale, especially in terms of hydrogen yield (HY) and production rate (HPR) (Boodhun et al., 2017; O-Thong et al., 2008). Higher HYs are provided under thermophilic operating conditions (Balachandar et al., 2013; Chou et al., 2008; Gupta et al., 2016). Indeed, *Thermotoga neapolitana*, a hyperthermophilic organism has been repeatedly shown to reach values approaching the theoretical maximum of 4 mol H₂/mol glucose (d'Ippolito et al., 2010; Munro et al., 2009). This microorganism has

gained increasing attention for hydrogen production featuring high conversion and growth rates, a large substrate range and a low risk of contamination due to growth at high temperatures (Pradhan et al., 2015). Previous studies provide valuable information, including nutrient requirements or the optimum ranges of temperature and pH (Pradhan et al., 2015), but have so far been limited to batch (d'Ippolito et al., 2010; Munro et al., 2009; Nguyen et al., 2008) or fed batch (Ngo et al., 2011a; Ngo and Bui, 2013) cultivation.

However, some crucial parameters still require further investigation. The biomass concentration observed in dark fermentation by thermophilic cultures is commonly low (Lee et al., 2011, p. 8399), limiting the process due to the direct correlation with the HPR (Gupta et al., 2016, p. 140; Show et al., 2012, p. 15625). End-products such as hydrogen or VFAs often accumulate in the reactor and are reported to be major inhibitors of dark fermentation. In particular, the supersaturation of hydrogen in the liquid phase is often disregarded in many dark fermentation studies. Furthermore, a continuous-flow operation is generally preferred in industrial applications (Balachandar et al., 2013; Kumar et al., 2014), which entails the optimization of the organic loading rate (OLR) and the hydraulic retention time (HRT).

This thesis focused on the improvement of hydrogen production via dark fermentation by a pure culture of *T. neapolitana*. The main objective was a substantial increase of the HPR while maintaining the conversion efficiency to hydrogen high. For the implementation of this process optimization, high biomass concentrations were initially established in batch reactors through biomass recycling. Furthermore, the accumulation of H_{2aq} was thoroughly investigated by applying a range of potential counteracting measures such as increased agitation, recirculation of H_2 -rich biogas and bubble induction by addition of K1 biofilm carriers. In a second stage, we aimed for the first time the development of a continuous-flow hydrogen production process using *T. neapolitana* evaluating the impact of the most fundamental parameters, i.e. HRT and feed substrate concentration.

2 State of the Art

2.1 Hydrogen

Hydrogen is the most common substance in the universe (Baykara, 2018), which exists in its elemental form as a colorless, odorless and highly combustible gas under standard conditions. The energy density of hydrogen is with 1.22 kJ/g, more than 2.7 times higher compared to other hydrocarbon fuels (Gupta et al., 2013), which can be efficiently converted to usable power (Marbán and Valdés-Solís, 2007). In contrast to most other current fuels, no CO₂ is formed upon its combusted (Marbán and Valdés-Solís, 2007) characterizing it as one of the most promising, non-polluting fuel of the future (Elsharnouby et al., 2013; Gupta et al., 2013). Furthermore, hydrogen is a highly flexible energy carrier with a wide range of applications including the use as transport fuel, electricity generation or the chemical industry (Marbán and Valdés-Solís, 2007). The demand for hydrogen has continuously increased in the recent past and is expected to contribute 8–10% of the energy market by 2025 (Gupta et al., 2013). However, hydrogen in its gaseous form is not readily available on Earth (Baykara, 2018). It is therefore not considered a source but a carrier of energy, and consequently only as clean as the method used for its production (Marbán and Valdés-Solís, 2007).

Currently, hydrogen is generated primarily from fossil resources through steam reforming of natural gases and industrial oil as well as naphtha reforming. Coal gasification, electrolysis of water, and other sources are used to a much lower extent (Gupta et al., 2013). However, most of these conventional methods are highly energy-intensive processes and are of major economic and environmental concerns. Researchers have started to explore environmentally friendly hydrogen production alternatives such as biotechnological processes from renewable sources (Yasin et al., 2013).

2.2 Hydrogen production via dark fermentation

Dark fermentation refers to an incomplete biological degradation of organic material, where the final conversion of anaerobic digestion to methane and CO₂ is suppressed to produce hydrogen and VFAs. This process has gained increasing attention in the last decades as a

potential environmentally friendly and sustainable method to produce hydrogen from organic waste streams (Gupta et al., 2013). The performance of a dark fermentation process is determined by the HY (i.e. the conversion efficiency from glucose to hydrogen) and the HPR (i.e. the velocity of the process).

Both parameters are of the highest importance and require a further enhancement before dark fermentation can be cost-effectively applied at an industrial scale (Gupta et al., 2016; Karakashev and Angelidaki, 2011; Lee et al., 2011; de Vrije et al., 2007).

2.3 Challenges in dark fermentation

The process performance is affected by a large range of factors such as temperature, pH, microbial competition (for instance, in the presence of hydrogen consumers), end product inhibition, and substrate concentration. Comprehensive knowledge regarding the effect of these factors is crucial to keep them in an optimal range. Previous studies provide invaluable information and determine the current state of knowledge. For a further optimization, the factors currently limiting the hydrogen production must be identified to allow the implementation of counteracting measures.

Due to the complex nature of such a process, most factors are closely linked to each other rendering individual optimization difficult. For example, a variation of the substrate concentration will simultaneously influence the concentration of end products, the pH, the HPR which consequently affects the H_{2aq} (to name only the most important parameters).

2.4 Pure culture vs. mixed culture in dark fermentation

The efficiency of hydrogen production greatly depends on the microbial species used (Arimi et al., 2015). Either mixed or pure cultures can be applied, both providing specific benefits and drawbacks in dark fermentation.

In mixed culture cultivation, biohydrogen producers are subjected to competition. The presence of hydrogen-consuming bacteria, including methanogens, acidogens and sulfate-reducing anaerobes, as well as substrate consumption by non-hydrogen-producing microorganisms often results in a reduced HY (Arimi et al., 2015; Tapia-Venegas et al., 2015). Pretreatment is generally required to reduce the presence of unwanted species (Ghimire et al., 2015). On the other hand, mixed cultures are generally more practical for environmental

engineering applications, exhibiting a more stable operation, a more efficient degradation of complex substrates (Balachandar et al., 2013) and eliminating the necessity of costly mechanisms to prevent contamination (Ghimire et al., 2015).

In contrast, the major drawback of using a pure microbial culture is the risk of contamination under non-sterile conditions (Lee et al., 2011). However, the application of a single strain allows the targeted selection of an efficient hydrogen producer with regard of the substrate available. Furthermore, the use of a pure culture allows a customized adjustment of the operational conditions to direct its metabolism and promote an efficient conversion to hydrogen (Tapia-Venegas et al., 2015). From a scientific point of view, excluding the effect of competition and shifts in the microbial structure commonly occurring in mixed culture fermentations (Dessi et al., 2017; Yun and Cho, 2016) provides valuable information on the impact of the studied parameters on the process performance of the microorganism.

2.5 Production organism - *Thermotoga neapolitana*

Thermotoga neapolitana (briefly *T. neapolitana*) is a hyperthermophilic, strictly heterotrophic, strictly anaerobic, gram-negative bacterium obtained from a submarine thermal vent at Lucrino near Naples, Italy (Belkin et al., 1986; Huber and Hannig, 2006), which has revealed a great potential for hydrogen production through dark fermentation (Pawar and van Niel, 2013; Suellen A. Van Ooteghem et al., 2002). *Thermotogae* are rod-shaped cells with an outer sheath-like envelope ('toga') occurring worldwide in volcanically or geothermally heated environments. Hyperthermophilic cultures possess a superior ability to form hydrogen (Lee et al., 2011) partially caused by a decreased Gibbs free energy according to the second law of thermodynamics at elevated temperatures (Karakashev and Angelidaki, 2011).

Accordingly, *T. neapolitana* was selected for its ability to achieve HYs close to the theoretical 4 mol H₂/mol glucose (d'Ippolito et al., 2010; Elsharnouby et al., 2013, p. 4947; Pradhan et al., 2015, p. 6; Schröder et al., 1994; Thauer et al., 1977). The bacterium was attributed an efficient hydrolytic capacity (de Vrije et al., 2009) and is capable of metabolizing a wide range of substrates including glucose, fructose, maltose, starch (Huber and Hannig, 2006; Pradhan et al., 2015), xylose (Ngo et al., 2012), sucrose (Ngo et al., 2011b) but also more complex substrates like molasses (Frasconi et al., 2013), cheese whey (Cappelletti et al., 2012), algal biomass (Dipasquale et al., 2012), carrot pulp (de Vrije et al., 2010), *Miscanthus*

hydrolysate (de Vrije et al., 2009, p. 12) and glycerol (Eriksen et al., 2011). Furthermore, *T. neapolitana* does not suffer from catabolite repression and can simultaneously degrade and utilize different substrates (de Vrije et al., 2009, p. 12; Eriksen et al., 2008). The capability to tolerate low concentrations of oxygen has likewise been reported (Eriksen et al., 2008; Van Ooteghem et al.), whereas the risk of contamination is severely reduced due to the extreme cultivation temperature of 80°C (Nguyen et al., 2010, S39).

The discussed qualities and characteristics of *T. neapolitana* highlight its potential use as a suitable production organism for hydrogen via dark fermentation from organic waste streams (Eriksen et al., 2008; Pradhan et al., 2015). Its optimum range of temperature and pH, which can easily be controlled have been studied extensively were identified to be between 70 - 85 °C (Munro et al., 2009; Nguyen et al., 2008) and 7 and 7.5 (Ngo and Sim, 2012; Nguyen et al., 2008), respectively. However, up until now, *T. neapolitana* has been exclusively applied in batch or fed batch operation (Eriksen et al., 2008; Pradhan et al., 2015).

However, *T. neapolitana* grows in low biomass concentration which is commonly observed for hyperthermophilic cultures (Balachandar et al., 2013). The biomass concentration inside a reactor is in turn directly correlated to the production rate of the process. Hence, low HPRs were obtained in previous *T. neapolitana* studies (Pradhan et al., 2015) representing a major obstacle for an industrial application.

2.5.1 Metabolism

Fermentation is a catabolic process of organic material occurring in anaerobic conditions, which generates energy in form of ATP via substrate level phosphorylation. To maintain a redox balance inside the cell and allow the continuation of the fermentation, end products such as alcohols, VFAs and molecular H₂ are produced and excreted (Madigan et al., 2015). In mixed culture dark fermentation, the substrate is metabolized via numerous pathways producing a wide variety of end products in addition to the desired formation of hydrogen (Balachandar et al., 2013).

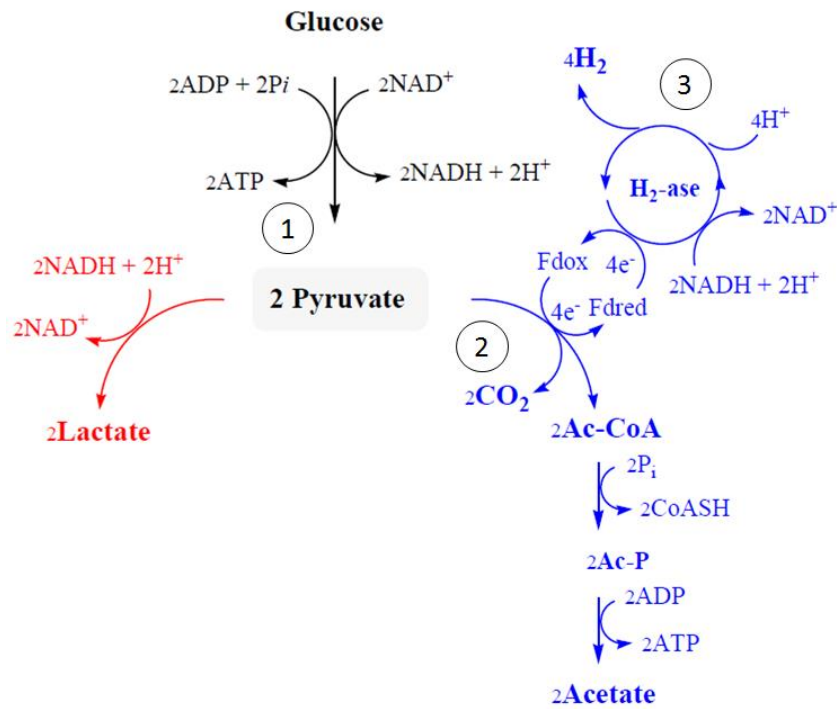
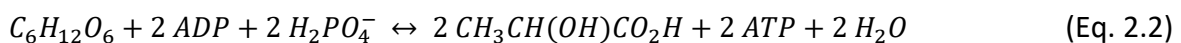
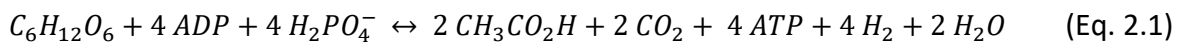


Fig. 2.1: Simplified biochemical pathway for dark fermentative H₂ production from glucose by *Thermotoga neapolitana* (Pradhan et al., 2015).

T. neapolitana uses 2 metabolic routes to a relevant extent in the degradation of glucose (Fig. 2.1). Energy is gained in the form of ATP when degrading glucose to pyruvate, transferring electrons to the electron carrier NADH (Pradhan et al., 2015). Subsequently NADH is recycled in either the AA pathway (Eq. 2.1), which yields 4 moles of hydrogen and 2 additional moles of ATP per mole glucose, or the LA pathway (Eq. 2.2) without the formation of hydrogen (Balachandar et al., 2013; Pradhan et al., 2015). Therefore, hydrogen is tightly linked to the production of AA in a molar ratio of 2 mol H₂/mol AA.



The energy required to recycle NADH, depicted by the Gibbs free energy under standard conditions (ΔG) is much higher in the AA pathway (+81 KJ/mol), transferring electrons to H₂ compared to the LA pathway (-25 KJ/mol) transferring electrons to LA (Balachandar et al., 2013). Although the AA pathway represents a higher energy gain for the microorganism, its

occurrence is determined by the thermodynamics of the reaction and will be replaced by the LA pathway if conditions are unfavorable to allow the fermentation to continue. Hence, high HYs can only be achieved when the reactor conditions allow the recycling of reducing equivalents via the AA pathway.

2.6 Operating mode: Continuous-flow

Hydrogen production has been studied extensively in batch, semi-continuous and continuous bioreactors (Show and Lee, 2013). Batch operation is usually more simple and requires less time, which makes it more suitable for research purposes, in particular for initial optimization studies (Balachandar et al., 2013; Elsharnouby et al., 2013; Ntaikou et al., 2010). However, a less efficient performance in batch compared to semi-continuous or continuous operation with lower HPRs is often reported (Show et al., 2011; Show and Lee, 2013). A continuous-flow process operation offers a more stable process and is generally preferred in applications at industrial scale, (Balachandar et al., 2013; Kumar et al., 2014; Ntaikou et al., 2010; Show et al., 2011; Show and Lee, 2013). The continuous process is externally controlled by the HRT and OLR (Arimi et al., 2015; Ntaikou et al., 2010; Sivagurunathan et al., 2016), as more in detail discussed in the following two sections.

2.6.1 Effect of Hydraulic retention time

HRT describes the mean residence time for a defined volume of substrate to pass through the reactor system before being discharged (Arimi et al., 2015) and is therefore defined by the working volume divided by the dilution rate.

A decrease of HRT, which entails an increase of OLR at constant feed substrate, is generally beneficial for hydrogen production. Numerous studies have reported an increase of HPR when decreasing the HRT (O-Thong et al., 2008; Palomo-Briones et al., 2017; de Vrije et al., 2007; Whang et al., 2011; Zhang et al., 2013a; Zhang et al., 2006). Furthermore, short HRTs may reduce reactor size and capital cost, by treating more substrate in the same period of time (Hawkes et al., 2007).

However, especially in CSTRs, where biomass is not artificially retained, the decrease of HRT is limited by washout of the active culture, when the dilution rate surpasses the specific growth rate (Ghimire et al., 2015; Gupta et al., 2016; Karakashev and Angelidaki, 2011). On

the other hand, washout can be used as a tool in mixed cultures dark fermentation to selectively wash out methane producers and other hydrogen consumers, which exhibit lower specific growth rates compared to hydrogen producers (Balachandar et al., 2013; Ghimire et al., 2015; Gupta et al., 2016; Hawkes et al., 2007; Palomo-Briones et al., 2017). The result is a microbial community rich in hydrogen-producing bacteria.

Also, the HY is highly influenced by the HRT (Arimi et al., 2015), whereas it is not entirely clear whether the impact is positive or negative. Several studies have reported diverging results with increasing yields with pure (Zhang et al., 2013a) and mixed (Palomo-Briones et al., 2017) cultures as well as decreasing yields with pure (de Vrije et al., 2007) and mixed (Kumar et al., 2014; Silva-Illanes et al., 2017) cultures, when decreasing the HRT. The effect of an HRT change depends on a range of factors, including the microbial culture as described in the previous paragraph, the gas-liquid mass transfer of the system as discussed in section 2.7.1 or the nature of the substrate (Ghimire et al., 2015). Therefore, a more profound understanding is important to allow optimization towards a more efficient hydrogen production.

2.6.2 Effect of Organic loading rate

The OLR refers to the amount of organic material per unit of reactor volume, which is subjected to degradation in the reactor in a period of time (Arimi et al., 2015). Consequently, the OLR is defined by the substrate feed concentration and the HRT of the process (Lin et al., 2012).

Higher OLRs are considered advantageous in industrial applications as they minimize the energy requirements for operation (Elbeshbishy et al., 2017; Jung et al., 2011). In addition, the increase of substrate concentration within a certain range generally improves the HPR in dark fermentation (Arimi et al., 2015; Elbeshbishy et al., 2017; Hawkes et al., 2007; Lin et al., 2012; Sivagurunathan et al., 2016). However, an excessive increase of the substrate concentration leads to an aggravation of several inhibitory factors including the accumulation of volatile fatty acids (VFAs), low pH, and high H₂ partial pressure (Elbeshbishy et al., 2017) until hydrogen production becomes unfavorable. These phenomena might also explain why higher HYs were generally obtained at lower substrate concentrations (Lin et al., 2012). Thus, similar to HRT, the ideal substrate concentration depends on a variety of parameters such as the microbial culture and the nature of the substrate (Lin et al., 2012).

2.7 Inhibitory parameters

Keeping potentially inhibitory factors within their optimum working range is very important in biotechnological production processes (Elbeshbishy et al., 2017). Apart from toxic compounds present in the substrate, the most common inhibitors of dark fermentation are the end products of the process, such as hydrogen (Ghimire et al., 2015) and VFAs (Arimi et al., 2015; Balachandar et al., 2013; Elbeshbishy et al., 2017). Naturally, any kind of inhibition needs to be prevented to increase the economic viability of dark fermentation. The effect of hydrogen and VFA accumulation on *T. neapolitana* have hardly been studied and require further investigation to keep them below inhibitory concentrations.

2.7.1 Hydrogen

Inhibitory mechanism

In the majority of hydrogen producing pathways, protons are reduced to elemental H₂ by an internal electron carrier (EC), namely NADH and reduced ferredoxin (Eq. 2.3) (Balachandar et al., 2013; Bundhoo and Mohee, 2016). This reaction is energetically challenging as protons are poor electron acceptors (E°_{H₂} = -414 mV) (Thauer et al., 1977; Verhaart et al., 2010). According to Le Chatelier's principle, the equilibrium of the reaction will shift to the left if the concentration H₂ increases (Mandal et al., 2006). In line, the impact of hydrogen on the Gibbs free energy change (ΔG') of this reaction is shown in (Eq. 2.4) (Verhaart et al., 2010). Hence, elevated concentrations of hydrogen thermodynamically restrain the reduction of protons to elemental H₂ (Eq. 2.3) (Balachandar et al., 2013; Bundhoo and Mohee, 2016; Verhaart et al., 2010).



$$\Delta G' = \Delta G'_0 + RT * \ln \left(\frac{[EC_{ox}][H_2]}{[EC_{red}][H^+]} \right) \quad (\text{Eq. 2.4})$$

ΔG'₀ is the Gibbs energy change under standard conditions (25°C, 1 atm and equal concentration of reactants and products) and R is the universal gas constant.

If the formation of hydrogen and, therefore, degradation via the AA pathway becomes thermodynamically unfavorable, the microorganisms shift their metabolism toward more

reduced end products (Balachandar et al., 2013; Mandal et al., 2006). This ensures the continuation of fermentation, however, with a reduced HY of the process as described in section 2.5.1. According to the rate law of chemical reactions, increasing the hydrogen concentration does not only shift the equilibrium balance of the reaction (Eq. 2.3) but also affects the rate at which it occurs.

Numerous studies have been conducted confirming the inhibition by hydrogen theoretically described above on the yield (Kraemer and Bagley, 2006; Ljunggren et al., 2011) and the rate of dark fermentation (Beckers et al., 2015; Kim et al., 2012; Kraemer and Bagley, 2006; Mandal et al., 2006; Nualsri et al., 2017; Obazu et al., 2012).

Hydrogen supersaturation

Due to its simple measurement, the hydrogen partial pressure in the gas phase has widely been used to study its effect on dark fermentation, assuming that liquid and gas phase hydrogen are in equilibrium as suggested by Henry's law (Balachandar et al., 2013; Sivagurunathan et al., 2016). However, hydrogen in the liquid phase (H_{2aq}) is the factor which directly acts on and inhibits the microbial culture. The concentration of H_{2aq} has thereby shown to supersaturate, reaching concentrations multiple-fold surpassing the equilibrium depending on the gas-liquid mass transfer (Pauss et al., 1990; Beckers et al., 2015; Kraemer and Bagley, 2006). Consequently, numerous studies have identified the H_{2aq} concentration a crucial parameter to be considered in dark fermentation (Gupta et al., 2016; Kraemer and Bagley, 2006; Ljunggren et al., 2011; Zhang et al., 2013b).

Counteracting measures to prevent inhibition by hydrogen

Even though many studies did not measure nor consider H_{2aq} , numerous studies have successfully applied a variety of techniques counteracting the inhibition by hydrogen (Sivagurunathan et al., 2016). Most commonly, the hydrogen partial pressure was reduced by headspace sparging with N_2 (Kraemer and Bagley, 2006) or CO_2 (Kim et al., 2012), but also increasing the area for gas exchange (Beckers et al., 2015), higher agitation (Zhang et al., 2012), reducing the total reactor pressure (Mandal et al., 2006), bubble induction (Fritsch et al., 2008; Sonnleitner et al., 2012) and recirculating H_2 -rich biogas (Bakonyi et al., 2017) proved to be effective. Independent from the method applied, counteracting hydrogen inhibition generally leads to an improvement of the HYs and/or HPRs.

However, only some of those measures can realistically be applied in an industrial process. Sparging with inert gases results in the dilution of the produced gas, which subsequently requires a more intense down streaming process and increases the production costs (Sonnleitner et al., 2012). High agitation and a decreasing reactor pressure below ambient pressure increase the operating costs and are challenging to be implemented at larger scale. On the other side, the recirculation of H₂-rich biogas and the application of solid elements inside the reactor to induce heterogeneous nucleation, appear more promising. H₂-rich biogas recirculation provides a higher surface area for an increased gas-liquid mass transfer without biogas dilution, whereas heterogeneous nucleation decreases the energy required for the formation of bubbles and thereby reduces the degree of supersaturation.

2.7.2 Feed substrate and VFAs

As indicated in 2.6.2, an increase of the substrate concentration surpassing the beneficial range for hydrogen production leads an inhibitory effect (Ciranna et al., 2014; Elbeshbishy et al., 2017; Jung et al., 2011; Sivagurunathan et al., 2016). In continuous-flow operation, a direct impact of substrate concentration can be excluded as it is low in a functioning process as a consequence of degradation inside the reactor. However, due to this conversion, the increase of substrate concentration inevitably entails an enhanced formation of fermentation end products, primarily VFA in dark fermentation. As a result, feedback inhibition, is widely considered to be responsible for inhibition at high substrate concentrations (Boodhun et al., 2017; Elbeshbishy et al., 2017), acting on the HY as well as the HPR (Tang et al., 2012). The inhibition by VFA includes several mechanisms such as inhibition by undissociated acids or ionic strength as well as a thermodynamic limitation according Le Chatelier's principle as described for hydrogen in section 2.7.1 (Jones et al., 2017). Furthermore, the production of VFA and LA can lead to a drop of pH below the operating range of the culture. However, inhibition by pH is generally counteracted by the addition of buffers or bases or the selection of an acidophilic culture. On the contrary, preventing the buildup of fermentation end products in the fermentation broth is challenging and cost-intensive. Hence, it is essential to find the most suitable substrate concentration allowing an efficient hydrogen production and preventing inhibition.

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3 Enhancement of hydrogen production rate by high biomass concentrations of *Thermotoga neapolitana*

(Paper I)

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Abstract

The objective of this study was to enhance the hydrogen production rate of dark fermentation in batch operation. For the first time, the hyperthermophilic pure culture of *Thermotoga neapolitana* cf. *capnolactica* was applied at elevated biomass concentrations. The increase of the initial biomass concentration from 0.46 to 1.74 g cell dry weight/L led to a general acceleration of the fermentation process, reducing the fermentation time of 5 g glucose/L down to 3 h with a lag phase of 0.4 h. The volumetric hydrogen production rate increased from 323 (\pm 11) to 654 (\pm 30) mL/L/h with a concomitant enhancement of the biomass growth and glucose consumption rate. The hydrogen yield of 2.45 (\pm 0.09) mol H₂/mol glucose, the hydrogen concentration of 68% in the produced gas and the composition of the end products in the digestate, i.e. 62.3 (\pm 2.5)% acetic acid, 23.5 (\pm 2.9)% lactic acid and 2.3 (\pm 0.1)% alanine, remained unaffected at increasing biomass concentrations.

Keywords: Hydrogen; *Thermotoga neapolitana*; Biomass concentration; Dark fermentation; Hyperthermophilic;

Abbreviations

CDW	Cell dry weight [g/L]
HPR	Hydrogen production rate
AA	Acetic acid
LA	Lactic acid

3.1 Introduction

Hydrogen (H₂) is a non-polluting and clean fuel of the future with a wide range of applications [1,2]. The demand for hydrogen is continuously increasing and expected to contribute 8–10% to the energy market by 2025 [1]. Biotechnological hydrogen production processes have advanced in recent years and revealed promising results for an environmentally friendly production route [1,3]. Dark fermentation is thereby considered as the most attractive process due to its simplicity, independence of light and the potential to use substrates from renewable sources. However, the low H₂ production rate observed in dark fermentation still remains a fundamental challenge [4–6]. The hydrogen production rate (HPR) is crucial for the production at industrial scale [7] and a considerable increase is required for the establishment of an economically viable process [8,9].

Thermotoga neapolitana is a hyperthermophilic organism with a great potential for hydrogen production through dark fermentation [10]. This bacterium grows on a wide range of substrates including glucose, fructose, xylose, maltose, starch, glycogen, glycerol, molasses, cheese whey, algal biomass and carrot pulp [11–15]. The microorganism has fast growth kinetics [16], oxygen tolerance [14] and low contamination risks due to the extreme culture conditions at 80°C [17]. Previous studies using *Thermotoga neapolitana* have primarily focused on the optimization of the hydrogen yield in batch fermentation [14,18–20]. Yields approaching the theoretical 4 mol H₂/mol glucose have been reached when applying 5 – 10% of inoculum (v/v) [2,14,21]. Promising results of *Thermotoga neapolitana* in attached growth [22–24] further emphasize the potential of the organism, indicating towards a possible use in a biofilm reactor system. However, low HPRs between 23 and 50 mL/L/h observed in simple batch cultivation using *Thermotoga neapolitana* [14] demonstrate the need for further development.

Despite the lower H₂ yields, the highest HPRs are currently reached under mesophilic conditions due to their capability to grow in high biomass concentrations [25]. A positive correlation was demonstrated between the HPR and the biomass concentration in the reactor in continuous dark fermentations [6]. Hyperthermophilic cultures reach much higher hydrogen yields [26], but they commonly grow in low cell densities [5] leading to low H₂ production rates. A substantial improvement of the HPR is expected if hyperthermophilic cultures are cultivated at high biomass concentrations [25,27]. In batch cultivation of

suspended cell, high biomass concentrations are generally implemented by the recycling of biomass, which enables a faster and more robust process [27].

In the present study, a suspended culture of pure *Thermotoga neapolitana* biomass was used at different concentrations ranging from 0.46 – 1.74 g/L to mimic a sequential batch fermentation described by Basso et al. [28]. The main objective was to induce an acceleration of the dark fermentation process, in particular the HPR. Simultaneously, the effect of elevated biomass concentrations on the efficiency of the process and the kinetics of hydrogen production, glucose consumption and biomass growth were evaluated.

3.2 Material and Methods

3.2.1 Culture medium

A modified ATCC 1977 culture medium as described by d'Ippolito et al. [19] was used for the cultivation of *Thermotoga neapolitana* containing the following components (in g/L): 10 NaCl; 5 glucose; 2 yeast extract; 2 tryptone; 1 cysteine; 1 NH₄Cl; 0.3 K₂HPO₄; 0.3 KH₂PO₄; 0.2 MgCl₂·6 H₂O; 0.1 KCl; 0.1 CaCl₂·2 H₂O; 0.001 resazurin dissolved in distilled water, supplemented with 10 mL/L of vitamin and 10 mL/L of trace element solutions (DSM medium 141).

3.2.2 Bacterial strain – Cultivation and storage

A pure culture of *Thermotoga neapolitana* cf. *capnolactica* [29] (hereafter briefly *Thermotoga neapolitana*) was used in all experiments. For the conservation of the culture, 120 mL serum bottles containing 25 mL of culture medium were prepared. Prior to the inoculation, the medium was heated to remove excess oxygen until losing the characteristic resazurin color, sealed immediately with butyl rubber stoppers and sterilized by autoclaving for 5 min at 110°C [19]. The medium was inoculated using 6% (v/v) of stored cell cultures. After the cultivation at 80°C without agitation overnight, the grown culture was stored at 4°C [30].

3.2.3 Preparation of inoculum

The inoculum was produced in two 3 L continuously stirred tank reactors (Applikon Biotechnology, The Netherlands) each containing 2000 mL of culture medium. The medium was heated to 80°C and sparged with CO₂ for 5 min to remove the dissolved oxygen. Subsequently, the pH was adjusted to 7 by addition of 1 M NaOH and the reactors were inoculated using 6% v/v of stored culture (described in 2.2). The cultivation was performed at 80°C and 200 rpm for 14.5 h to obtain a culture at the end of the stationary phase where the amount of active biomass reaches its maximum. The biomass was harvested by centrifugation at 3750 rpm for 15 min and resuspended in an isotonic solution (10 g/L NaCl in distilled water) to produce a highly concentrated inoculum (1 mL of inoculum contains biomass of 50 mL of grown culture). The biomass concentration in g CDW/L of each sampling point was estimated from the optical density at 540 nm (OD₅₄₀) using the relation $CDW [g/L] = 0.27 * OD_{540} - 0.06$ (R² = 0.98) [20,31].

3.2.4 Experimental design

Eight 250 mL Schott flasks, each containing 200 mL of culture medium were used to investigate the hydrogen production from a *Thermotoga neapolitana* culture at increasing biomass concentrations. Prior to inoculation, the medium was heated to 80°C, sparged with CO₂ for 5 min to remove oxygen and the pH adjusted to 7 by addition of 1 M NaOH, being the optimum pH for hydrogen production by *Thermotoga neapolitana* [32]. A volumetric ratio of inoculum to culture medium (v/v) is commonly used to describe the amount of inoculum applied. Considering this ratio, the concentrated inoculum (described in 2.3) was used to inoculate the reactors between 100 and 400% v/v (100% corresponding to biomass harvested from 200 ml of grown culture in 200 ml of fresh medium): C1 - 100% (= 0.46 g CDW/L), C2 - 200% (= 0.91 g CDW/L), C3 - 300% (= 1.33 g CDW/L) and C4 - 400% (= 1.74 g CDW/L). The medium was maintained at 80°C applying 300 rpm agitation by magnetic stirring (STIRRING DRYBATH 15-250, 2mag AG, Germany). 1.5 mL of liquid samples were taken at 1 h (C3, C4) and 30 min (C1, C2) intervals. The pH was manually adjusted to 7 by after each sampling. The produced gas was released continuously and quantified with 500 mL water displacement systems. The fermentation was completed when the gas production

stopped, and the pH remained constant. Each experimental condition was conducted in duplicate.

3.2.5 Analytical Methods

Liquid samples were centrifuged at 10000 rpm for 5 min to collect the supernatant for the determination of glucose, acetic acid (AA), lactic acid (LA) and alanine concentrations. The glucose concentration was measured by the dinitrosalicylic acid method calibrated on a standard solution of 1 g/L [33]. AA, LA and alanine were quantified by ^1H Nuclear Magnetic Resonance (NMR) on a 600 MHz spectrometer (Bruker Avance 400) equipped with a Cryoprobe using 3.8 mM trimethylamine hydrochloride (TMA) as internal standard [34]. The biomass concentration was determined by measuring optical density at 540 nm (OD_{540}) (UV/Vis spectrophotometer DU 730, Beckman Coulter Inc, Brea, USA) of the liquid samples and the CDW via lyophilization after the completion of the fermentation. For this purpose, 200 mL of culture broth was centrifuged at 3750 rpm for 20 min. The pellet was subsequently resuspended in 25 mL of 10 g/L NaCl in distilled water and centrifuged at 6000 rpm for 20 min to remove remaining media components. The pellet was stored at -20°C and lyophilized overnight.

Produced gas was quantified by water displacement using 500 mL glass containers. At the end of each experiment, the H_2 -containing gas was sampled and analyzed by gas chromatography as described by Dipasquale et al. [13]. The molar H_2 production was calculated using the ideal gas law [7]. The hydrogen concentration in the produced gas was calculated considering a dilution of the measured gas with the CO_2 initially in the headspace of the reactor.

3.2.6 Kinetic study of glucose consumption, biomass growth and biohydrogen production

To evaluate and compare glucose consumption, biomass growth and hydrogen production at the different biomass concentrations investigated, models based on the Gompertz equation [14,35] were applied to fit the experimental data and calculate the kinetic rates and lag phases. To validate the suitability of the modified Gompertz model, the models of glucose consumption, biomass growth and hydrogen production were plotted against the experimental data. The quality of the fitting was determined by calculating the coefficients

of determination (R^2). The specific rates were calculated referring the volumetric rates to the initial biomass concentration of each experimental condition.

For glucose consumption, Eq. 3.1 was applied, where G [g/L] is the glucose concentration at fermentation time t [h]; G_0 [g/L] is the glucose concentration at time 0 h; G_m [g/L] is the glucose consumed throughout the fermentation; R_G is the volumetric glucose consumption rate [g/L/h]; λ_G is the lag phase of glucose consumption [h]; and e is the Euler's number, i.e. 2.72.

For biomass growth, Eq. 3.2 was applied, where B [g CDW/L] is the biomass concentration at fermentation time t [h]; B_0 [g CDW/L] is the biomass concentration at time 0 h; B_m [g CDW/L] is the gain of biomass concentration throughout the fermentation; R_B is the volumetric growth rate [g CDW/L/h]; and λ_B is the lag phase of biomass growth [h].

Eq. 3.3 was applied for hydrogen production, with H [mL] being the cumulative hydrogen at time t [h]; H_m [mL] the hydrogen produced throughout the fermentation; R_H [mL/L/h] the volumetric HPR; and λ_H the lag phase of hydrogen production [h]. For the calculation of the HPR, the gas remaining in the headspace of the reactor at the end of the fermentation was equally distributed throughout the length of the batch experiment.

$$G = G_0 - G_m \exp \left\{ -\exp \left[\frac{R_G e}{G_m} (\lambda_G - t) + 1 \right] \right\} \quad (\text{Eq. 3.1})$$

$$B = B_0 + B_m \exp \left\{ -\exp \left[\frac{R_B e}{B_m} (\lambda_B - t) + 1 \right] \right\} \quad (\text{Eq. 3.2})$$

$$H = H_m \exp \left\{ -\exp \left[\frac{R_H e}{H_m} (\lambda_H - t) + 1 \right] \right\} \quad (\text{Eq. 3.3})$$

Table 3.1: Hydrogen yield, cumulative hydrogen, final biomass and biomass yield fermenting 5 g/L glucose at different initial biomass concentrations of *Thermotoga neapolitana*.

	Initial biomass [g CDW /L]	Hydrogen yield [mol H ₂ /mol glucose]	Cumulative hydrogen [mL/L]	Final biomass [g CDW/L]	Biomass yield [g CDW/g glucose]
C1	0.46	2.39	1462 (± 12)	1.10 (± 0.03)	0.14
C2	0.91	2.44	1477 (± 3)	1.43 (± 0.09)	0.12
C3	1.33	2.58	1516 (± 10)	1.89 (± 0.06)	0.13
C4	1.74	2.37	1456 (± 5)	2.10 (± 0.15)	0.08

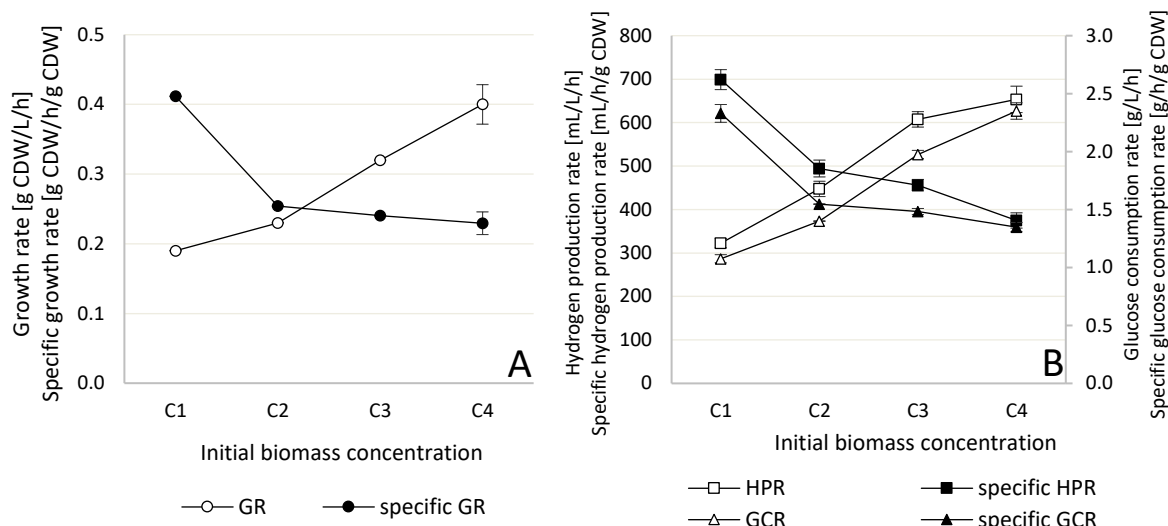


Fig. 3.1: A - Volumetric and specific growth rate; B - Volumetric as well as specific glucose consumption and hydrogen production rate at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. Specific rates were calculated per g initial cell dry weight. (GR – Growth rate; HPR – Hydrogen production rate; GCR – Glucose consumption rate). Error bars depict the standard deviation.

3.3 Results and Discussion

3.3.1 Hydrogen yield and production rate

A change of the initial biomass concentration between 0.46 and 1.74 g/L did not affect the hydrogen yield i.e. 2.45 (\pm 0.09) mol H₂/mol glucose (Table 3.1) or the composition of the biogas that maintained a constant level of hydrogen at 67.6 (\pm 2.4)% (data not shown). The HPR observed at the lowest initial biomass concentration of 0.46 g CDW/L (C1) reached 323 (\pm 11) mL/L/h (Table 3.2). A further increment of the biomass concentration to 0.91 (C2), 1.33 (C3) and 1.74 g CDW/L (C4) increasingly enhanced the volumetric HPR to 448 (\pm 18), 608 (\pm 18) and 654 (\pm 30) mL/L/h (Fig. 3.1B; Table 3.2), respectively. On the whole, a fourfold expansion of the biomass concentration caused an approximately twofold increase of the volumetric production rate. This is consistent with previous studies on dark fermentation by anaerobic sludge in closed serum bottles, where a general increase of HPR was obtained by raising the biomass concentrations [36,37]. On the other hand, Ngo and coworkers reported a reverse correlation between the two parameters in a fed batch process using *T. neapolitana*. In 4 feeding cycles, the authors observed an increase of the initial biomass from

1.3 ± 0.1 to 2.4 ± 0.1 g/L yielding a mild reduction of HPR from 114 to 106 mL/L/h [32]. Simultaneously, acetic acid and lactic acid accumulated in the fermentation broth up to 123 ± 7 and 28 ± 1 mM, respectively, which could potentially have caused an inhibitory effect. While hyperthermophilic cultures are capable to achieve even higher hydrogen yields (Table 3.3) the volumetric HPR observed in this work exceeds those achieved in similar studies using pure hyperthermophilic cultures at low biomass concentrations (Table 3.3). The highest production rate of 654 (± 30) mL/L/h (C4) (Table 3.2) depicts a roughly 13-fold increase to the maximum production rate of 50 mL/L/h achieved in similar experiments using *Thermotoga neapolitana* in batch cultures with 6% (v/v) of inoculum and 5 g/L of glucose as a substrate [19]. Only two studies achieved HPRs in a similar range. Mars et al. [31] and de Vrije et al. [11] reached production rates of 269 and 304 mL/L/h, respectively, by continuously flushing the reactor headspace with N₂ gas at 7 L/h both using 10% (v/v) of preculture as inoculum and 10 g glucose/L as a substrate. While gas sparging is a common method to counteract hydrogen inhibition [25,44], it is an unsatisfying solution due to a manifold dilution of the produced hydrogen gas. The resulting need to separate the sparging gas from the hydrogen creates additional operating cost [45].

Table 3.2: *Thermotoga neapolitana* cultivated on 5 g/L of glucose as a main substrate using different initial biomass concentrations. Rates and lag phase determined through curve fitting to a modified Gompertz model. Fit quality illustrated through coefficient of determination (R^2).

	Initial biomass	Volumetric rate	Specific rate	Lag phase	R^2
Hydrogen production					
	[g CDW/L]	[mL/L/h]	[mL/h/g initial CDW]	[h]	
C1	0.46	323 (± 11)	699 (± 23)	1.50 (± 0.07)	0.99
C2	0.91	448 (± 18)	494 (± 20)	0.79 (± 0.01)	0.99
C3	1.33	608 (± 18)	456 (± 13)	0.52 (± 0.01)	0.99
C4	1.74	654 (± 30)	375 (± 17)	0.40 (± 0.02)	0.99
Biomass growth					
	[g CDW/L]	[mg CDW/L/h]	[mg CDW/h/g initial CDW]		
C1	0.46	190 (± 0)	412 (± 0)		0.99
C2	0.91	230 (± 0)	254 (± 0)		0.98
C3	1.33	320 (± 0)	240 (± 0)		0.96
C4	1.74	400 (± 28)	229 (± 16)		0.98
Glucose consumption					
	[g CDW/L]	[g Glucose/L/h]	[g Glucose/h/g initial CDW]		
C1	0.46	1.08 (± 0.04)	2.33 (± 0.08)		0.99
C2	0.91	1.40 (± 0.00)	1.55 (± 0.00)		0.98
C3	1.33	1.98 (± 0.04)	1.48 (± 0.03)		0.98
C4	1.74	2.35 (± 0.07)	1.35 (± 0.04)		0.99

Table 3.3: Hydrogen production rate and hydrogen yield of selected studies of batch fermentations by various hyperthermophilic pure cultures using glucose as a substrate.

Microorganism	Hydrogen production rate [mL/L/h]	Hydrogen yield [mol H ₂ /mol glucose]	Reference
<i>Caldicellulosiruptor saccharolyticus</i>	264	2.5	[38]
	296	3.4	[31]
	277	3.2	[11]
<i>Thermotoga elfi</i>	200	3.3	[39]
<i>Thermotoga maritima</i>	170	4	[40]
<i>Thermotoga neapolitana</i>	304	2.9	[31]
	269	3.5	[11]
	21	3.9	[18]
	252	1.8	[20]
	50	3.9	[19]
<i>Thermoanaerobacter mathranii</i> A3N	100	2.6	[41]
<i>Thermoanaerobacterium</i>	287	2.4	[42]
<i>thermosaccharolyticum</i> PSU-2			
<i>Thermoanaerobacterium</i>	309	2.4	[43]
<i>thermosaccharolyticum</i> W16			
<i>Thermotoga neapolitana</i>	654	2.5	Present study

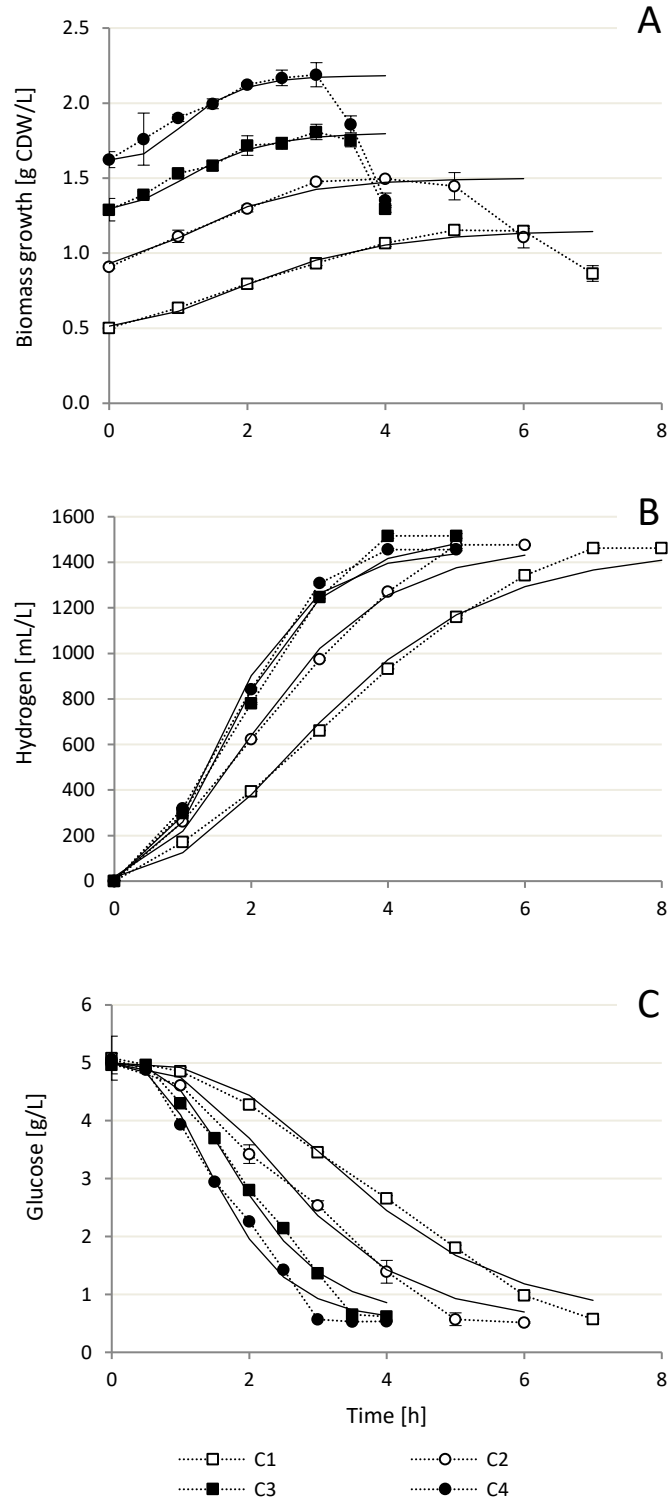


Fig. 3.2: A – Biomass growth; B – Cumulative hydrogen and C – Glucose consumption production at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. The solid line represents the fitting to the Gompertz model. Error bars depict the standard deviation.

3.3.2 Volumetric and specific rates of glucose consumption, biomass growth and hydrogen production

Volumetric (per L of working volume) and specific (per g CDW) glucose consumption, biomass growth and HPRs (Fig. 3.1, Table 3.2) were calculated via the Gompertz model (described in 2.6)(Fig. 3.2). The increase of the volumetric HPR with increasing biomass concentrations was coupled to a general acceleration of the process, indicated by an increase of the volumetric biomass growth and glucose consumption rate (Table 3.2). The calculation of the Pearson correlation coefficient confirmed a positive linear correlation of volumetric glucose consumption rate and volumetric growth rate ($r=0.99$) as well as volumetric HPR ($r=0.99$). In contrast, all specific rates (Fig. 3.1, Table 3.2) exhibited a decreasing trend with increasing biomass concentrations. This suggests that the overall process was considerably accelerated, while the individual cells were partially repressed at increased biomass concentration. The decrease in specific rates was particularly distinct between C1 and C2. The specific glucose consumption rate (g glucose/h/g CDW) dropped by 34% from 2.33 (± 0.08) of C1 to 1.55 (± 0) of C2 and by 9% to 1.35 (± 0.04) of C4 (Table 3.2). The specific growth rate (mg CDW/h/g CDW) decreased by 38% from 412 (± 0) of C1 to 254 (± 0) of C2 and merely by 6% to 229 (± 16) of C4 (Table 3.2). The specific HPR (mL/h/g CDW) decreased by 29% from 699 (± 23) of C1 to 494 (± 20) of C2 and by 17% to 375 (± 17) of C4 (Table 3.2). A similar trend of decreasing specific HPR was observed in previous studies when the initial biomass was increased using mixed cultures [36,37]. Kargi et al. [37] observed a drop of the specific HPR from 48 to approximately 3 mL/h/g when the biomass concentration was increased from 0.48 to 2.88 g/L. Substrate limitation caused by flock formation [37] and hydrogen consuming homo-acetogenic bacteria [36] were presumed to be responsible for the decrease of specific HPR at higher initial biomass concentrations.

In the present study, the formation of flocks was not observed, and hydrogen was not consumed, indicated by the ratio of hydrogen to acetic acid being consistent with the dark fermentation model Eq. 3.4. However, an increase of HPR induces the accumulation of hydrogen in the liquid phase which mainly depends on the HPR and the mass transfer rate of the system [46]. The concentration of liquid phase hydrogen can reach multiple fold the equilibrium concentration suggested by Henry's Law even in hyperthermophilic stirred reactor systems [46,47], acting as a potent inhibitor of hydrogen production by dark fermentation [44,48]. In the present study, the accumulation of hydrogen was observed at

the highest biomass concentration (C4) where the highest HPR was obtained. After 3 h of fermentation, the glucose consumption of C4 was completed (Fig. 3.2C), while hydrogen continued to be produced by the reactor (Fig. 3.2B), due to the transport of accumulated hydrogen from the liquid to the gas phase. According to Ljunggren et al. [46], cultures of *Caldicellulosiruptor saccharolyticus* respond to increasing concentrations of liquid phase hydrogen by adjusting the specific growth rate to reduce the HPR and prevent hydrogen from reaching inhibitory concentrations. Similarly, a reduction of specific HPRs was observed in the presented study when the volumetric production rates increased. This is supported by the results obtained in dark fermentation by *Thermotoga neapolitana*, achieving the highest hydrogen yields and production rates, when hydrogen was removed through headspace sparging with N₂ continuously [11,31] or in regular intervals [19].

3.3.3 Fermentation time and lag phase

The lag phase was determined via the Gompertz model, while the fermentation time was estimated from the moment of inoculation to the completion of the fermentation. An initial biomass concentration of 0.46 g CDW /L (C1) induced a lag phase of 1.50 (\pm 0.07) h (Table 3.2) and the completion of the fermentation within approximately 7 h (Fig. 3.2). In previous studies, the duration of the batch fermentation with *Thermotoga neapolitana* was longer than 18 h [11,23,31] when applying a 5-10% (v/v) inoculum. By increasing the initial biomass concentration to 1.74 g CDW /L (C4), the fermentation time and the lag phase were reduced to approximately 3 h (Fig. 3.2) and 0.4 (\pm 0.02) h (Table 3.2), respectively. This is in agreement with observations made in bioethanol production plants [28], where yeast cultures were recycled at high densities in sequential batch fermentations resulting in the reduction of the fermentation time and the unproductive lag phase [27]. In all conditions, independent from the initial biomass concentration a substrate consumption of 88.8 (\pm 0.9)% (data not shown) was reached at the end of the fermentation.

3.3.4 Biomass production

The biomass concentration at the end of a batch fermentation is defined by the initial biomass concentration, the biomass yield (g CDW/g glucose) and the amount of glucose converted. A similar formation of biomass was observed using initial biomass concentrations

between 0.46 (C1) and 1.33 (C3), with a biomass yield ranging between 0.12 - 0.14 g CDW/g glucose (Table 3.1). In contrast, at the highest biomass concentration of 1.74 g CDW/L (C4) the biomass production was lower, corresponding to a biomass yield of 0.08 g CDW/g glucose (Table 3.1), similar to what observed in previous studies with lower inoculum concentrations of *Thermotoga neapolitana*. De Vrije et al. [11] and Mars et al. [31] reported biomass yields of 81.6 and 87.1 mg CDW/ g glucose, respectively, using 10% inoculum (v/v) with additional headspace sparging. Ngo et al. [23] obtained 0.71 (\pm 0.04) g CDW/ L with 10 g/L pure glycerol as main substrate and 10% of inoculum (v/v). Van Niel et al. [39] reached 0.89 g CDW/L by fermenting 10 g/L glucose with 10% inoculum of *Thermotoga elfi*. At the end of each fermentation *Thermotoga neapolitana* is morphologically adapting to the nutrient limitation causing the OD₅₄₀ to decrease (Fig. 3.2) [49], while the CDW remains high (Table 3.1). Consequently, the curve fitting to the Gompertz model for biomass growth was done exclusively until the highest value of turbidity was reached (Fig. 3.2).

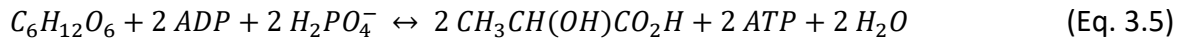
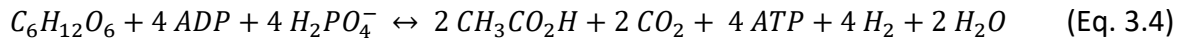
Table 3.4: Composition of broth after completed fermentation of 5 g/L glucose by different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g cell dry weight/L) of *Thermotoga neapolitana*.

	AA [mM] {yield [mol/mol glu]}	LA [mM] {yield [mol/mol glu]}	Ratio LA / AA	Alanine [μ M] {yield [mol/mol glu]}	Glucose [mM]
C1	34.3 (\pm 0.6) {1.39}	10.9 (\pm 0.4) {0.44}	0.32	1190 (\pm 27) {0.05}	3.2 (\pm 0.04)
C2	32.9 (\pm 0.8) {1.32}	12.2 (\pm 0.8) {0.49}	0.37	1260 (\pm 1) {0.05}	2.9 (\pm 0.06)
C3	32.3 (\pm 0.2) {1.33}	11.5 (\pm 0.5) {0.47}	0.36	1220 (\pm 18) {0.05}	3.4 (\pm 0.01)
C4	31.4 (\pm 1.1) {1.27}	14.7 (\pm 0.7) {0.59}	0.47	1260 (\pm 43) {0.05}	3.0 (\pm 0.04)

AA - acetic acid; LA - lactic acid; glu – glucose;

3.3.5 Production of fermentation products

The distribution of end products obtained in the digestate (62.3 (\pm 2.5)% AA; 23.5 (\pm 2.9)% LA; 2.3 (\pm 0.1)% alanine) was similar in the range of biomass concentrations investigated (Table 3.4), corresponding to yields of 1.33 (\pm 0.05) mol/mol glucose for AA, 0.50 (\pm 0.06) mol/mol glucose for LA and 0.05 (\pm 0.001) mol/mol glucose for alanine (Table 3.4). As observed in previous studies [14], *Thermotoga neapolitana* primarily ferments glucose via the hydrogen producing acetic acid (Eq. 3.4) or the lactic acid pathway (Eq. 3.5).



Considering a production of 2 mol of end product per mol of glucose (Eq. 3.4 and Eq. 3.5), an average of 95 (± 1)% (data not shown) of the initial substrate could be accounted for in the fermentation broth (Table 3.4). Similar results were obtained by Mars et al. [31] and de Vrije et al. [11] when low inoculum concentrations were used, reporting AA yields of 1.6 and 1.4 mol/ mol glucose and LA yields of < 0.1 and 0.03 mol/ mol glucose, respectively. The highest initial biomass concentration (C4) showed a slightly higher LA/ AA ratio (Table 3.4) coupled with a slightly lower hydrogen yield (Table 3.1). This is in accordance to the dark fermentation model suggesting a negative correlation between hydrogen yield and LA/AA ratio [48].

Additionally, an impact of capnophilic lactic acid fermentation caused by sparging with CO₂ needs to be considered resulting in elevated amounts of LA without significant loss in hydrogen yield [30,34]. Dipasquale et al. [30] observed LA/AA ratio of 0.28 by N₂ sparging and 0.56 by CO₂ sparging twice throughout the batch experiment. LA/AA ratios between 0.32 and 0.47 observed in this study suggest a similar influence of capnophilic lactic acid fermentation.

Conclusion

This study identified the use of high initial biomass concentrations of *Thermotoga neapolitana* (from 0.46 to 1.74 g cell dry weight/L) as a suitable method to accelerate the dark fermentation process and increase the HPR. The four-fold increase in biomass concentration led to the consumption of 5 g/L of glucose within 3 h and accelerated the hydrogen production rate by approximately 50% reaching a maximum of 654 (± 30) mL/L/h. The variation of the biomass concentration had no effect on the yield (2.45 (± 0.09) mol H₂/mol glucose), the concentration of hydrogen in the produced gas (68%) or the composition of fermentation end products (i.e. 62.3 (± 2.5)% AA, 23.5 (± 2.9)% LA and 2.3 (± 0.1)% alanine). To continue optimizing the rate of dark fermentation processes more research is required to understand the role of hydrogen in the liquid phase as an inhibitor at elevated hydrogen production rates. Furthermore, economical and environmentally friendly

substrates like organic waste streams need to be investigated for their suitability in large-scale applications of the proposed process.

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Conflicts of interest: none.

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4 H₂-rich biogas recirculation prevents hydrogen supersaturation and enhances hydrogen production by *Thermotoga neapolitana* cf. *capnolactica*

(Paper II)

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Abstract

This study focused on the inhibitory effect of hydrogen in the liquid phase (H_{2aq}) on dark fermentation by *Thermotoga neapolitana* cf. *capnolactica*. We investigated the impact of agitation (from 100 to 500 rpm) and recirculation of H_2 -rich biogas (GaR) on the accumulation of H_{2aq} . At low cell concentrations, both 500 rpm and GaR reduced the H_{2aq} from 30.1 (\pm 4.4) mL/L to the lowest values of 7.4 (\pm 0.7) mL/L and 7.2 (\pm 1.2) mL/L, respectively. However, when the rate of the process was increased by using high cell concentrations (0.79 g CDW/L), only GaR was successful in preventing the inhibition by H_{2aq} . This was revealed by the 271% increase in hydrogen production rate when GaR was added at 300 rpm, compared to a 136% increase when the agitation was increased from 300 to 500 rpm. While H_{2aq} primarily acted on the rate of dark fermentation, GaR concomitantly increased the hydrogen yield up to 3.5 mol H_2 /mol glucose. Hence, our results show that H_{2aq} supersaturation highly depends on the gas-liquid mass transfer and the hydrogen production rate of the system and can strongly inhibit both the rates and yields of dark fermentation. Furthermore, we demonstrated for the first time that GaR is an efficient counteracting measure with high potential to improve the fermentative production of hydrogen.

Key words: *Thermotoga neapolitana*; Dark fermentation; Gas recirculation; Sparging; Hydrogen supersaturation; End product inhibition

Declarations of interest: none

Abbreviations

AA	acetic acid
BGR	biomass growth rate
BMV	biomass yield
CSTR	continuously stirred tank reactor
GaR	recirculation of H_2 -rich biogas
GCR	glucose consumption rate
H_{2aq}	liquid phase hydrogen
HPR	hydrogen production rate
HY	hydrogen yield
LA	lactic acid

4.1 Introduction

Hydrogen, a nonpolluting energy carrier can be produced from organic residues in an environmental-friendly biological process called dark fermentation [1,2]. Hydrogen production has been reported in a wide range of temperatures, with thermophilic conditions being generally correlated to higher hydrogen yields (HY) [1,2]. *Thermotoga neapolitana* (hereafter *T. neapolitana*) is a hyperthermophilic microorganism with a high potential for hydrogen production via dark fermentation [3,4]. Some of the key characteristics exhibited by this organism include fast growth kinetics [4], a reduced risk of contamination [5] and efficient degradation of a wide range of substrates, e.g. glucose, fructose, xylose, maltose, starch [4,6], molasses and cheese whey [7], algal biomass [8], and carrot pulp [9]. Moreover, *T. neapolitana* is capable to approach the theoretical HY of 4 mol H₂/mol glucose by optimizing the conditions for the hydrogen forming acetate pathway [10].

In dark fermentative hydrogen production, the presence of hydrogen is one of the most crucial factors, as high concentrations of hydrogen are a major inhibitor of the process. To reduce the effect of hydrogen on the process, previous experimental studies (Table 4.1) have employed a variety of techniques, e.g. sparging the headspace, reducing the total pressure, applying vigorous stirring, optimizing the reactor design, or simply increasing the headspace/solution ratio. Regardless of the method used, alleviation of hydrogen inhibition results in increased HYs and/or hydrogen production rates (HPR).

The headspace hydrogen concentration has been widely used (Table 4.1) to predict hydrogen in the liquid phase (H_{2aq}), which *de facto* acts on the microbial cultures. However, poorly soluble gases, such as hydrogen, supersaturate in solution during anaerobic processes [26]. Studies, including a direct measurement of H_{2aq} (Table 4.1), have observed that H_{2aq} can exceed the equilibrium concentration suggested by Henry's law multiple times. As a result, the measurement of headspace hydrogen is considered inappropriate to predict the H_{2aq} value [1,11–13,26].

Our study focused on the effect of agitation and biogas recirculation on the accumulation of H_{2aq} and the consequent impact on dark fermentation by *T. neapolitana*. The main goal was to demonstrate that the recirculation of the H₂-rich biogas (GaR) into the culture broth is an effective method to improve the gas-liquid mass transfer and prevent H_{2aq} from supersaturating, without diluting the H₂ percentage in the biogas. Furthermore, we wanted

to show that keeping the H_{2aq} in equilibrium with the gas phase is sufficient to achieve high hydrogen production yields and rates. In a first assay, the effect of GaR and agitation speed on hydrogen production was evaluated using a highly concentrated *T. neapolitana* culture. In a second assay, a direct measurement of H_{2aq} was included to demonstrate the correlation between hydrogen supersaturation and the performance of dark fermentation.

Table 4.1: Different techniques applied to decrease the H_{2aq} and reduce its inhibition on dark fermentation. A “x” was used to indicate if H_{2aq} was directly measured in the referenced study or an effect on H_2 yield and production rate was observed.

Counteracting measures	H_{2aq} measurement	Effect on yield	Effect on rate	Reference
Headspace sparging with N_2	x	x		[11–13]
Headspace sparging with CO_2		x	x	[14]
Headspace sparging with N_2/H_2 , stirring		x		[15]
Sparging with biogas (CH_4)		x	x	[16]
CO_2/N_2 and internal gas injection		x		[17]
Internal CO_2 enriched gas injection		x	x	[18]
N_2 gas injection	x	x	x	[19]
Control of reactor pressure		x		[10]
Control of reactor pressure		x	x	[20,21]
Stirring and organic loading rate	x	x	x	[22]
Bubble induction		x	x	[23]
Stirring and surface enlargement	x	x	x	[24]
Recycling of degassed effluent		x	x	[25]

4.2 Material and methods

4.2.1 Bacterial culture and medium

A pure culture of *Thermotoga neapolitana cf. capnolactica* [27] was used in all experiments. The cultivation and storage conditions for the culture, the preparation of inoculum for elevated cell concentrations (assay 1) and the modified ATCC 1977 medium used (containing 27.8 mM of glucose) were as described by Dreschke et al. [28]. The pH of the medium was adjusted to 7.5 prior to being autoclaved at 110 °C for 5 min. Prior to the inoculation, the medium was heated at 80 °C for 30 min and sparged with N_2 for 5 min to remove the dissolved oxygen.

4.2.2 Experimental design

All experiments were run in a 3-L fully controlled continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The operating temperature was kept at a constant 80 °C and pH was automatically adjusted to 7 by adding 1M NaOH. GaR was applied by continuously pumping the produced biogas from the headspace to a gas dispersion device at the base of the reactor at a flow rate of 350 mL/min. The produced biogas was released into 500 mL water displacement systems to avoid pressure build-up and quantified every hour. Liquid samples of 2 mL were withdrawn each 30 or 60 min. The fermentation was considered completed when the reactors ceased to produce biogas. Fermentation time was approximated from the inoculation to the end of the fermentation. Each condition was conducted in duplicate to demonstrate reproducibility.

4.2.2.1 Assay 1 – Effect of agitation speed and gas recirculation on dark fermentation at high cell concentrations

Assay 1 was designed to study the effect of GaR and agitation speed on the dark fermentation process at high cell concentrations of *T. neapolitana*. The reactor was inoculated with 80 mL of concentrated inoculum [28] resulting in a biomass concentration of 0.79 (\pm 0.03) g cell dry weight (CDW)/L. The following operating conditions were investigated: 300 rpm agitation (300); 300 rpm agitation with gas recirculation (300 + GaR); 500 rpm agitation (500); 500 rpm agitation with gas recirculation (500 + GaR).

4.2.2.2 Assay 2 – Effect of GaR and agitation speed on H_{2aq}

Assays 2A and 2B were designed to study the effect of agitation speed and GaR on the retention of hydrogen in the liquid phase and its influence on the dark fermentation process. The reactor was inoculated with 20 mL of storage culture (1% v/v) and maintained for 15 h at 100 rpm to acclimatize the culture. Afterwards, a sequence of operating conditions was used with each condition being operated for 2 h as described in Table 4.2. In assay 2A, 5 min of 500 rpm + GaR (Table 4.2 – shaded grey cells) was used before each operating condition to remove the accumulated hydrogen from the liquid phase. After 5 or 30 min and at the end of each operating condition, 20 mL of broth was withdrawn to monitor H_{2aq}.

Table 4.2: Operating conditions during assay 2. 100, 200, 300 and 500 represent the agitation speed in rpm.

Time [h]	0 – 15	15 – 17	17 – 19	19 – 21	21 – 23
Assay 2A	100	GaR 300	GaR 500	GaR 300	GaR 500
2A (duplicate)	100	GaR 500	GaR 300	GaR 500	GaR 300
Assay 2B	100	200	200 + GaR	200	200 + GaR
2B (duplicate)	100	200 + GaR	200	200 + GaR	200

GaR = recirculation of the produced biogas

4.2.3 Analytical Methods

4.2.3.1 Assay 1

Cell growth was determined by measuring spectrophotometrically (Lambda 365, Perkin Elmer, USA) optical density (OD_{540}) of the liquid samples at 540 nm. Liquid samples were centrifuged at 10,000 rpm for 5 min to collect the supernatant for the determination of glucose, acetic acid (AA), lactic acid (LA) and alanine concentration as described by Dreschke et al. [28]. The glucose concentration was measured by the dinitrosalicylic acid method [29], while AA, LA and alanine were quantified by ^1H Nuclear Magnetic Resonance (NMR) with a 600 MHz spectrometer (Bruker Avance 400) as described by Dipasquale et al. [8]. After the completion of the fermentation, 500 mL of culture broth was centrifuged at 3750 rpm for 20 min for the determination of CDW via freeze drying. The concentration of hydrogen in the produced gas was analyzed by gas chromatography as described by Dipasquale et al. [8]. The conversion from volumetric to molar H_2 production was performed using the ideal gas law.

4.2.3.2 Assay 2

Glucose, AA and LA concentrations were determined using an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors, with the method described by Mancini et al. [30] with 0.0065 M of sulfuric acid as the mobile phase. The concentration of hydrogen in the biogas was determined with a Varian 3400 gas chromatograph (GC), equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas.

For the determination of H_{2aq} , a modified method of Kraemer and Bagley [11,12,31] was applied. Vials with a total volume of 31 mL were closed with silicon septa and depressurized using a plastic syringe. 20 mL of sample was injected into the vials and immediately placed upside down in the fridge to equilibrate the liquid and the gas phases. After around 20 h, the sample was heated at room temperature and the negative pressure equilibrated by adding air to the gas phase of the sample. The concentration of hydrogen in the gas phase was measured by the GC described above and referred to the 20 mL of liquid sample.

4.2.4 Kinetic study of dark fermentation

The rates and lag phases of dark fermentation were evaluated in terms of biomass growth, hydrogen production and glucose consumption and calculated by fitting the experimental data with a modified Gompertz model as described by Dreschke et al. [28].

4.2.5 Statistical analysis

The statistical significance of the experimental data was determined by the calculation of the p-value applying an unpaired t-test with Microsoft Excel 2016 (Microsoft Corporation, USA).

4.3 Results

4.3.1 Effect of agitation speed and GaR on dark fermentation at high cell concentrations (assay 1)

Fig. 4.1 shows the glucose consumption (A), cumulative hydrogen (B) and biomass growth (C) during batch fermentation with an initial cell concentration of $0.79 (\pm 0.03)$ g CDW/L of *T. neapolitana* using an agitation of 300 or 500 rpm with and without GaR.

Increasing the agitation significantly enhanced the specific rates of biomass growth [mg CDW/h/g CDW], hydrogen production [mL H_2 /h/g CDW], and glucose consumption [mmol glucose/h/g CDW] from $73 (\pm 11)$, $294 (\pm 44)$, and $4.4 (\pm 1.3)$ at 300 rpm to $266 (\pm 9)$ (p-value: 0.003), $695 (\pm 46)$ (p-value: 0.012), and $10.1 (\pm 0.6)$ (p-value: 0.028) at 500 rpm (Fig. 4.2, Table 4.3), respectively. Due to the accelerated process, the fermentation time and lag phase of hydrogen production were reduced from 11 h and $2.9 (\pm 0.2)$ h at 300 rpm to 6 h and from $1.3 (\pm 0.1)$ h at 500 rpm (Table 4.3), respectively. The HY increased from $3.0 (\pm 0.0)$

at 300 rpm to 3.2 (± 0.1) mol H₂/mol glucose at 500 rpm, while the biomass yield (BMY) increased from approximately 16.7 to 21.9 g CDW/mol glucose (Table 4.4). The composition of the end products, expressed in terms of an LA/AA ratio, decreased from 0.31 at 300 rpm to 0.21 at 500 rpm (Table 4.5).

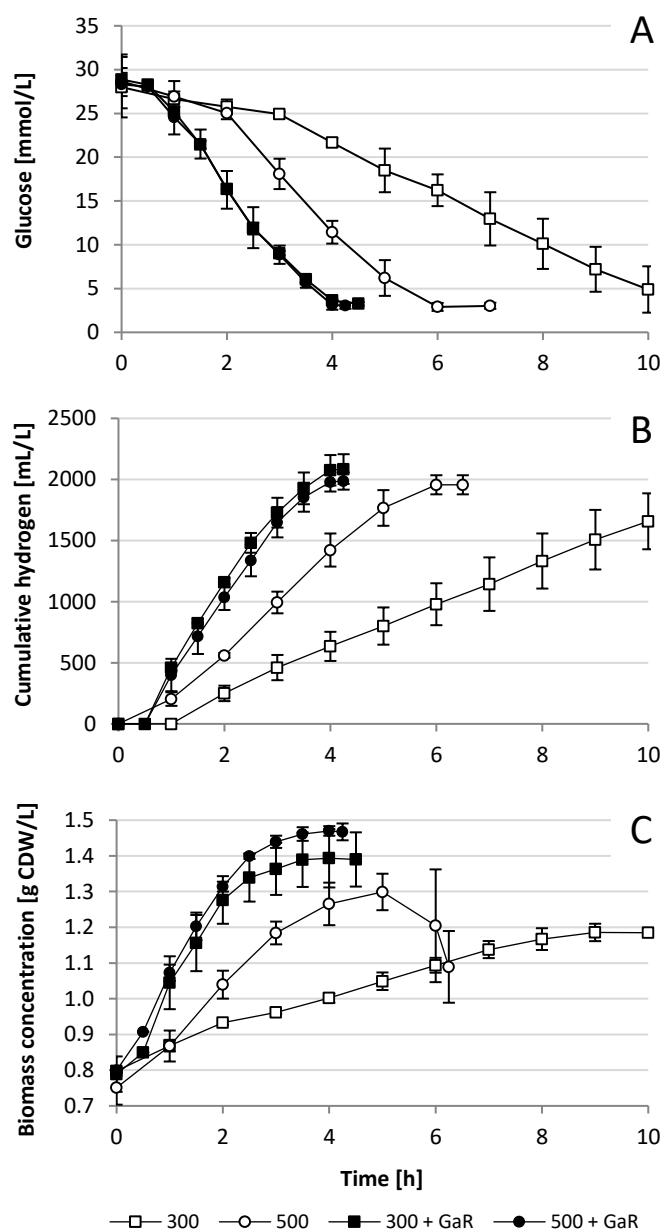


Fig. 4.1: Glucose consumption (A), cumulative hydrogen production (B) and biomass growth (C) throughout the dark fermentation of 27.8 mM of glucose by *T. neapolitana* cf. *capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR). Error bars depict the standard deviation.

A combination of GaR and agitation speed at 300 rpm further accelerated the dark fermentation. The specific biomass growth rate reached 423 (± 9) mg CDW/h/g CDW (p -value: 0.004), while the specific rates of hydrogen production and glucose consumption

increased up to 1090 (± 91) mL/h/g CDW (p-value: 0.032) and 13.3 (± 0.8) mmol glucose/h/g CDW (p-value: 0.040), respectively, over those obtained at 500 rpm without GaR (Fig. 4.2, Table 4.3). The fermentation was completed within 4 h with a lag phase of 0.4 (± 0.0) h (Table 4.3), a HY of 3.5 (± 0.2) mol H₂/mol glucose, an approximate BMY of 24.8 g CDW/mol glucose (Table 4.4), and a LA/AA ratio of 0.11 (Table 4.5).

Table 4.3: Hydrogen production, biomass growth and glucose consumption rates during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* at 300 and 500 rpm, excluding or including recirculation of the produced gas (GaR). The rates and lag phase were determined through data fitting with a modified Gompertz model. The adequateness of the fit is illustrated by the coefficient of determination (R²). Specific rates are calculated based on the initial biomass concentration in the reactor.

	Specific rate	Volumetric rate	Lag phase	R ²
Hydrogen production	[mL H ₂ /h/g CDW]	[mL H ₂ /L/h]	[h]	
300 rpm	294 \pm 44	235 \pm 35	2.9 \pm 0.2	0.98
500 rpm	695 \pm 46	535 \pm 35	1.3 \pm 0.1	0.99
300 rpm + GaR	1090 \pm 91	850 \pm 71	0.4 \pm 0.0	0.99
500 rpm + GaR	1016 \pm 22	813 \pm 18	0.5 \pm 0.1	0.99
Biomass growth	[mg CDW/h/g CDW]			
300 rpm	73 \pm 11			0.98
500 rpm	266 \pm 9			0.99
300 rpm + GaR	423 \pm 9			0.99
500 rpm + GaR	431 \pm 9			0.99
Glucose consumption	[mmol glucose/h/g CDW]			
300 rpm	4.4 \pm 1.3			0.99
500 rpm	10.1 \pm 0.6			0.99
300 rpm + GaR	13.3 \pm 0.8			0.99
500 rpm + GaR	12.7 \pm 0.2			0.99

Table 4.4: Hydrogen yield (HY), biomass yield (BMY) and glucose consumption obtained in a 2 L batch reactor inoculated with 0.79 (± 0.03) g CDW/L of *T. neapolitana cf. capnolactica*, performing the dark fermentation of 27.8 mM of glucose at 300 and 500 rpm agitation speed, in the presence or absence of recirculation of the produced gas (GaR).

Operating condition	HY [mol H ₂ /mol glucose]	BMY [g CDW/mol glucose]	Glucose consumption [%]
300 rpm	3.0 \pm 0.0	16.7	82
500 rpm	3.2 \pm 0.1	21.9	89
300 rpm + GaR	3.5 \pm 0.2	24.8	88
500 rpm + GaR	3.3 \pm 0.1	27.7	89

Increasing the agitation to 500 rpm while applying GaR had no significant effect on the process, as shown by the similar performance obtained at 300 rpm + GaR and 500 rpm + GaR (Fig. 4.1). At 500 rpm + GaR, the specific rates of biomass growth, hydrogen production and glucose consumption were 431 (± 9) mg CDW/h/g CDW, 1016 (± 22) mL H₂/h/g CDW and 12.7 (± 0.2) mmol glucose/h/g CDW (Fig. 4.2, Table 4.3), respectively. The HY reached 3.3 (± 0.1) mol H₂/mol glucose (Table 4.4) coupled with a LA/AA ratio of 0.13 (Table 4.5). The fermentation time and lag phase of hydrogen production remained at 4 h and 0.5 (± 0.1) h (Table 4.3), respectively, with an approximate BMY of 27.7 g CDW/mol glucose.

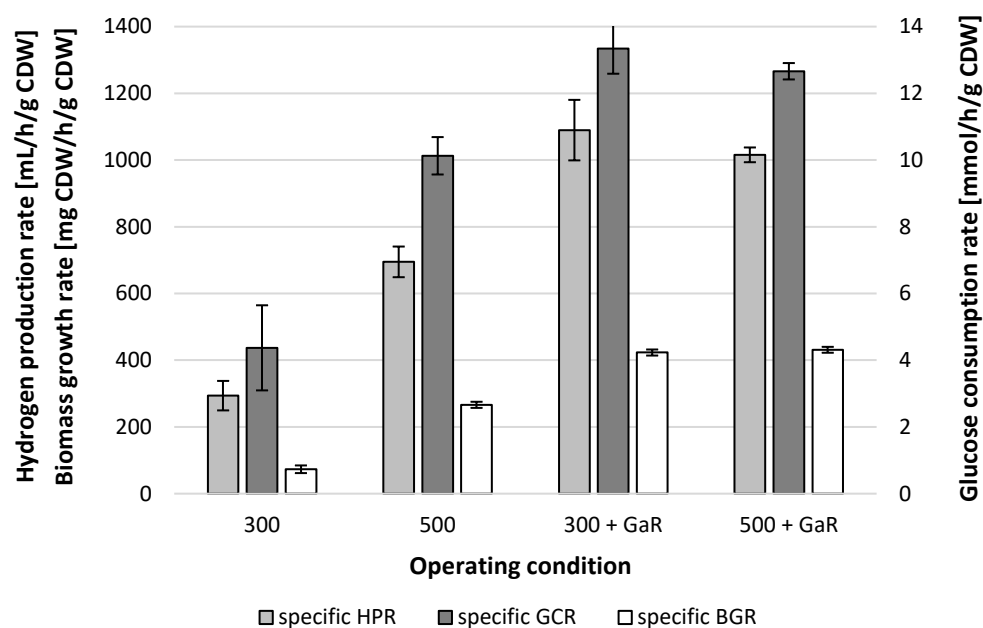


Fig. 4.2: Specific rates of biomass growth (BGR), glucose consumption (GCR) and hydrogen production (HPR) obtained during the dark fermentation of 27.8 mM of glucose by 0.79 (± 0.03) g CDW /L of *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR). Specific rates were calculated based on the initial biomass concentration in the reactor expressed as cell dry weight (CDW). Error bars depict the standard deviation.

The highest volumetric HPRs of 850 (± 71) and 813 (± 18) mL/h/L were obtained when GaR was applied at 300 and 500 rpm, respectively (Table 4.3). The hydrogen concentration in the biogas produced was not affected by the use of GaR or the agitation speed and remained constant at 65 (± 2)% (data not shown) under all studied conditions. In all bioassays, glucose was consumed up to 82 - 89 % (Table 4.4) with AA and LA as the main fermentation end products in the digestate and alanine constituting a minor proportion of 2% (Table 4.5). A mass balance based on the stoichiometric equations 1 and 2 was performed to validate the

experimental results. The dark fermentation model suggests the formation of two moles of fermentation end products (AA and LA) per mole of glucose with the concomitant production of 2 moles of hydrogen per mole of AA. Under all operating conditions, the sum of fermentation products in the digestate reached 92% or more of the theoretical value calculated from the glucose consumption and 2.08 (± 0.01) moles H₂ per mole of AA were averagely produced.

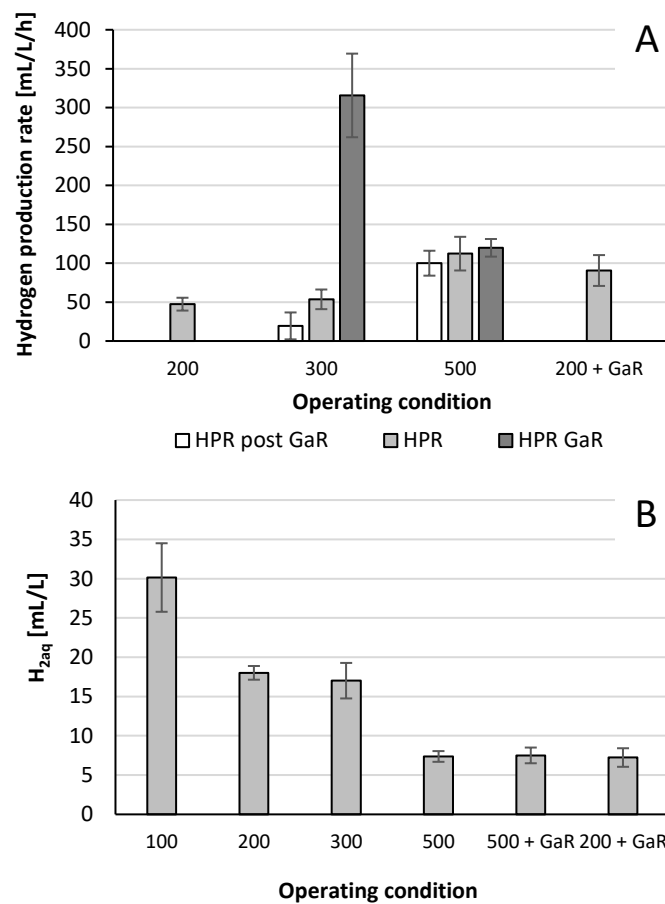


Fig. 4.3: Hydrogen production rate (HPR) (A) and concentration of hydrogen in the liquid phase (H_{2aq}) (B) at different operating conditions using 100, 200, 300 and 500 rpm as well as 200 rpm and 500 rpm with gas recirculation (GaR) for 2 h during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica*. (“HPR post GaR” – retention phase, i.e. 25 min after applying GaR; “HPR” – equilibrium phase, i.e. 90 min before the subsequent GaR; “HPR GaR” – removal phase, i.e. when applying GaR for 5 min) Error bars depict the standard deviation.

Table 4.5: Composition of the digestate at the end of the fermentation of 27.8 mM of glucose by *T. neapolitana* cf. *capnolactica* using 300 and 500 rpm agitation speed with or without the recirculation of the produced gas (GaR).

Operating condition	AA [mM] (yield [mol/mol glu])	LA [mM] (yield [mol/mol glu])	Alanine [mM]	Ratio LA/AA	Residual glucose [mM]	End product balance [%] ¹
300 rpm	32.3 ± 4.3 (1.4)	10.0 ± 1.0 (0.44)	1.1 ± 0.1	0.31	4.9 ± 2.7	96
500 rpm	37.7 ± 2.7 (1.5)	8.1 ± 0.2 (0.33)	1.0 ± 0.1	0.21	3.0 ± 0.4	95
300 rpm+GaR	39.2 ± 1.2 (1.6)	4.4 ± 0.1 (0.18)	0.9 ± 0.0	0.11	3.3 ± 0.2	92
500 rpm+GaR	38.7 ± 2.2 (1.6)	5.1 ± 0.5 (0.21)	0.8 ± 0.0	0.13	3.1 ± 0.4	92

¹ End product balance was calculated by summing AA, LA, alanine and residual glucose and referring it to the theoretical end product formation associated with the initial glucose concentration.

4.3.2 Effect of GaR and agitation speed on H_{2aq} and HPR (assay 2)

4.3.2.1 Assay 2A – Use of different agitation speeds

Assay 2A assessed the effect of the agitation speed on H_{2aq} and HPR. After the initial 15 h at 100 rpm, H_{2aq} accumulated up to 33.4 (± 0.5) mL/L (Fig. 4.3B). Then, using 500 rpm + GaR for 5 min before each operating condition reduced H_{2aq} to an average of 7.5 (± 1.0) mL/L (Fig. 4.4A). The subsequent agitation of the culture at 300 and 500 rpm without GaR for 2 h led to a H_{2aq} of 17.0 (± 2.3) and 7.4 (± 0.7) mL/L, respectively (Fig. 4.3B). To better demonstrate the effect of retention of hydrogen in the liquid phase on the HPR, the HPR was calculated in 3 phases of each operating condition: 1 – retention phase, i.e. 25 min after applying GaR (“HPR post GaR”); 2 – equilibrium phase, i.e. 90 min before the subsequent GaR (“HPR”); 3 – removal phase, i.e. when applying GaR for 5 min (“HPR GaR”) (Fig. 4.3A). At 300 rpm, a significantly higher “HPR GaR” of 316 (± 54) mL/L/h (Fig. 4.3A) was observed than “HPR” of 54 (± 13) mL/L/h (p-value: 0.0002) and “HPR post GaR” of 19 (± 17) mL/L/h (p-value: 0.0017), respectively. In contrast, at 500 rpm, the HPR remained similar before, during, and after GaR. The “HPR” reached 112 (± 22) mL/L/h, which was not significantly different from “HPR post GaR” (100 (± 16) mL/L/h, p-value: 0.397) and “HPR GaR” (120 (± 11) mL/L/h, p-value: 0.614) (Fig. 4.3A). Changing the agitation from 300 to 500 rpm roughly doubled the “HPR”, and concomitantly increased the glucose consumption rate (GCR) from 3.6 (± 0.4) to 4.7 ± (0.3) mmol/L/h (p-value: 0.016) (data not shown).

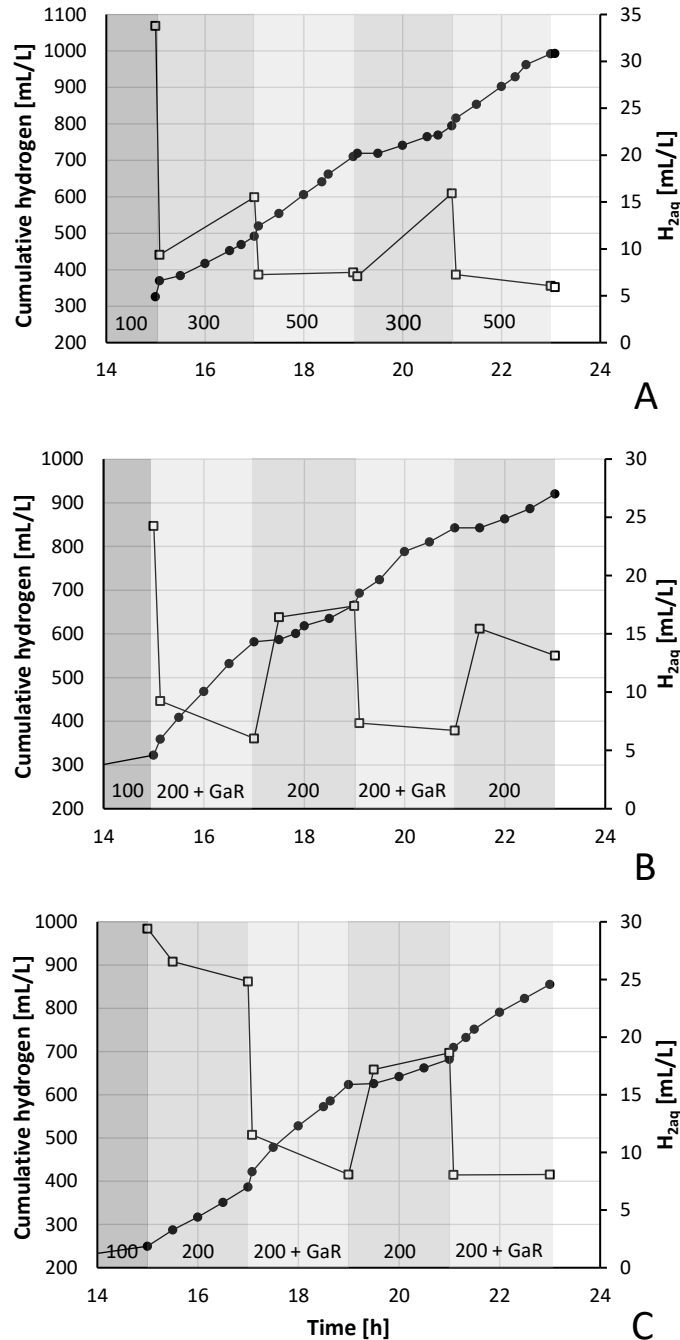


Fig. 4.4: Cumulative hydrogen production (●) and H_{2(aq)} evolution (□) during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* under different operating conditions: (A) 100, 300 and 500 rpm agitation speed with 5 min of gas recirculation (GaR) between each condition; (B) and (C) 100, 200 and 200 rpm + GaR.

4.3.2.2 Assay 2B – Gas sparging

Assay 2B was run to assess the effect of GaR on H_{2(aq)} and HPR at constant agitation at 200 rpm. Similar to assay 2A, the highest H_{2(aq)} of 26.8 (± 3.6) mL/L (Fig. 4.4B and C) was obtained after 15 h of operation at 100 rpm. Applying 200 rpm + GaR rapidly decreased the H_{2(aq)} to 9.0

(± 1.8) mL/L in 5 min (Fig. 4.4B and C) reaching a final value of 7.2 (± 1.2) mL/L after 2 h (Fig. 4.4B). The 200-rpm agitation without GaR caused an accumulation of H_{2aq} up to a concentration of 16.4 (± 0.9) mL/L in 30 min, which was 91% of 18.0 (± 0.9) mL/L reached after 2 h (Fig. 4.4B and C). The “HPR” at 200 rpm + GaR of 91 (± 20) mL/L/h was significantly higher than 47 (± 8) mL/L/h (p-value: 0.017) obtained at 200 rpm without GaR (Fig. 4.3A). Concomitantly, the GCR reached values of 4.2 (± 0.1) and 3.1 (± 0.8) mmol/L/h (p-value: 0.077), at 200 rpm + GaR and 200 rpm without GaR, respectively (data of GCR not shown). When agitation was increased from 100 to 200 rpm after 15 h in the absence of GaR, H_{2aq} decreased from 29.4 to 24.8 mL/L in 2 h (Fig. 4.4C), which was higher than 18.7 (± 4.6) mL/L obtained at 200 rpm agitation at 21 h after applying GaR between 17 and 19 h.

4.4 Discussion

4.4.1 Effect of agitation speed and gas recirculation on H_{2aq} concentration

In this study, GaR and agitation speed were examined as strategies to lower H_{2aq} and, thus, stimulate H_2 production by *T. neapolitana*. Agitating at 100 rpm led to the highest H_{2aq} , i.e. 30.1 (± 4.4) mL/L, which was approximately 3 times higher than the theoretical dissolved hydrogen concentration (i.e. 9.7 mL/L) in equilibrium with a gas phase containing 65% H_2 at 80 °C according to Henry's Law (Eq. 4.1):

$$H(T) = H^\theta * \exp\left(\frac{d \ln(H)}{d(1/T)} * \left(\frac{1}{T} - \frac{1}{T^\theta}\right)\right) \quad (\text{Eq. 4.1})$$

where $H(T)$ and H^θ are Henry's constants expressed in mol/m³/Pa at temperature T and T^θ (= 298.15 K), respectively; H^θ equals 7.7×10^{-6} [32]; $d \ln(H)/d(1/T)$ equals 500 K [32] and is the temperature dependence factor of Henry's constant; and T is the temperature in K.

Increasing the agitation speed effectively improved the gas-liquid mass transfer, decreasing the H_{2aq} concentration (Fig. 4.4 and Fig. 4.3B) until reaching values below the theoretical equilibrium concentration at 500 rpm. Similarly, GaR maintained the hydrogen partial pressure of gas and liquid phase in equilibrium (Fig. 4.3B).

The extent of H_{2aq} accumulation is determined by the HPR and the mass transfer from liquid to gas phase (Eq. 4.2) [11,26].

$$\frac{dH_{aq}}{dt} = HPR - k_L a (H_{aq} - H_{dis}) \quad (\text{Eq. 4.2})$$

with H_{2aq} being the hydrogen concentration in the liquid phase [mL/L]; H_{dis} the concentration of dissolved hydrogen at the thermodynamic equilibrium; HPR the volumetric production of hydrogen in the liquid phase [mL/L/h]; $k_L a$ the global mass transfer coefficient [1/h] made up of two terms, i.e. k_L (the "film" coefficient) and a (the specific interfacial area per unit of liquid volume in the reactor).

When the HPR exceeds the gas-liquid mass transfer of the system, H_{2aq} supersaturates. However, elevated H_{2aq} concentrations inhibit the HPR, which again reduce the degree of H_{2aq} supersaturation (Eq. 4.2) until HPR and H_{2aq} reach an equilibrium. Because of this feedback inhibition, the HPR of a production system is in many cases primarily determined by an insufficient gas-liquid mass transfer instead of the kinetic potential of the culture.

The evolution of H_{2aq} accumulation and its dependence on the HPR and the mass transfer was clearly shown in the 3 phases of assay 2A at 300 rpm (Fig. 4.4A). In the retention phase, a low "HPR post GaR" of 19 (\pm 17) mL/L/h (Fig. 4.3A) was obtained as HPR was strongly affected by the fast retention of hydrogen in the liquid phase (Fig. 4.4A). The "HPR" during the equilibrium phase was instead higher, reaching 54 (\pm 13) mL/L/h (Fig. 4.3A). Contrary to the "HPR post GaR", "HPR" was no longer affected by hydrogen retention but rather by the prevalent H_{2aq} of 17.0 (\pm 2.3) mL/L which was determined by the mass transfer of the system (Fig. 4.3B). The subsequent application of GaR after 2 h removed the accumulated hydrogen from the liquid phase, leading to a high "HPR GaR" of 316 (\pm 54) mL/L/h and a decrease of H_{2aq} to 7.5 (\pm 1.0) mL/L (Fig. 4.3A and B). The length of the retention phase was based on the results of assay 2B, where a cultivation at 200 rpm after GaR increased the H_{2aq} to 16.4 (\pm 0.9) mL/L in 30 min, i.e. 91% of 18.0 (\pm 0.9) mL/L measured after 2 h (Fig. 4.4B and C). The accumulation of H_{2aq} was, therefore, considered completed at the end of the retention phase and the HPR in equilibrium with H_{2aq} as explained in section 2.2 (Eq. 4.2). At 500 rpm, hydrogen did not accumulate in the liquid phase as demonstrated by the low H_{2aq} of 7.4 (\pm 0.7) mL/L after 2 h (Fig. 4.3B) and the statistically similar values of "HPR post GaR", "HPR" and "HPR GaR", i.e. 110 (\pm 18) mL/L/h (Fig. 4.3A).

In conclusion, at low cell concentrations the sole agitation at 500 rpm as well as GaR provided an adequate mass transfer to remove the produced hydrogen from the liquid to the gas phase and prevent hydrogen supersaturation.

4.4.2 Enhancement of dark fermentation kinetics

Independent of the technique applied, the reduction of H_{2aq} in assay 2 resulted in a significant increase of the HPR. Adding GaR increased the “HPR” by 94% from 47 (± 8) to 91 (± 20) mL/L/h at 200 rpm, whereas increasing the agitation from 300 to 500 rpm enhanced the “HPR” by 107% from 54 (± 13) to 112 (± 22) mL/L/h (Fig. 4.3A), both accompanied by a simultaneous increase of the GCR.

In assay 1, we accelerated the dark fermentation process by using a higher cell concentration (0.79 g CDW/L). At the lowest agitation of 300 rpm, we observed a specific HPR of 294 (± 44) mL H_2 /h/g CDW. Increasing the agitation to 500 rpm accelerated the dark fermentation process, enhancing the specific HPR by 136% to 695 (± 46) mL H_2 /h/g CDW. As observed in assay 2, the higher HPR obtained at 500 rpm was likely due to a decreased H_{2aq} concentration. However, the addition of GaR was capable to further increase the HPR to 1090 (± 91) and 1016 (± 22) mL H_2 /h/g CDW at 300 + GaR and 500 + GaR, respectively, i.e. 271 and 246% higher than the HPR achieved at 300 rpm in the absence of GaR. This indicates that, in contrast to what was observed at low cell concentrations, 500 rpm agitating was insufficient to completely prevent hydrogen supersaturation at high cell concentrations. In contrast, GaR effectively enhanced the gas-liquid mass transfer (by increasing the specific interfacial area a in Eq. 4.2) and maintained the H_{2aq} low, leading to the highest values of specific HPR. The similar reactor performance at 300 + GaR and 500 + GaR furthermore shows that hydrogen supersaturation was the crucial factor limiting the process as, once it was prevented by GaR, the agitation speed had no influence on the process.

In line, Beckers et al. [24] obtained an enhancement of the HPR from 0.14 to 0.26 L/h when agitation was increased from 0 to 400 rpm in 2-L reactors using *Clostridium butyricum* at 30°C and glucose as a substrate. However, they observed the H_{2aq} to remain high at approximately 4 times the equilibrium concentration despite 400 rpm agitation.

In our experiments, the specific rates of glucose consumption and biomass growth were closely linked to the HPR (Table 4.3), showing a similar enhancement when the different measures to prevent H_{2aq} supersaturation were applied. The specific GCR and biomass growth rate (BGR) at high cell concentrations increased by 202% and 479% from 4.4 (± 1.3) to 13.3 (± 0.8) mmol glucose/h/g CDW and from 73 (± 11) to 423 (± 9) mg CDW/h/g CDW at 300 rpm and 300 rpm + GaR, respectively. This acceleration of the process led to a reduction

of the total fermentation time from 11 and 6 h with the sole agitation at 300 and 500 rpm, respectively, to 4 h when GaR was applied independent from the agitation speed.

Ljunggren et al. [11] successfully increased the HPR by decreasing H_{2aq} through N_2 headspace sparging using *Caldicellulosiruptor saccharolyticus*. According to them, the culture self-regulates the growth rate as a response to high H_{2aq} to slow down the process, decrease the HPR and consequently prevent the H_{2aq} from reaching inhibitory levels (Eq. 4.2). However, in our study the effect on the specific HPR, BGR and GCR was simultaneous (Fig. 4.2), indicating that H_{2aq} directly acts on all rates of dark fermentation.

4.4.3 Hydrogen and biomass yields under different operating conditions and H_{2aq} concentrations

Despite the inhibition by H_{2aq} discussed in section 4.2, the HY remained high at 3.0 (\pm 0.0) mol H_2 /mol glucose (Table 4.4) when applying 300 rpm agitation without GaR in assay 1. The HY increased even further to 3.5 mol H_2 /mol glucose coupled with a decrease of the LA/AA ratio (Table 4.4) when GaR maintained the H_{2aq} in equilibrium with the gas phase.

T. neapolitana predominately ferments glucose via the AA and the LA pathways, with the former leading to hydrogen production and one additional mole of ATP [4].

The AA pathway is thereby energetically more challenging [2], because the formation of hydrogen requires a high free Gibbs energy change as protons are poor electron acceptors [33]. The reaction to gaseous hydrogen is favored by low hydrogen concentrations and high temperatures as explained in detail by Verhaart et al. [33]. This indicates that the temperature was most likely responsible for the high HYs at sole agitation of 300 rpm. The additional decrease of H_{2aq} by GaR caused an additional energetical advantage and allowed the further shift towards the AA pathway leading to the increase of the HY.

A similar effect on the HY was obtained by Beckers et al. [24], measuring H_{2aq} in 200 mL serum bottles inoculated with *Clostridium butyricum* at 30°C. Unstirred cultures experienced a supersaturation up to 7 times the equilibrium concentration resulting in a reduced HY of 1.16 mol H_2 /mol glucose, in comparison to 1.52 mol H_2 /mol glucose obtained when agitating at 120 rpm. The inhibition by hydrogen has also been demonstrated previously in *T. neapolitana* cultivation using closed 120 mL serum bottles. d'Ippolito et al. [10] were able to increase the HY by reducing the culture/headspace ratio leading to a lower hydrogen partial pressure in the gas phase.

When GaR was applied at high cell concentrations, a lower LA/AA ratio also explains the BMY increase. More energy was gained by the culture per unit of substrate. Therefore, biomass production did not compete with the final product formation, as often assumed, but rather increased simultaneously when the environmental conditions were optimized. In our study, the variation of HY and BMY were much lower than that of the HPR under different operating conditions, indicating that hydrogen inhibition primarily acts on the process rates. We assume that a reduction of the dark fermentation rate by the culture at high H_{2aq} concentrations aims to prevent the inhibition of the AA pathway which allows the higher energy yield.

4.4.4 Biogas recirculation as a strategy to prevent hydrogen supersaturation

A large number of studies used headspace sparging with gases such as N_2 or CO_2 (Table 4.1) to counteract hydrogen inhibition by lowering H_{dis} (Eq. 4.2). For instance, Sonnleitner et al. [20] observed an increase of HPR from 25 to 119 mL/L/h when sparging the headspace of a *Caldicellulosiruptor saccharolyticus* culture with N_2 at 1 L/L/h. However, while sparging with external gases is generally successful to improve the process performance (Table 4.1), it inevitably leads to an undesired dilution of the produced hydrogen and consequently an increase of costs for gas purification. In contrast, when GaR is applied the concentration of hydrogen in the produced gas remains high.

To our knowledge, only two studies have so far focused on the recirculation of the H_2 -rich biogas in dark fermentation. Kim et al. [17] used GaR at flow rates ranging from 100 to 400 mL/min in a CSTR with a mixed culture at 35°C. They obtained similar HPRs and HYs, i.e. 0.77–0.86 and 0.77 mol H_2 / mol hexose, respectively, with and without GaR. This suggests that at the prevalent reactor conditions, hydrogen did not supersaturate in the liquid phase which rendered GaR ineffective. Bakonyi et al. [18] recirculated the internal biogas at two different H_2 concentrations into a CSTR using a mixed culture at 35°C. The recirculation of the less concentrated gas (50% H_2) resulted in a HPR of 8.9 – 9.2, whereas the use of a more concentrated gas (66% H_2) reduced the HPR to 2.7 – 3.0 L H_2 /L/d, compared to 7.4 L H_2 /L/d obtained without recirculation. Unfortunately, a direct measurement of H_{2aq} was not included in any of the two studies, making the correlation of the reactor performance to H_{2aq} impossible. In our study, we directly demonstrate the impact of stirring speed and GaR on H_{2aq} and correlate it to the process performance. GaR has proven highly effective to provide

an adequate gas-liquid mass transfer, that can be readily adjusted by adapting the recirculation flow to meet the requirements of the system. Furthermore, the results confirm that maintaining H_{2aq} in equilibrium with the gas phase is sufficient to reach high HY up to 3.5 mol H_2 /mol glucose (Table 4.4) and a high specific HPR of 1090 ± 91 mL/h/g CDW (Table 4.3).

Conclusions

This study confirms that hydrogen supersaturates in the liquid phase and strongly inhibits dark fermentation by *T. neapolitana cf. capnolactica*. GaR and the sole agitation at 500 rpm efficiently reduced the H_{2aq} (i.e. 30.1 ± 4.4 mL/L) observed at low cell concentration and agitation of 100 rpm to below the equilibrium value suggested by Henry's law (i.e. 9.7 mL/L). At high cell concentrations (i.e. 0.79 g CDW/L), 500 rpm agitation was not did not provide sufficient gas-liquid mass transfer to prevent H_{2aq} supersaturation, which was instead counteracted by GaR. High H_{2aq} concentrations led to thermodynamic constraints and the reciprocal influence of H_{2aq} and HPR, which directly hampered the dark fermentation rates. In this line, we observed the specific HPR increasing by 271% when adding GaR at 300 rpm, as well as a HY increase from 3.0 to 3.5 mol H_2 /mol glucose. Hence, we conclude that recirculation of H_2 -rich biogas is an efficient method to prevent hydrogen supersaturation and allow high production rates and yields without negatively effecting the hydrogen content of the produced biogas.

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5 Influence of liquid-phase hydrogen on dark fermentation by *Thermotoga neapolitana*

(Paper III)

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Abstract

Hydrogen is a strong inhibitor of dark fermentation. We aimed at directly correlating the hydrogen production by *Thermotoga neapolitana* with the supersaturation of hydrogen in the liquid phase (H_{2aq}), which is often disregarded. Different agitation speeds, biogas recirculation and bubble induction by AnoxKaldnes™ K1 carrier were tested to prevent the supersaturation of H_{2aq} . At 100 rpm agitation, the H_{2aq} was $29.7 (\pm 1.4)$ mL/L, which is 3-times higher than 9.7 mL/L, i.e. the equilibrium concentration given by Henry's law. Increasing the agitation speed up to 600 rpm reduced the H_{2aq} until $8.5 (\pm 0.1)$ mL/L in 2 h and increased the hydrogen production rate (HPR) from $39 (\pm 2)$ at 0 rpm to $198 (\pm 4)$ mL/L/h at 600 rpm. Similar to 600 rpm, biogas recirculation and the presence of K1 carrier at 200 rpm maintained the H_{2aq} below the equilibrium concentration. This study demonstrates the reciprocal influence of HPR and H_{2aq} and reveals an inverse nonlinear correlation between the two parameters. Therefore, we conclude that an adequate gas-liquid mass transfer, efficiently provided by biogas recirculation or the presence of solid materials (e.g. a biomass carrier), is essential to remove H_2 from the liquid phase and prevent H_2 supersaturation.

Key Words: *Thermotoga neapolitana*, hyperthermophilic, dark fermentation, gas recirculation, hydrogen inhibition, supersaturation

Abbreviations

AA	acetic acid
BGR	biomass growth rate
GaR	recirculation of H_2 -rich biogas
H_{2aq}	liquid phase hydrogen
HPR	hydrogen production rate
HY	hydrogen yield
LA	lactic acid

5.1 Introduction

The continuing rise of the worldwide energy consumption, the depletion of fossil fuels and climate change due to CO₂ emissions emphasize the need to change the current energy system. Hydrogen is predicted to play a major role in this transition as it is a highly flexible clean energy carrier which can be effectively transformed and used in many applications [1]. However, while the use of hydrogen has major advantages, its current production is primarily based on fossil resources. To successfully introduce hydrogen as the energy carrier of the future, a sustainable production process from renewable sources needs to be established [1].

Amongst the environmentally friendly hydrogen production processes, dark fermentation represents one of the most promising [2]. Dark fermentation refers to the conversion of organic material to mainly hydrogen, CO₂ and organic acids [3]. To reach the economic viability of this H₂ production process, low hydrogen yields (HY) and hydrogen production rates (HPR) are major constraints that still need to be overcome [4,5]. In dark fermentation, higher yields are generally achieved under thermophilic compared to mesophilic conditions [6]. *Thermotoga neapolitana* is a hyperthermophilic microorganism which has repeatedly been reported to approach the theoretical yield of 4 moles H₂/mol of glucose [7–9]. In addition, the potential of this culture for hydrogen production is emphasized by an efficient hydrolysis of a wide range of substrates combined with fast growth kinetics [8].

The HPR primarily depends on the biomass concentration in the reactor [10,11]. However, the HPR is strongly inhibited by the presence of hydrogen in the liquid phase (H_{2aq}) [12]. H_{2aq} is often wrongly considered in equilibrium with the gas phase [2,13], as it has been demonstrated to supersaturate up to multiple times the equilibrium concentration suggested by Henry's law [14,15]. H_{2aq} has been identified as the crucial parameter for hydrogen inhibition as this product inhibition directly acts on the bioconversion capacity of microbial cultures [3].

According to Pauss et al. [12], H_{2aq} supersaturates if HPR exceeds the gas-liquid mass transfer potential of the system. High levels of H_{2aq} thermodynamically constrain the further production of hydrogen [16,17] and hamper the HPR and the HY of the system until an equilibrium between the HPR and the H_{2aq} concentration is reached [12]. As a result, high HPRs can only be achieved if an adequate gas-liquid mass transfer is provided. In this regard,

a variety of techniques have been applied to counteract the inhibition by hydrogen [13,16], including headspace sparging with N₂ [15] or CO₂ [18], reduction of the total pressure [19], increase of agitation [20], increase of the surface area [14] and recirculation of the H₂-rich biogas (GaR) [21]. While most of the studies focused on the effect of such techniques on HPR and HY, only a few included a direct measurement of H_{2aq} [14,15,22,23], which allows the direct correlation between H_{2aq} and the inhibition of dark fermentation.

The present study, therefore, focused on the effect of hydrogen accumulation in the liquid phase on dark fermentation by *Thermotoga neapolitana*. The main objective was to directly correlate the H_{2aq} concentration to HPR and HY. Different agitation speeds between 0 and 600 rpm were investigated, as well as the recirculation of the H₂-rich biogas and the addition of AnoxKaldnes™ K1 carriers as potential counteracting measures against hydrogen supersaturation.

5.2 Materials and Methods

5.2.1 Bacterial culture and medium

A pure culture of *Thermotoga neapolitana* (briefly *T. neapolitana*) purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used in all experiments. The protocol for cultivation and storage of the culture as well as the composition and preparation of the culture medium (modified ATCC 1977) with a glucose concentration of 27.8 mmol/L are described in detail in [10].

5.2.2 Experimental design

All batch experiments were conducted in a 3 L fully controlled continuously stirred tank reactor (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The medium was heated to 80 °C for 30 min and sparged with N₂ for 5 min to remove dissolved oxygen before the inoculation with 20 mL of storage culture (1% v/v). Throughout the experiments, the temperature was kept at 80 °C and the pH was automatically controlled to 7 by adding 1 M NaOH.

The culture was maintained at 100 rpm of agitation for 15 h (phase 1) to be acclimatized prior to applying the following operating conditions (phase 2): 0, 200, 400 and 600 rpm

agitation as well as biogas recirculation without agitation (0 + GaR). GaR was applied by continuously pumping the produced biogas from the headspace to a gas dispersion device at the base of the reactor at a flow rate of 350 mL/min. Furthermore, the presence of AnoxKaldnes™ K1 carriers (Fig. 5.1) (1 L packed volume, Veolia Water Technologies, Italy) in the fermentative solution was investigated for their ability to enhance the gas-liquid mass transfer by inducing bubble formation. Due to the anaerobic conditions in the gastight reactor, the K1 carriers were added before the inoculation and agitated at 100 rpm for 15 h in phase 1 and at 200 rpm in phase 2 (200 + K1).

The produced biogas was continuously released into 500 mL water displacement systems to avoid pressure build-up and quantified after 15 h and subsequently every hour. Liquid samples of 2 mL were withdrawn every hour for the analysis of biomass concentration and metabolite composition (i.e. glucose, acetic acid (AA) and lactic acid (LA)). At selected time points (i.e. after 15, 17, 20 and 23 h), 20 mL of liquid sample was taken for the determination of H_{2aq} . The fermentation was considered completed when a reactor ceased to produce biogas. Each operating condition was investigated in duplicate.

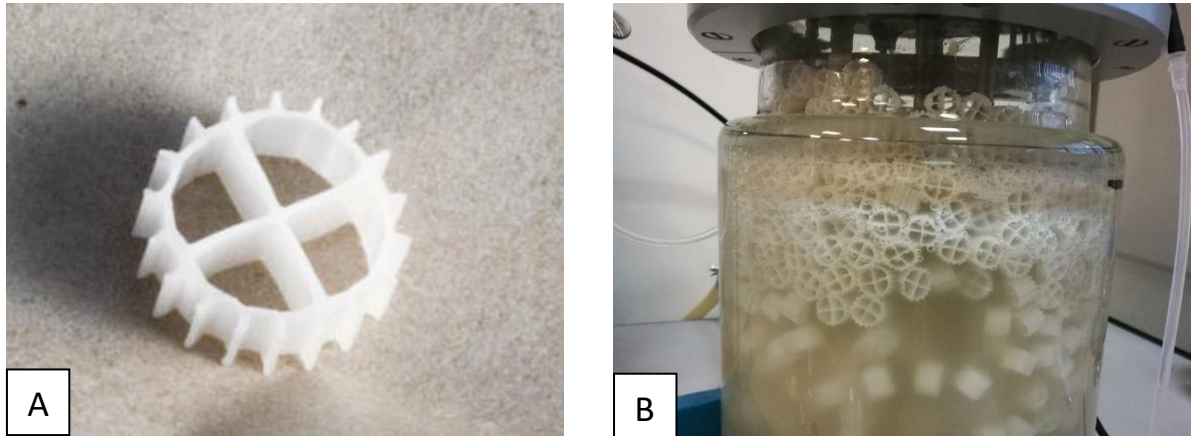


Fig. 5.1: A - K1 biomass carrier (<https://prozesstechnik.industrie.de>) manufactured by AnoxKaldnes (Sweden) out of polyethylene (density 0.95 g/cm³) with a diameter of 9.1 mm, a height of 7.2 mm and a specific biofilm surface area of 500 m²/m³. B – Carrier applied inside the 3 L bioreactor.

5.2.3 Analytical methods

Cell growth was determined by measuring spectrophotometrically the optical density (OD₅₄₀) of the liquid samples at 540 nm. Liquid samples were centrifuged at 10000 rpm for 5 min to collect the supernatant for the determination of the glucose, AA and LA concentration. The

glucose, AA and LA concentrations were determined using an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors, using the method described by Mancini et al. 2016 [24] with 0.0065 M sulfuric acid as the mobile phase. The concentration of hydrogen in the biogas was determined with a Varian 3400 gas chromatograph (GC), equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas. The conversion from volumetric to molar H₂ production was performed using the ideal gas law.

For the determination of H_{2aq}, a modified method of Kraemer and Bagley [15] was applied. Vials with a total volume of 31 mL were closed with silicon septa and depressurized using a plastic syringe. 20 mL of sample was injected into the vials and immediately placed upside down in the fridge to equilibrate the liquid and the gas phases. After approximately 20 h, the sample was heated at room temperature and the negative pressure equilibrated by adding air to the gas phase of the sample. The hydrogen concentration in the gas phase was measured by the GC described above and referred to the value of the liquid sample.

5.2.4 Kinetic study of biomass growth and hydrogen production

The rates of biomass growth and hydrogen production during phase 2 were calculated by fitting the experimental data with a modified Gompertz model based on the calculations by Dreschke et al. [10]. For biomass growth, Eq. 5.1 was applied, where B [OD₅₄₀] is the biomass concentration at fermentation time t [h]; B_0 [OD₅₄₀] is the biomass concentration at time 15 h (beginning of phase 2); B_m [OD₅₄₀] is the gain of biomass concentration throughout phase 2; R_B is the volumetric biomass growth rate (BGR) in phase 2 [OD₅₄₀/h]; λ_B is the lag phase of cell growth [h]; and e is the Euler's number, i.e. 2.72. As *T. neapolitana* morphologically adapts to the exhaustion of the substrate by decreasing its size resulting in a lower OD₅₄₀ [10], the fitting of the data with the Gompertz model was performed until the highest biomass concentration was reached.

Eq. 5.2 was applied for hydrogen production, with H [mL/L] being the cumulative hydrogen concentration at time t [h]; H_0 [mL/L] is the hydrogen produced after 15 h (beginning of phase 2); H_m [mL/L] is the hydrogen produced throughout phase 2; R_H [mL/L/h] is the volumetric HPR in phase 2; and λ_H the lag phase of hydrogen production [h]. The gas-liquid mass transfer of the system determines the removal or retention of hydrogen in the liquid

phase and consequently has a high impact on the HPR [12]. To avoid the alteration of the HPR, the experimental data from 16 h onwards were used for the Gompertz model of hydrogen production (Fig. 5.1B).

$$B = B_0 + B_m \exp \left\{ -\exp \left[\frac{R_B e}{B_m} (\lambda_B - t) + 1 \right] \right\} \quad (\text{Eq. 5.1})$$

$$H = H_0 + H_m \exp \left\{ -\exp \left[\frac{R_H e}{H_m} (\lambda_H - t) + 1 \right] \right\} \quad (\text{Eq.5.2})$$

5.2.5 Statistical analysis

The statistical analysis of the experimental data was performed using Microsoft Excel 2016 (Microsoft Corporation, USA). To determine significance, we applied a paired t-test for the decrease of H_{2aq} due to a change of the operating condition and an unpaired t-test with Benjamini Hochberg correction for the differences at varying operating conditions.

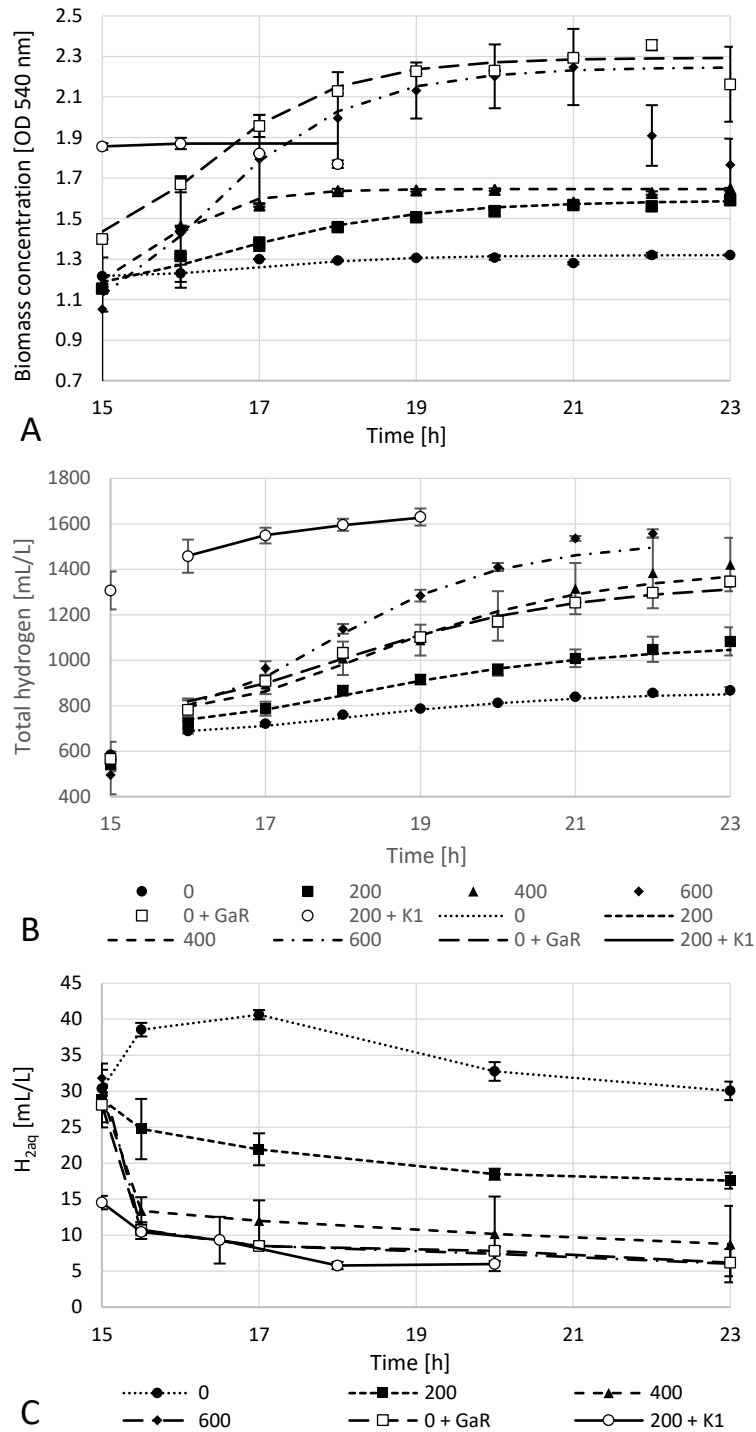


Fig. 5.1: Fermentation of 27.8 mM of glucose by *T. neapolitana* in batch operation at 100 rpm and 80 °C for 15 h (phase 1) and subsequent application of different operating conditions: 0 rpm, 200 rpm, 400 rpm, 600 rpm, 0 rpm with gas recirculation (GaR) and 200 rpm with K1 carriers from 15 to 23 h (phase 2). Biomass growth (A), hydrogen production (B) and hydrogen in the liquid phase – H_{2aq} (C) at the end of phase 1 after 15 h and their evolution during phase 2 from 15 to 23 h. In A and B, the symbols represent the average of the experimental data, while the lines depict the corresponding Gompertz model. Error bars depict the standard deviation.

5.3 Results

5.3.1 Effect of agitation speed on H_{2aq} accumulation and dark fermentation

5.3.1.1 Phase 1: Culture growth and acclimatization

In phase 1, excluding the bioassay with the K1 carriers, a similar performance was obtained in all experiments (Fig. 5.1 and Fig. 5.2). Approximately 33% of glucose consumption, a cumulative hydrogen production of 552 (\pm 36) mL/L (Fig. 5.1B), a biomass concentration of 1.20 (\pm 0.13) (OD₅₄₀) (Fig. 5.1A) and a H_{2aq} of 29.7 (\pm 1.4) mL/L (Fig. 5.1C) were obtained after 15 h. In contrast, when K1 carriers were used, approximately 80% of the glucose was consumed in the first 15 h resulting in a hydrogen production of 1307 (\pm 84) mL/L, a H_{2aq} of 14.5 (\pm 0.9) mL/L and a biomass concentration (OD₅₄₀) of 1.86 (\pm 0.02) (Fig. 5.2).

5.3.1.2 Phase 2: Application of different operating conditions

From 15 h onwards, increasing the agitation speed to 200, 400 and 600 rpm rapidly decreased the H_{2aq} concentration in 0.5 h (Fig. 5.1C). This decrease continued linearly but more slowly, reaching a significant difference compared to 15 h and values of 21.9 (\pm 2.2) (p-value: 0.031), 12.0 (\pm 2.9) (p-value: 0.041) and 8.5 (\pm 0.1) (p-value: 0.024) mL/L at 17 h (Fig. 5.1C). At the end of the experiment (i.e. after 23 h), agitation at 200, 400 and 600 rpm led to a H_{2aq} of 17.6 (\pm 1.1), 8.8 (\pm 5.3) and 6.0 (\pm 0.1) mL/L, respectively (Fig. 5.1C). When the agitation was omitted (i.e. 0 rpm) in phase 2, the H_{2aq} concentration increased to a maximum of 40.6 (\pm 0.6) mL/L at 17 h before decreasing again to 30.1 (\pm 1.3) mL/L after 23 h incubation (Fig. 5.1C).

The total production of hydrogen and biomass increased with agitation speed. The hydrogen produced reached 868 (\pm 15), 1083 (\pm 62), 1421 (\pm 118) and 1557 (\pm 18) mL/L with a maximum biomass concentration (OD₅₄₀) of 1.31 (\pm 0.01), 1.59 (\pm 0.01), 1.65 (\pm 0.01) and 2.41 (\pm 0.19) at 0, 200, 400 and 600 rpm (Fig. 5.1A and B) at 23 h, respectively. Concomitantly, the HPR and the BGR were 39 (\pm 2), 67 (\pm 1), 131 (\pm 16) and 198 (\pm 4) mL/L/h and 0.03, 0.11, 0.29 and 0.39 OD₅₄₀/h at 0, 200, 400 and 600 rpm, respectively (Fig. 5.3). Similarly, the glucose consumption accelerated at increasing agitation speeds and was complete at 400 and 600 rpm (Fig. 5.2A).

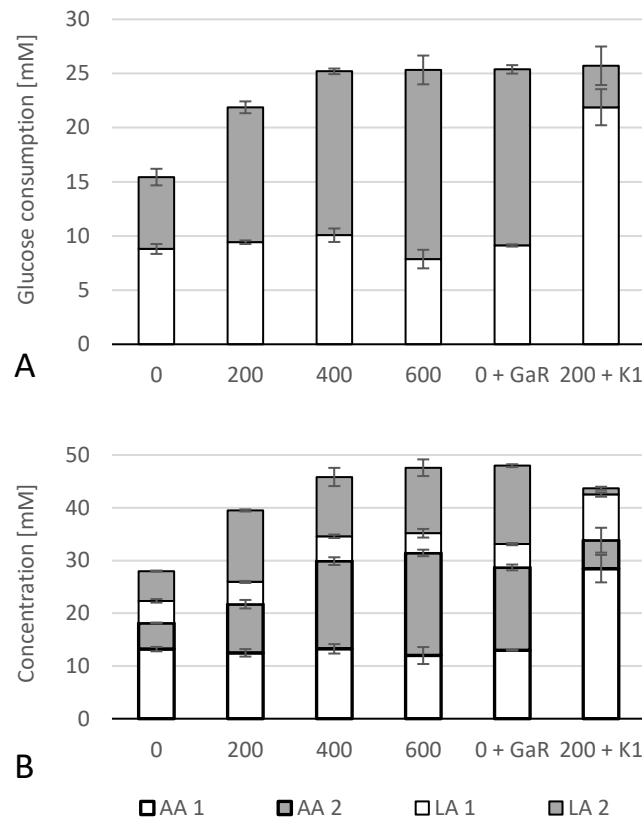


Fig. 5.2: Dark fermentation of 27.8 mM of glucose by *T. neapolitana* in batch operation at 100 rpm and 80 °C for 15 h (phase 1) and subsequent application of different operational conditions: 0 rpm, 200 rpm, 400 rpm, 600 rpm, 0 rpm with gas recirculation (GaR) and 200 rpm with K1 carriers from 15 to 23 h (phase 2). Glucose consumption (A) and production of fermentation end products (AA – acetic acid; LA – lactic acid) during phase 1 (white bars) and phase 2 (grey bars). Error bars depict the standard deviation.

5.3.2 Use of H₂-rich biogas recirculation and addition of K1 carriers

The effect of GaR on H_{2aq} was similar to that observed with agitation at 600 rpm, with the H_{2aq} concentration dropping significantly from 28.1 (± 2.4) to 10.6 (± 1.2) mL/L (p-value: 0.032) in 0.5 h (Fig. 5.1C). Glucose was completely consumed in 23 h and led to a hydrogen production of 1345 (± 23) mL/L and a biomass concentration (OD₅₄₀) of 2.39 (± 0.03) (Fig. 5.1). The BGR and HPR reached approximately 0.32 OD₅₄₀/h and 113 (± 3.5) mL/L/h (Fig. 5.3), respectively, which was significantly different to the HPR at 0 rpm (p-value: 0.0015).

When K1 carriers were used, increasing the agitation to 200 rpm from 15 h onwards (phase 2) decreased H_{2aq} to 10.5 (± 0.1) mL/L in 0.5 h and led to the completion of the fermentation in about 19 h. The final values of H_{2aq}, hydrogen production and biomass concentration reached 5.8 (± 0.5) mL/L, 1630 (± 38) mL/L and 1.87 OD₅₄₀, respectively.

5.3.3 Gas composition and biomass and hydrogen production yields

Independent from the operating conditions used, the concentration of hydrogen in the produced biogas reached 65 – 70%, the hydrogen yield was between 2.0 and 2.5 mol H₂/mol glucose and an average production of 1.95 (± 0.5) mol H₂/mol AA was obtained (Table 5.1). The sum of AA and LA at the end of the fermentation accounted for 94 (± 5)% of the glucose consumed in all conditions, except for 200 + K1 where it reached 85% (Fig. 5.2C). The biomass yields ranged from 0.065 and 0.093 OD₅₄₀/mol glucose (Table 5.1).

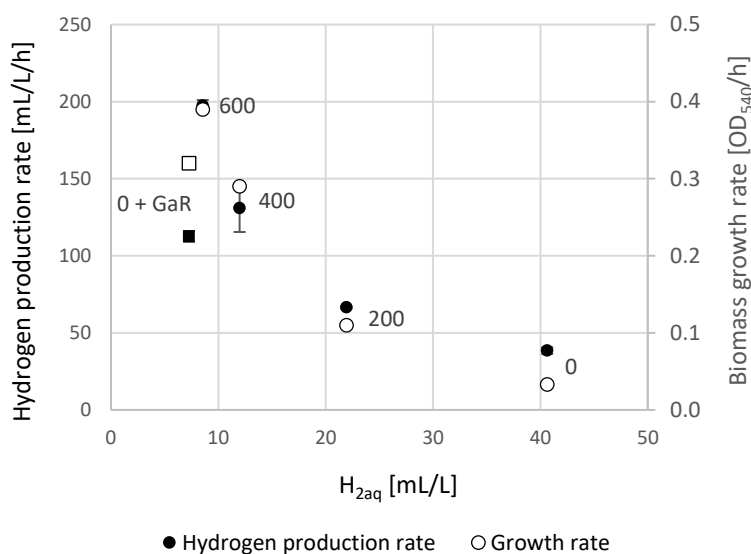


Fig. 5.3: Batch dark fermentation by *T. neapolitana* (at 80 °C) using different operational conditions (agitation at 0, 200, 400 and 600 rpm, no agitation with gas recirculation and 200 rpm with K1 carriers) after an acclimatization phase of 15 h at 100 rpm. Hydrogen production rate (full symbols – circles: 0 – 600 rpm; square: 0 + re) and biomass growth rate (hollow symbols – circles: 0 – 600 rpm; square: 0 + re). Error bars depict the standard deviation.

Table 5.1: Final yields of hydrogen, biomass and acetic acid, as well as the hydrogen/acetic acid ratio after fermentation of 27.8 mM glucose by *T. neapolitana* at different operating conditions.

Operating condition	Hydrogen yield [mol/mol glu]	Biomass yield [OD ₅₄₀ /mol glu]	Acetic acid yield [mol AA/mol glu]	Ratio H ₂ /AA [mol/mol]
0	2.3 (± 0.1)	0.086	1.18	1.94
200	2.0 (± 0.0)	0.073	0.99	2.02
400	2.3 (± 0.1)	0.065	1.19	1.93
600	2.5 (± 0.1)	0.089	1.24	2.01
0 + GaR	2.1 (± 0.1)	0.093	1.13	1.90
200 + K1	2.5 (± 0.0)	0.073	1.32	1.92

Glu – glucose; AA – acetic acid;

5.4 Discussion

5.4.1 Techniques to enhance hydrogen gas-liquid mass transfer

This study showed the importance of an efficient gas-liquid mass transfer to prevent the supersaturation of H_{2aq} and its inhibitory effect on dark fermentation, which consequently increased the HPR and BGR considerably (Fig. 5.3). Despite often disregarding the supersaturation of H_{2aq} , many studies have investigated the inhibition of dark fermentation by hydrogen and proposed a variety of counteracting measures [13,16]. While most of the applied measures successfully diminished the inhibitory effect of hydrogen at lab scale, only few have the potential to be applied at a larger scale. More specifically, headspace sparging with N_2 or CO_2 leads to an undesired dilution of the produced biogas [25], while the reduction of the total reactor pressure and vigorous stirring are extremely challenging in industrial applications and entail an inevitable increase of the reactor operation costs. Recirculation of biogas [21,26] has proven promising as it increases the area for gas-liquid mass transfer similar to inert gas sparging, without dilution of the final biogas. In addition, the application of bubble inducing objects in the reactor has revealed a high potential. The underlying principle of this technique is that the energy required for the formation of bubbles in a supersaturated liquid is much lower on a surface (heterogeneous nucleation), compared to bubble formation in a homogenous solution (homogeneous nucleation) and can consequently occur at lower levels of supersaturation [27]. As a result, the bubble formation is facilitated by providing a high surface area in the reactor, which increases the gas-liquid mass transfer without the need to change other operating conditions [28].

5.4.1.1 Enhancement of H_2 production at increasing agitation speeds

In the absence of K1 carriers, agitation at 100 rpm during the first 15 h (phase 1) led to an accumulation of H_{2aq} to 29.7 (± 1.4) mL/L (Fig. 5.1C), i.e. 206% higher than the theoretically calculated value of 9.7 mL/L in equilibrium with the gas phase containing 65% H_2 at 80 °C as suggested by Henry's law [29]. Increasing the agitation from 15 h onwards (phase 2) induced a higher turbulence and improved the gas-liquid mass transfer of hydrogen, causing the decrease of H_{2aq} (Fig. 5.1C) and a significant increase of HPR (Fig. 5.3) in proportion to the agitation speed. In particular, the agitation at 600 rpm resulted in a H_{2aq} of 8.5 (± 0.1) mL/L

after 17 h (Fig. 5.1C), demonstrating that the required mass transfer for an equilibrium between the gas and liquid phase was provided.

No agitation (0 rpm) from 15 h onwards resulted in a further accumulation of H_{2aq} up to 40.6 (± 0.6) mL/L at 17 h (Fig. 5.1C). Between 15 and 17 h, approximately 70 mL H_2 /L/h was released to the gas phase despite the retention of hydrogen in the liquid phase. With the continuation of the process at 0 rpm, the H_{2aq} declined back to 32.8 (± 1.3) and 30.1 (± 1.3) mL/L after 20 and 23 h (Fig. 5.1C), respectively, indicating a net removal of hydrogen from the liquid phase. Regardless, the HPR decreased from 17 h onwards to approximately 30 mL H_2 /L/h, due to the self-regulatory bilateral interaction of H_{2aq} and HPR [12]. The time required by the culture to adapt to the change in mass transfer and reach the equilibrium between H_{2aq} and HPR was about 2 h.

5.4.1.2 Biogas recirculation to effectively reduce H_{2aq}

Similar to the highest agitation speed investigated (i.e. 600 rpm), the use of GaR decreased the H_{2aq} concentration below the theoretical equilibrium concentration. Concomitantly, dark fermentation was significantly accelerated, indicated by an increase of the HPR and BGR by 192% and 870%, respectively, in comparison to the condition without GaR (0 rpm) (Fig. 5.3), while the total cumulative hydrogen increased to 1345 (± 23) mL/L and more biomass (i.e. 2.36 (± 0.03) OD_{540}) was produced (Fig. 5.1A and B).

Similarly, Frigon et al. [26] studied H_{2aq} in an anaerobic upflow sludge bed and filter reactor. They reported the reduction of the H_{2aq} concentration from 1.1 μ M without recirculation to 0.4 μ M when biogas was recirculated at a flow of 85 L/L/d resulting in a higher gas production rate from approximately 4.5 to 12.0 L/L/d and an increase of COD removal. In line, Bakonyi et al. [21] reported an increase of HPR from 7.4 to 8.9 – 9.2 L H_2 /L/d by recirculating internal biogas with 50% H_2 in a continuously stirred tank reactor using a mixed culture.

The use of GaR to prevent the supersaturation of H_{2aq} is a technique rarely applied in dark fermentation [21,30]. Compared to inert gas sparging, the lower limit of H_{2aq} is defined by the equilibrium concentration with the gas phase when the hydrogen-rich biogas is recirculated. Therefore, an improvement by GaR can only be expected when hydrogen supersaturation occurs hampering the hydrogen production in the control condition. This

might explain why GaR has sometimes been reported ineffective when H_{2aq} was not directly monitored in the experiments [30].

5.4.1.3 K1 carriers to enhance hydrogen bubble formation

The addition of the K1 carriers at the beginning of the fermentation induced a better process performance in phase 1. Despite the same agitation speed of 100 rpm, the hydrogen production, glucose consumption and biomass growth were significantly higher (Fig. 5.1 and Fig. 5.2) compared to the other operating conditions. Several studies reported a faster process under batch conditions when different solid materials were placed inside the reactor [31,32]. Ngo et al. [32] ascribed the better performance to a higher efficiency of hydrogen production by immobilized compared to suspended cells. In our study, the attachment of cells onto the carrier was unlikely due to the short cultivation time, and the higher fermentation rates were rather induced by the approximately 50% lower H_{2aq} at 15 h (Fig. 5.1C). The lower H_{2aq} concentration was more likely caused by an enhanced bubble formation due to heterogeneous nucleation of the supersaturated hydrogen at the surface of the carrier material. Similarly, Fritsch et al. [28] obtained a higher production rate when placing a static monolith column inside a continuous dark fermentation system, ascribing the enhanced nucleation at the high surface area of the column as the responsible factor for the improved reactor performance. Also, Wu et al. [33] could achieve an HPR as high as $15.1 (\pm 0.3)$ L/L/h with a yield of $3.2 (\pm 0.1)$ mol H_2 /mol glucose, when applying silicone gel particles inside the reactor that promoted heterogeneous nucleation and improved the gas-liquid mass transfer. Unfortunately, Wu et al. [33] did not directly measure the H_{2aq} concentration in their study.

When the agitation speed was increased to 200 rpm after 15 h, the H_{2aq} concentration further decreased to $10.5 (\pm 0.1)$ mL/L in 0.5 h reaching a final value of $5.8 (\pm 0.5)$ mL/L after 19 h (Fig. 5.1C), which was similar to the value observed at 600 rpm in the absence of K1 carriers. This indicates that a similar gas-liquid mass transfer can be obtained at a 67% lower agitation speed by inducing bubble formation through heterogeneous nucleation, which would entail a considerable reduction of energy consumption and operating costs.

5.4.2 Impact of the H_{2aq} concentration on the hydrogen yield and rate of the process

When the release of H_2 from the fermentation broth is lower than the potential HPR of the system, hydrogen accumulates in the liquid phase. Elevated concentrations of H_{2aq} repress the HPR by product inhibition [16,17], which consequently results in a lower H_{2aq} concentration until an equilibrium is reached, primarily determined by the gas-liquid mass transfer of the system [12]. Ljunggren et al. [22] attributed this to a self-regulatory mechanism within microbial cultures, which decrease the growth rate and, consequently, the HPR to avoid hydrogen supersaturation.

Fig. 5.3 shows the H_{2aq} after 17 h of cultivation, which was considered to evaluate the impact on the cultures performance, revealing an inverse nonlinear correlation between the H_{2aq} and the HPR as well as the BGR. In the studied range of H_{2aq} , the HPR and the BGR increased by 413 and 1082%, respectively, when the H_{2aq} concentration was decreased from 40.6 to 8.5 mL/L. This correlation confirms that elevated H_{2aq} concentrations act as a strong inhibitor on the rates of dark fermentation. Furthermore, the variation of H_{2aq} at low (i.e. between 8.5 and 21.9 mL/L) H_{2aq} concentrations had a greater impact on the process compared to a change at high (i.e. between 21.9 and 40.6 mL/L) H_{2aq} concentrations.

Contrary to the HPR, the HY at different H_{2aq} concentrations only ranged between 2.0 and 2.5 mol H_2 /mol glucose with the highest value observed when the K1 carriers were used (Table 5.1). A slightly negative correlation between HY and H_{2aq} was obtained when applying agitation between 200 and 600 rpm (Table 5.1). On the other hand, the HY at 0 rpm was 2.3 (± 0.1) mol/mol glucose (Table 5.1), but it needs to be considered that only about 50% of the glucose was consumed at the end of the experiment (Fig. 5.2).

The H_{2aq} concentration had a more severe impact on the HPR compared to the HY. We assume that the HPR responds immediately to changes of the H_{2aq} concentration, due to a direct impact on the thermodynamics of the hydrogen producing reaction [16,17]. In contrast, a change in the yield of the process implies the culture to shift its metabolism to another pathway, requiring more energy and time. In agreement, Ljunggren et al. [22] reported the importance of the culture growth history for the performance of dark fermentation. We hypothesize that a prolonged cultivation at low H_{2aq} would have a positive effect on the hydrogen yield. This is supported by the highest total yield achieved when the H_{2aq} concentration was reduced in phase 1 by the use of K1 carriers.

Independent from the operating condition used, an average of $1.95 (\pm 0.5)$ mol H_2 /mol AA (Table 5.1) was produced. This is in accordance with the theoretical value of 2 mol H_2 /mol AA suggested by the dark fermentation model. Furthermore, dark fermentation predicts the formation of 2 moles of fermentation end products per mole of glucose consumed. The sum of AA and LA at the end of the fermentation accounted for 94 (± 5)% in the absence of K1 carriers and 85% at 200 + K1 (Fig. 5.2C). This discrepancy might be due to the production of alanine, an end product which is often produced to a minor fraction without formation of hydrogen [8].

5.4.3 Outlook

This study showed the importance of an efficient mass transfer of hydrogen in dark fermentation. Agitation at 600 rpm effectively kept H_{2aq} low, reaching high HPR and HY. However, accomplishing the required turbulence by agitation at larger scale is challenging and create an elevated economic burden. Furthermore, based on the proportionate increase of mass transfer with agitation speed, 600 rpm might be insufficient to prevent hydrogen supersaturation if the HPR would further increase, e.g. by higher biomass concentrations. Biogas recirculation is a promising alternative to agitation by stirring and has shown to effectively enhance the gas-liquid mass transfer [21,26]. The potential of GaR is further emphasized by the possibility to adjust the recirculation flow rates or apply intermittent GaR to meet the required mass transfer without unnecessary energy consumption. Further research is required to compare the operating costs for GaR and stirring at a larger scale and investigate the slightly lower hydrogen yields and rates despite a similar H_{2aq} concentration compared to 600 rpm.

The application of a solid material mixed in the liquid solution has also revealed potential to maintain a high gas-liquid mass transfer through bubble formation by heterogeneous nucleation at a much reduced agitation speed. The bubble formation largely depends on the area and the nature of the provided surface, whereat bubble formation is favored by a rougher surface [27]. Therefore, K1 carrier might not be the best option, especially considering the high capital costs for their application at a larger scale. Different, cheaper materials should be further investigated for their bubble formation capacity. Commonly, solid materials are used in biological processes to promote biofilm growth and consequently high biomass concentrations in the reactor, leading to an acceleration and a better stability

of the process. The combined positive effect of biomass retention and improved mass transfer strongly suggests the use of immobilized cell reactors for dark fermentation. However, many aspects of such a process, including the effect of biofilm formation on bubble formation and the influence of attached biomass on the required agitation speed, still need to be investigated. Only then, the savings in energy consumption and operating costs indicated by the lab scale experiments in the present work can be confirmed.

Conclusion

This study has shown the importance of the hydrogen gas-liquid mass transfer in dark fermentation. Hydrogen accumulated up to 29.7 (\pm 1.4) mL/L at 100 rpm, resulting in a supersaturation of 206% compared to the equilibrium concentration of 9.7 mL/L suggested by Henry's law. Stopping the agitation further increased the H_{2aq} to 40.6 (\pm 0.6) mL/L after 17 h, which decreased back to 30.1 (\pm 1.3) mL/L at 23 h due to the equilibrium reached between H_{2aq} and HPR. The inhibitory effect on HPR decreased nonlinearly with decreasing H_{2aq} . Hence, the low H_{2aq} concentration of 8.5 (\pm 0.1) mL/L at 600 rpm led to a 413% increase of HPR compared to that obtained in the absence of agitation. The use of GaR at 0 rpm and the addition of the K1 carriers at 200 rpm similarly reduced the H_{2aq} to 8.5 (\pm 0.4) and 9.3 (\pm 3.2) mL/L, respectively. In particular, the use of a solid carrier indicates a high potential to keep the H_{2aq} concentration low by increasing the gas-liquid mass transfer through heterogeneous nucleation of the supersaturated hydrogen, without requiring additional energy for mixing or recirculating the gas.

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6 Effect of feed glucose and acetic acid on continuous biohydrogen production by *Thermotoga neapolitana*

(Paper IV)

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Abstract

This study focused on the biohydrogen production under continuous-flow conditions using a pure culture of *Thermotoga neapolitana*. We investigated the effect of different feed glucose and acetic acid concentrations on the dark fermentation at a hydraulic retention time of 24 h. Increasing the feed glucose concentration from 11.1 to 41.6 mM decreased the hydrogen yield from 3.6 (\pm 0.1) to 1.4 (\pm 0.1) mol H₂/mol glucose. The hydrogen production rate concomitantly increased until feed glucose was risen to 27.8 mM. At feed glucose of 41.6 mM, the hydrogen production rate remained stable due to the conversion of additional glucose exclusively via the non-hydrogen producing lactic acid pathway. Increasing the acetic acid concentration in batch bioassays from 0 to 240 mM decreased the cumulative hydrogen production from 1739 (\pm 12) to 950 (\pm 29) mL/L as well as the hydrogen production rate from 265 (\pm 7) to 115 (\pm 0) mL/L/h. In contrary, raising the feed acetic acid concentration up to 240 mM during continuous operation did not inhibit the process resulting in a hydrogen yield of 3.0 (\pm 0.2) mol H₂/mol glucose. The overall operation led to an enhancement of the process performance, as indicated by the 47% higher hydrogen yield obtained on day 110 at 27.8 mM feed glucose.

Key words: *Thermotoga neapolitana*; Hydrogen; Continuous-flow dark fermentation; Acetic acid; Feed concentration; Inhibition

Abbreviations

AA	Acetic acid
BGR	Biomass growth rate
CDW	Cell dry weight
CSTR	Continuous stirred tank reactor
Glu	Glucose
HPR	Hydrogen production rate
HY	Hydrogen yield
LA	Lactic acid
VFAs	Volatile fatty acids

6.1 Introduction

In the past decades, our society has been primarily fueled by fossil resources, which are non-sustainable and polluting energy sources, releasing green-house gases and other toxic compounds upon combustion (Elbeshbishy et al., 2017). The resulting aggravation of air pollution, global warming and extreme weather phenomena in recent years have indicated that a continuing excessive use of fossil fuels will have devastating results on the climate, sea levels and the quality of living for a large part of the population worldwide. To counteract this negative trend, an increasing amount of research is being dedicated to find and establish sustainable sources for clean energy.

Hydrogen has been identified as a highly versatile energy carrier, providing high energy density, a good conversion efficiency, without creating further pollution upon combustion (Baykara, 2018). In this regard, biological processes have gained increased attention, representing a green and sustainable alternative to produce hydrogen. Among these, dark fermentation is considered the most promising (Arimi et al., 2015), resulting in a high productivity with a flexible and simple operation (Sivagurunathan et al., 2016), while allowing the use of waste streams as a substrate.

Thermotoga neapolitana is a hyperthermophilic bacterium which has a high potential for dark fermentative hydrogen production (Chou et al., 2008; Pradhan et al., 2015). Hydrogen yields (HY) approaching the theoretical value of 4 mol H₂/mol hexose, fast growth kinetics and a large range of potential substrates are its main advantages (Pradhan et al., 2015). Up to now, *T. neapolitana* has been exclusively studied in batch or semi batch operation with the aim of identifying the optimal range of operating parameters such as temperature, pH and mixing (Munro et al., 2009; Nguyen et al., 2008; Pradhan et al., 2015).

However, in large scale applications, a continuous-flow process is generally preferred (Balachandar et al., 2013; Kumar et al., 2014). Besides the hydraulic retention time (HRT), one of the most important parameters in a continuous process is the organic loading rate (OLR), which is defined by the ratio between the influent substrate concentration and the HRT (Arimi et al., 2015; Elbeshbishy et al., 2017; Sivagurunathan et al., 2016). The increase of the OLR within a certain range allows a more energy-efficient operation (Jung et al., 2011) and has shown to enhance H₂ production in dark fermentation (Arimi et al., 2015; Elbeshbishy et al., 2017; Hawkes et al., 2007; Lin et al., 2012; Sivagurunathan et al., 2016).

High feed substrate concentrations do not only lead to an increased hydrogen production, but also to higher concentrations of fermentation end products, e.g. volatile fatty acids (VFAs) and alcohols. When these products exceed a certain threshold level, which is specific to the microbial culture and the particular substrate used (Lin et al., 2012), inhibition of dark fermentation can occur (Lin et al., 2012; Sivagurunathan et al., 2016) resulting in changes of the H₂ producing pathways as well as the microbial activity (Ciranna et al., 2014; Jung et al., 2011). Feedback inhibition, which acts on the HY as well as the hydrogen production rate (HPR) (Tang et al., 2012), is considered one of the main challenges in dark fermentation (Boodhun et al., 2017). Therefore, it is essential for a dark fermentative hydrogen production process to find the substrate concentrations that allow for the highest HPR and efficiency, while minimizing the effect of inhibitory compounds.

In the present study, we established for the first time a continuous hydrogen production process using a pure culture of *T. neapolitana*. The main goal of this study was to initially determine the impact of different feed glucose concentrations on the process yields and rates. Secondly, the inhibition by acetic acid (AA), i.e. the main fermentation end product, on H₂ production and biomass growth was investigated in both batch and continuous experiments.

6.2 Material and Methods

6.2.1 Bacterial culture and medium

A pure culture of *Thermotoga neapolitana* purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was cultivated and stored according to Dreschke et al. (2018) and subsequently used in all experiments. The medium was based on a modified ATCC 1977 medium (Dreschke et al., 2018), in which glucose and AA concentrations were varied as specified in section 2.2. The medium was autoclaved at 110 °C for 5 min, pH-adjusted to 7 and sparged with N₂ for 10 min to establish anaerobic conditions.

6.2.2 Experimental design

Both continuous and batch experiments were run using a working volume of 2 L, a constant temperature of 80 °C and a pH of 7, automatically adjusted by adding 5M NaOH in a 3-L fully controlled, continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands). To avoid pressure build-up, the produced biogas was continuously released from the headspace of the reactor.

6.2.2.1 Continuous process

After the inoculation with 6% (v/v) of storage culture, the reactor was run in batch mode for 16 h to allow the culture to grow and acclimatize. After this initial phase, the feeding was started in continuous mode at a flow rate of 83.3 mL/h to maintain an HRT of 24 h. The feed medium was stored at 4 °C after autoclaving and removing the oxygen by sparging the headspace of the container with N₂ for 10 min. The working volume was controlled using a level probe. Twice a day, liquid samples were drawn for the determination of turbidity (OD₅₄₀), glucose, AA and lactic acid (LA) concentrations. Furthermore, 200 mL of effluent was taken to determine the cell dry weight (CDW). The biogas production rate was measured by measuring the time to fill a 500 mL water displacement system.

Initially, the reactor was operated to investigate the effect of the OLR on dark fermentation by varying the glucose concentration in the feed as reported in Table 6.1. From day 83 onwards, the feed glucose was maintained constant at 27.8 mM, while the AA concentration was gradually increased from 30 to 240 mM (Table 6.1) to evaluate the effect of increasing AA concentrations on the process. The feed glucose and AA concentrations were changed when a steady state was reached, determined by a variation of the hydrogen production by less than 10%.

Table 6.1: Feed glucose (Glu) and acetic acid (AA) concentrations used in the operation of the continuously stirred tank reactor aimed at dark fermentation by *T. neapolitana*. Seven (G1 - G7) and six (AA1 – AA6) experimental phases were used to assess the effect of Glu and AA, respectively. Feed Glu remained at 27.8 mM when increasing the feed AA.

Phase	Feed Glu [mM]	Operation period [d]	Phase	Feed AA [mM]	Operation period [d]
G1	27.8	0 - 14	AA1	30	83 - 87
G2	41.6	15 - 25	AA2	60	88 - 92
G3	27.8	26 - 36	AA3	90	93 - 96
G4	16.7	37 - 47	AA4	120	97 - 107
G5	11.1	48 - 54	AA5	180	102 - 106
G6	22.2	55 - 64	AA6	240	107 - 110
G7	27.8	65 - 82			

6.2.2.2 Batch bioassays

Simultaneously to the continuous-flow operation, batch bioassays were run in order to assess the effect of the AA concentration (i.e. 0, 30, 60, 90, 120, 180 and 240 mM) on dark fermentation by *T. neapolitana*. Prior to investigating each condition, the reactor was inoculated with 1% (v/v) of storage culture and stirred at 100 rpm for 15 h to allow the culture to grow and acclimatize. After 15 h, the agitation speed was increased to 500 rpm to accelerate the process. The produced biogas was captured in a 500 mL water displacement system and quantified every hour. Liquid samples of 2 mL were drawn every hour to measure turbidity as well as the glucose, AA and LA concentrations. The fermentation was terminated after 23 h or previously, when the reactor ceased to produce further biogas. Duplicates were used for each operating condition.

6.2.3 Analytical methods

The biomass concentration of batch bioassays was quantified by measuring the optical density (OD₅₄₀) at 540 nm (8453 UV-Visible Spectrophotometer, Agilent Technologies, USA), whereas in the continuous experiment 200 mL of effluent was dried at 105 °C until constant weight to determine the CDW. Subsequently, the samples were centrifuged (10,000 rpm at 5 min) and the supernatant was used to measure the concentrations of glucose, AA and LA applying the method described by Mancini et al. (2018) with an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors and 0.0065 M of sulfuric acid as the mobile

phase. The hydrogen concentration of the biogas was measured with a Varian 3400 gas chromatograph (GC), equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas. The hydrogen production was converted from volumetric to molar by using the ideal gas law (O-Thong et al., 2008).

6.2.4 Kinetic study of biohydrogen production and biomass growth

The rates of biomass growth and hydrogen production were determined by fitting the batch experimental data with the Gompertz model as described by Dreschke et al. (2018). Eq. 6.1 and Eq. 6.2 were applied for biomass growth and hydrogen production, respectively:

$$B = B_0 + B_m \exp \left\{ -\exp \left[\frac{R_B e}{B_m} (\lambda_B - t) + 1 \right] \right\} \quad (\text{Eq. 6.1})$$

$$H = H_m \exp \left\{ -\exp \left[\frac{R_H e}{H_m} (\lambda_H - t) + 1 \right] \right\} \quad (\text{Eq.6.2})$$

where B [OD₅₄₀] is the biomass concentration at fermentation time t [h]; B_0 [OD₅₄₀] is the biomass concentration at time 0 h; B_m [OD₅₄₀] is the gain of biomass concentration throughout the fermentation; R_B is the volumetric biomass growth rate (BGR) [OD₅₄₀/h]; and λ_B is the lag phase of biomass growth [h]; H [mL] is the cumulative hydrogen at time t [h]; H_m [mL] is the hydrogen produced throughout the fermentation; R_H [mL/L/h] is the volumetric hydrogen production rate; and λ_H is the lag phase of hydrogen production [h]; and e is the Euler's number, i.e. 2.72.

6.2.5 Microbial community analysis

On day 82 and 102 of the continuous operation, 3 mL of liquid sample were extracted for the determination of the microbial community. DNA was extracted, stored and sequenced as explained by Kostytsia et al. (2018). Quality filtering, sequence clustering, chimera removal and taxonomy assignment using the Silva (v.128) database (Glöckner et al., 2017; Pruesse et al., 2007) was applied on the raw sequence data before generating an operational taxonomic unit (OTU) table via the Quantitative Insight into Microbial Ecology (QIIME v1.9) pipeline (Caporaso et al., 2010). A threshold of 0.1% was employed to distinguish rare from abundant taxa.

6.3 Results and Discussion

6.3.1 Effect of glucose concentration in a continuous system

6.3.1.1 Limit of feed glucose concentration

Fig. 6.1 shows the evolution of dark fermentation in continuous operation at different feed glucose concentrations. During phase G1, a feed glucose concentration of 27.8 mM led to a stable process. Within 1 day, a HPR of 55.2 (\pm 4.7) mL/L/h (Table 6.2), a HY of 2.1 (\pm 0.2) mol H₂/mol glucose and a biomass yield of 26.9 (\pm 1.2) g CDW/mol glucose were reached (Fig. 6.1A) and the produced biogas contained 70 (\pm 4)% of H₂ (data not shown). The process remained stable for the subsequent 10 days of operation producing 30.9 (\pm 0.7) mM of AA and 17.6 (\pm 1.1) mM of LA (Fig. 6.1B), with an AA/LA ratio of 1.8 (\pm 0.2) (Fig. 6.2A) and a residual glucose concentration of 2.1 (\pm 0.1) mM (Fig. 6.1B).

Table 6.2: Hydrogen production rate (HPR) and biomass concentration (BM) obtained during the continuous dark fermentation by *T. neapolitana* at different feed glucose (phases G1 – G7) and acetic acid (phases AA1 – AA6) concentrations.

Phase	Feed Glu [mM]	HPR [mL/L/h]	BM [mg CDW/L]	Phase	Feed AA [mM]	HPR [mL/L/h]	BM [mg CDW/L]
G1	27.8	55.2 (\pm 4.7)	698 (\pm 39)	AA1	30	79.7 (\pm 2.6)	701 (\pm 34)
G2	41.6	53.7 (\pm 4.0)	703 (\pm 20)	AA2	60	78.3 (\pm 3.1)	678 (\pm 18)
G3	27.8	54.7 (\pm 4.9)	679 (\pm 21)	AA3	90	75.3 (\pm 2.9)	621 (\pm 19)
G4	16.7	50.3 (\pm 3.2)	574 (\pm 27)	AA4	120	78.5 (\pm 3.9)	694 (\pm 23)
G5	11.1	33.8 (\pm 0.8)	440 (\pm 26)	AA5	180	83.8 (\pm 2.6)	703 (\pm 47)
G6	22.2	61.2 (\pm 4.3)	655 (\pm 54)	AA6	240	80.8 (\pm 1.8)	710 (\pm 26)
G7	27.8	72.8 (\pm 3.4)	702 (\pm 36)				

Increasing the feed glucose concentration to 41.6 mM in phase G2 did not improve the reactor performance exhibiting an HPR of 53.7 (\pm 4.0) mL/L/h, similar to that observed in phase G1 (Table 6.2). The glucose concentration in the effluent increased to 4.2 (\pm 0.6) mM, while the AA concentration remained unaffected at 28.2 (\pm 1.1) mM and the LA concentration increased sharply to 43.2 (\pm 2.3) mM (Fig. 6.1B), entailing an AA/LA ratio of 0.7 (\pm 0.1) (Fig. 6.2A). In *T. neapolitana*, only 2 pathways are involved to a relevant extent in the dark fermentation of glucose, i.e. the AA pathway yielding 4 moles of hydrogen and 4 moles of ATP per mole of glucose and the energetically less challenging LA pathway producing no hydrogen but 2 moles of ATP (Balachandar et al., 2013; Pradhan et al., 2015). The AA/LA

ratio is, thus, tightly linked to the HY as shown in Fig. 6.2A and represents another indicator for the conversion efficiency to hydrogen. Consequently, the decrease of the AA/LA ratio in phase G2 was accompanied by the reduction of the HY to $1.4 (\pm 0.1)$ mol H₂/mol glucose (Fig. 6.1A and Fig. 6.2A).

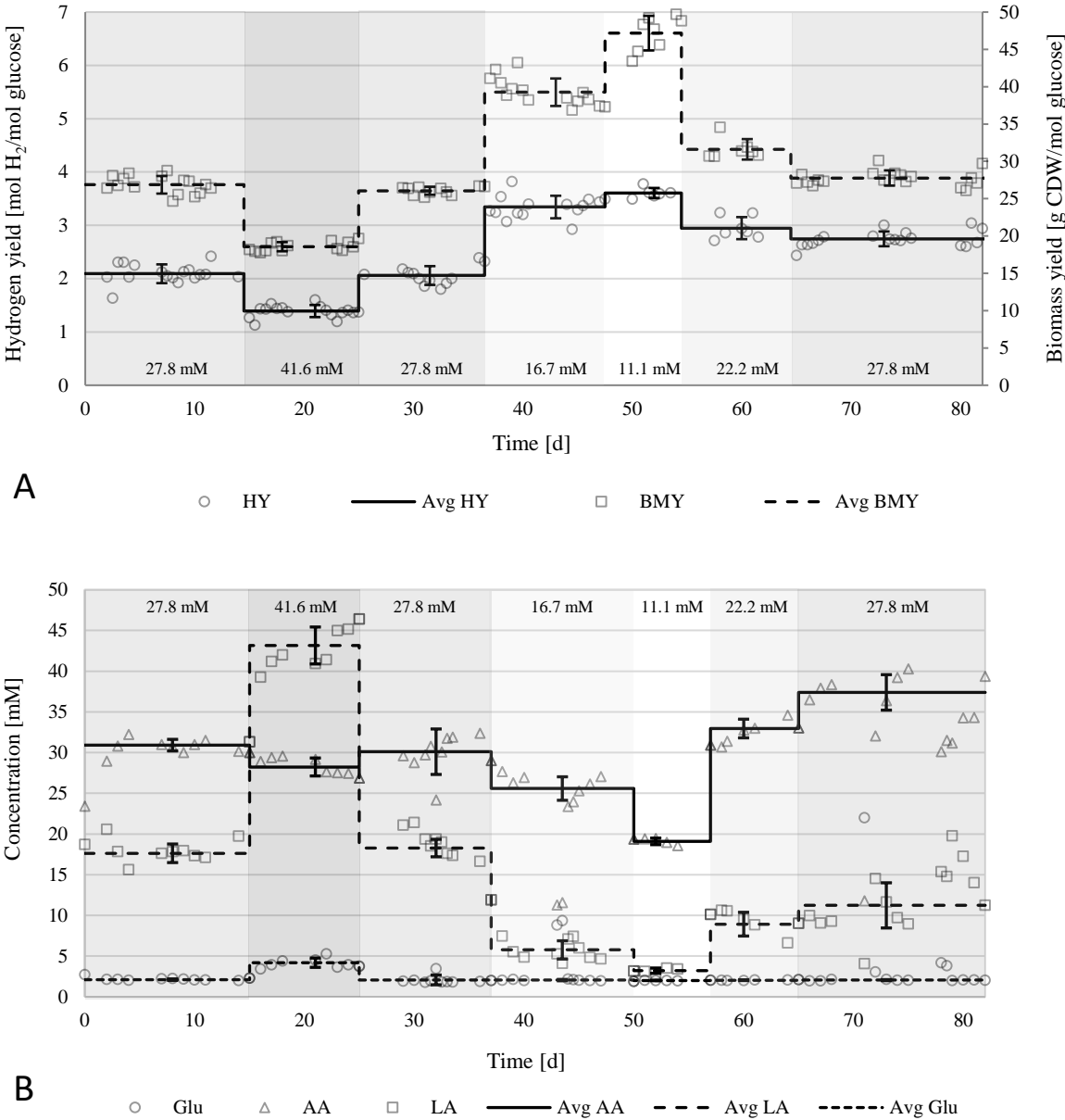


Fig. 6.1: Continuous hydrogen production by *T. neapolitana* at varying feed glucose (Glu) concentrations (11.1 – 41.6 mM) and a HRT of 24 h. Yields of biomass (BMY) and hydrogen (HY) (A) as well as residual Glu, acetic acid (AA) and lactic acid (LA) concentrations in the effluent (B). Error bars depict the standard deviation.

The lower H₂ production efficiency at 41.6 mM of feed glucose strongly suggests a substrate overload of *T. neapolitana*, which is commonly observed in dark fermentation (Akutsu et al., 2009; Hafez et al., 2010). For instance, Zhang et al. (2013) raised the feed glucose concentration from 5 to 15 g/L in a CSTR at an HRT of 6 h using *Clostridium bifermentans* 3AT-*ma*. Similar to our results, this induced a decrease of the HY and AA concentration from 1.1 to 0.7 mol H₂/mol glucose and from 10.0 to 6.8 mM, respectively, with a sharp increase of the LA and butyric acid concentrations. Zhang et al. (2013) assumed the VFA accumulation responsible for the HY decrease. Due to the low residual glucose concentration, i.e. between 2.2 (± 0.0) and 4.2 (± 0.6) mM in the effluent (Fig. 6.1B), we assume a similar effect (Elbeshbishy et al., 2017; Zhang et al., 2013) prevented the further AA formation and subsequently limited the hydrogen production.

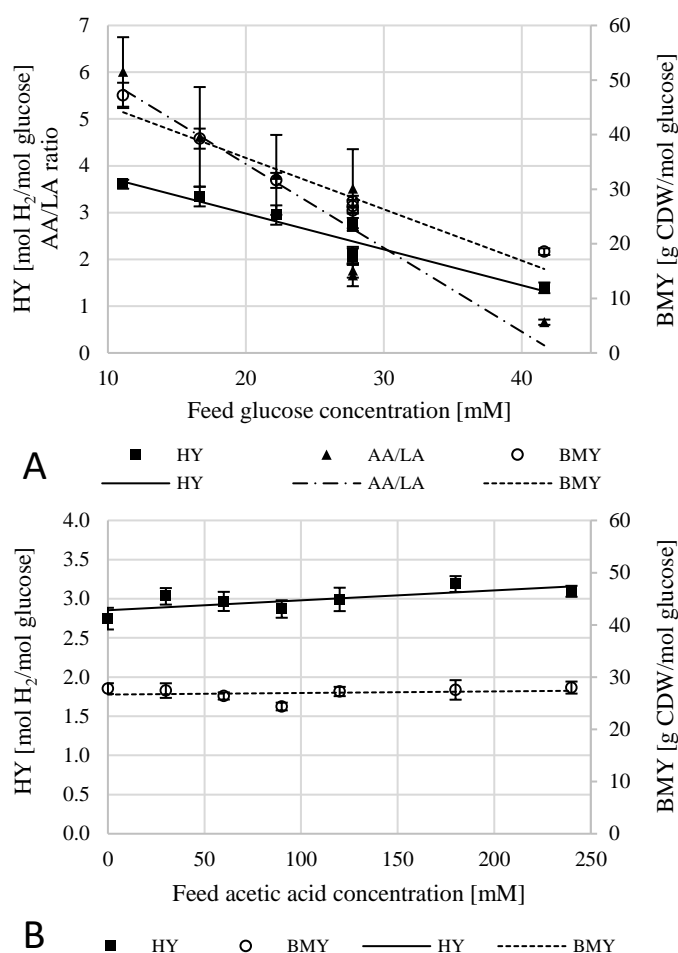


Fig. 6.2: Hydrogen yield (HY), biomass yield (BMY) and acetic acid to lactic acid ratio (AA/LA) of a continuous dark fermentation by *T. neapolitana* at (A) different feed glucose (11.1 – 41.6 mM) and (B) acetic acid (0 – 240 mM) concentrations. Error bars depict the standard deviation.

6.3.1.2 Effect of feed glucose concentration on the hydrogen and biomass yield

To better study the effect of the glucose concentration on dark fermentation, lower feed glucose concentrations (i.e. 16.7, 11.1 and 22.2 mM) were used in phases G4, G5 and G6, respectively. The reduction of the feed concentration increased the HY to 3.3 (\pm 0.2), 3.6 (\pm 0.1) and 2.9 (\pm 0.2) mol H₂/mol glucose and biomass yield to 39.3 (\pm 1.8), 47.2 (\pm 2.3) and 31.6 (\pm 1.4) g CDW/mol glucose, in phases G4, G5 and G6, respectively (Fig. 6.1A). Concomitantly, the AA/LA ratio increased to 4.6 (\pm 1.1), 6.0 (\pm 0.7) and 3.8 (\pm 0.9) (Fig. 6.2A). This revealed an almost linear negative correlation between the HY and feed glucose concentration (Fig. 6.2A), with a maximum yield of 3.6 (\pm 0.1) mol H₂/mol glucose at the lowest feed (i.e. 11.1 mM) and the concomitant shift from AA to LA at increasing feed glucose concentrations (Fig. 6.1B). A similar correlation was observed for the biomass yield (Fig. 6.2A). The biomass concentration increased with the feed glucose concentration until reaching a plateau at 687 (\pm 21) mg CDW/L above 22.2 mM of feed glucose (Table 6.2). It is unclear why the biomass concentration did not increase further at higher glucose concentrations.

Up to now, the effect of substrate concentration on *T. neapolitana* activity has exclusively been studied in batch operation, most commonly in 120 mL closed serum bottles without pH control (Ngo et al., 2012; Nguyen et al., 2008; Nguyen et al., 2010). While reporting a notable increase of the HY with increasing glucose (Nguyen et al., 2008; Nguyen et al., 2010) or xylose (Ngo et al., 2012) concentrations up to a certain threshold level, the results obtained under these conditions are generally highly affected by the decrease of pH (Brynjarsdottir et al., 2013) and the build-up of the hydrogen partial pressure (Ngo et al., 2012). Mars et al. (2010) used *T. neapolitana* in a pH-controlled reactor using headspace sparging. When increasing the glucose concentration from 10 to 27 g/L, the HY and HPR remained similar at 2.9 and 3.0 mol H₂/mol glucose as well as 12.3 and 12.4 mmol/L/h, respectively. However, the fermentation time increased from 20 to over 71 h. Similarly, the general HPR increased and HY decreased when increasing the substrate concentration of a continuous-flow reactor with mixed cultures, using glucose (van Ginkel and Logan, 2005b) or organic-containing wastewater (Lin et al., 2012) as substrates.

Table 6.3: Cumulative hydrogen production and hydrogen yield (HY), final concentrations of glucose (Glu), acetic acid (AA) and lactic acid (LA) in the effluent and the molar AA/LA ratio after the batch dark fermentation of 27.8 mM of glucose by *T. neapolitana* at different initial AA concentrations (0 – 240 mM).

Initial AA [mM]	Cumulative H ₂ [mL]	HY [mol/mol]	residual Glu [mM]	AA [mM]	LA [mM]	AA/LA ratio
0	1739 (± 12)	2.8 (± 0.0)	2.7 (± 0.1)	33.2 (± 0.2)	14.6 (± 0.9)	2.28
30	1474 (± 112)	2.4 (± 0.2)	2.7 (± 0.1)	29.2 (± 0.1)	17.5 (± 1.6)	1.67
60	1402 (± 27)	2.3 (± 0.0)	3.2 (± 0.2)	28.6 (± 2.1)	18.0 (± 2.4)	1.59
90	1273 (± 54)	2.1 (± 0.0)	3.2 (± 0.1)	27.6 (± 0.6)	20.7 (± 0.6)	1.34
120	1167 (± 34)	2.0 (± 0.0)	3.8 (± 1.0)	27.0 (± 1.1)	21.2 (± 2.3)	1.28
180	1066 (± 67)	2.1 (± 0.0)	7.8 (± 1.2)	18.4 (± 1.8)	15.4 (± 1.4)	1.20
240	950 (± 29)	2.3 (± 0.0)	11.0 (± 1.6)	16.5 (± 1.2)	11.2 (± 0.2)	1.48

6.3.2 Impact of the initial AA concentration in batch bioassays

6.3.2.1 Effect on hydrogen production and yield

The batch fermentation of 27.8 mM of glucose by *T. neapolitana* without AA addition resulted in a HY of 2.8 (± 0.0) mol H₂/mol glucose and an AA/LA ratio of 2.3 (Table 6.3). Increasing the initial AA concentration from 0 to 240 mM gradually reduced the total hydrogen production from 1739 (± 12) to 950 (± 29) mL/L (Table 6.3). Up to 120 mM of AA, glucose was completely consumed within 23 h with a 30% decrease of the HY to 2.0 (± 0.0) mol H₂/mol glucose, accompanied by a decline of the AA/LA ratio (Table 6.3).

The reduction of the HY at elevated concentrations of fermentation end products is commonly observed in dark fermentation (Jones et al., 2017; Tang et al., 2012; van Ginkel and Logan, 2005a; Wang et al., 2008). For example, Ciranna et al. (2014) reported the decrease of HY from 3.0 (± 0.2) to 0.6 (± 0.4) mol H₂/mol glucose when increasing the AA concentration from 40 to 225 mM in a batch fermentation of 55 mM glucose using *Caloramator celer* in 120 mL closed serum bottles.

In this study, a further increase of the feed AA to 180 and 240 mM led to an elevated residual glucose concentration, i.e. 7.8 (± 1.2) and 11.0 (± 1.6) mM, in the effluent after 23 h and a slight increase of the HY to 2.1 (± 0.0) and 2.3 (± 0.0) mol H₂/mol glucose, respectively (Table 6.3). A more detailed analysis revealed that the AA/LA ratio was 161 (± 84) % higher from 15 to 18 h than from 18 to 22 h in all batch bioassays. This indicates that the HY was higher in the early stages of the experiment. A possible cause for this effect is the higher

initial glucose concentration. Nguyen et al. (2010) reported a considerable increase of the HY when increasing the initial glucose concentration from 1 to 4 g/L in batch fermentation by *T. neapolitana*. Therefore, we assume that the slightly higher HY at 180 and 240 mM AA was not linked to the AA concentration, but rather caused by the evolution of the process.

6.3.2.2 Dark fermentation kinetics

In a continuous-flow operation, the process rate is strongly determined by the substrate feeding rate. Therefore, batch bioassays were performed to study the effect of AA on the biomass growth and hydrogen production kinetics of *T. neapolitana*.

As indicated by the incomplete glucose consumption at 180 and 240 mM AA, an elevated initial AA concentration notably decreased the dark fermentation rate. To better compare the results obtained at different AA feed concentrations, a modified Gompertz model was used to fit the data of hydrogen production and biomass growth (Fig. 6.3A and B). Under all operating conditions, the quality of the fit was confirmed by an R^2 of 0.99 (± 0.00) for hydrogen production and 0.98 (± 0.01) for biomass growth. Without AA in the medium, the HPR and BGR reached 265 (± 7) mL/L/h and 0.27 (± 0.03) OD₅₄₀/h, respectively (Fig. 6.4A). The low BGR obtained at 0 mM AA was presumably caused by the high biomass growth already achieved after 15 h (Fig. 6.3B), leading to a distorted value from the Gompertz model.

In the range between 30 - 240 mM, the initial AA concentration was found to be negatively correlated to the BGR and the HPR, which decreased from 0.42 (± 0.06) to 0.24 (± 0.04) OD₅₄₀/h and from 230 (± 7) to 115 (± 0) mL/L/h, respectively (Fig. 6.4A). This corresponds to a deceleration of hydrogen production and biomass growth by 50 and 43%, respectively. The slowdown of the fermentation was furthermore confirmed by an increase of the lag phase from 14.0 (± 0.3) to 15.7 (± 0.4) h for biomass growth and from 15.7 (± 0.1) to 18.0 (± 0.1) h for hydrogen production (Fig. 6.4B). Both lag phases were similarly affected by the AA concentration increase by approximately 2.5 h in the studied AA concentration range (i.e. 0 - 240 mM) (Fig. 6.4B). However, the hydrogen production initiated about 2.2 h after the biomass growth according to the Gompertz analysis.

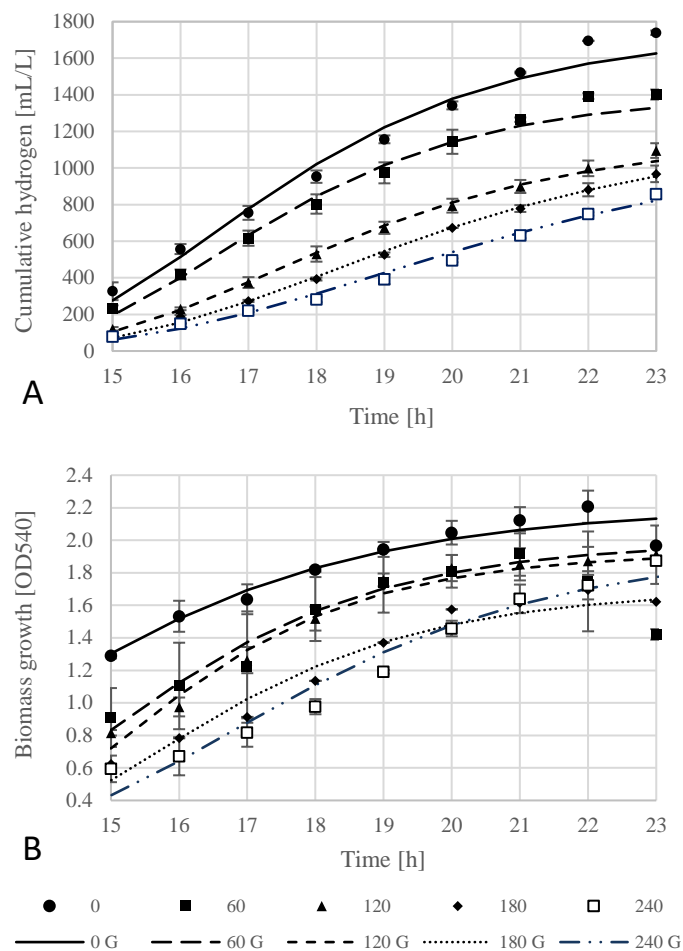


Fig. 6.3: Evolution of cumulative hydrogen (A) and biomass growth (B) in batch bioassays fermenting 27.8 mM glucose with *T. neapolitana* at different initial AA concentrations (0 – 240 mM). The symbols depict the experimental data while the lines exhibit the Gompertz model. Error bars depict the standard deviation.

Mars et al. (2010) previously investigated the effect of increased AA concentrations on the performance of *T. neapolitana* using closed 120 mL serum bottles without pH control. They reported biomass growth at up to 300 mM AA with a decreasing amount of total VFAs produced with increasing initial AA concentration. However, they did not present detailed information on the production of AA and hydrogen or the evolution of the fermentation.

The effect of AA on dark fermentation of pure cultures was studied in more detail by Van Niel et al. (2003) and Ciranna et al. (2014) in 120 mL closed serum bottles without pH control. Van Niel et al. (2003) added sodium acetate in the exponential growth phase of the

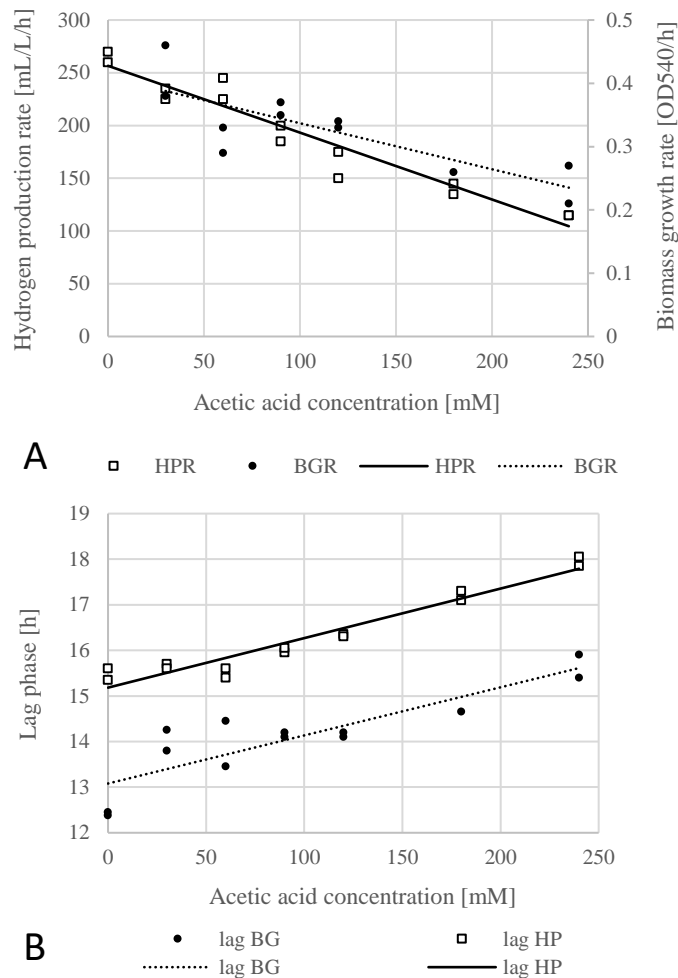


Fig. 6.4: Effect of different initial acetic acid (AA) concentrations on (A) biomass growth (BGR) and hydrogen production (HPR) rates and (B) the lag phases of hydrogen production (HP) and biomass growth (BG) during the batch dark fermentation of 27.8 mM of glucose as substrate with *T. neapolitana*.

extreme thermophile *Caldicellulosiruptor saccharolyticus* obtaining concentrations from 0 to 300 mM AA, while Ciranna et al. (2014) investigated the effect of an initial AA concentration up to 350 mM on *Caloramator celer*. Both studies observed a drastic decrease of the process rate, revealed by a reduction of the HPR and the BGR. Concomitantly, they reported no further biomass growth above 200 mM AA for *Caldicellulosiruptor saccharolyticus* (van Niel et al., 2003) and approximately 150 mM AA for *Caloramator celer* (Ciranna et al., 2014). In both studies, increasing the undissociated AA fraction in the fermentation broth due to a pH decrease did not alter the inhibitory effect. Furthermore, Van Niel et al. (2003) found the inhibition of sodium chloride and sodium acetate to be identical. Therefore, both studies concluded that ionic strength was responsible for the inhibition of dark fermentation rather than the free AA.

6.3.3 Effect of acetic acid concentration in a continuous system

In the second stage of the continuous process, the feed AA concentration was gradually increased from 0 to 240 mM. The HPR, biomass concentration and H₂ percentage in the produced biogas remained unaffected reaching 77.9 (± 4.5) mL/L/h, 694 (± 39) mg CDW/L (Table 6.3) and 69 (± 1)% (data not shown), respectively. Glucose was completely consumed under all operating conditions (Fig. 6.5B), entailing that also the hydrogen and biomass yields remained constant at 3.0 (± 0.2) mol H₂/mol glucose and 27.1 (± 1.6) g CDW/mol glucose, respectively (Fig. 6.2B and 5A). Hence, contrary to the results obtained in the batch bioassays, the increase of the feed AA concentration had no effect on the continuous-flow dark fermentation. In particular, the HY of the continuous process increased by approximately 6% at 0 mM AA and 48% at 120 mM AA compared to the batch experiments. We assume that the prolonged cultivation at high AA concentrations allows *T. neapolitana* to adapt its metabolism and continue to ferment via the energetically more challenging AA pathway, which results in a higher yield of ATP (Pradhan et al., 2015).

Van Ginkel (van Ginkel and Logan, 2005a) studied the effect of undissociated VFAs on continuous dark fermentation of a mixed culture operated at 30 °C, an HRT of 10 h and a stable pH of 5.5 with glucose (10 – 50 g/L) as a substrate. A total AA concentration of approximately 10, 100 and 165 mM, resulting in an undissociated AA concentration of 2, 15 and 25 mM, induced HYs of about 2.5, 2.4 and 2.0 mol H₂/mol glucose and HPRs of 0.29, 0.30 and 0.20 L/h, indicating a little effect of non-dissociated AA on H₂ yields. Jones et al. (2017) ran a continuous hydrogen production reactor with a mixed culture (35 °C; pH 5.5; HRT 48 h and 40 g sucrose/L as a substrate) applying electrodialysis to remove VFAs from the liquid phase. When the AA concentration was decreased from 3.08 to 1.77 g/L, the HY increased from 0.24 to 0.90 mol H₂/mol hexose and the carbohydrate consumption from 12 to 25%, indicating a higher dark fermentation rate.

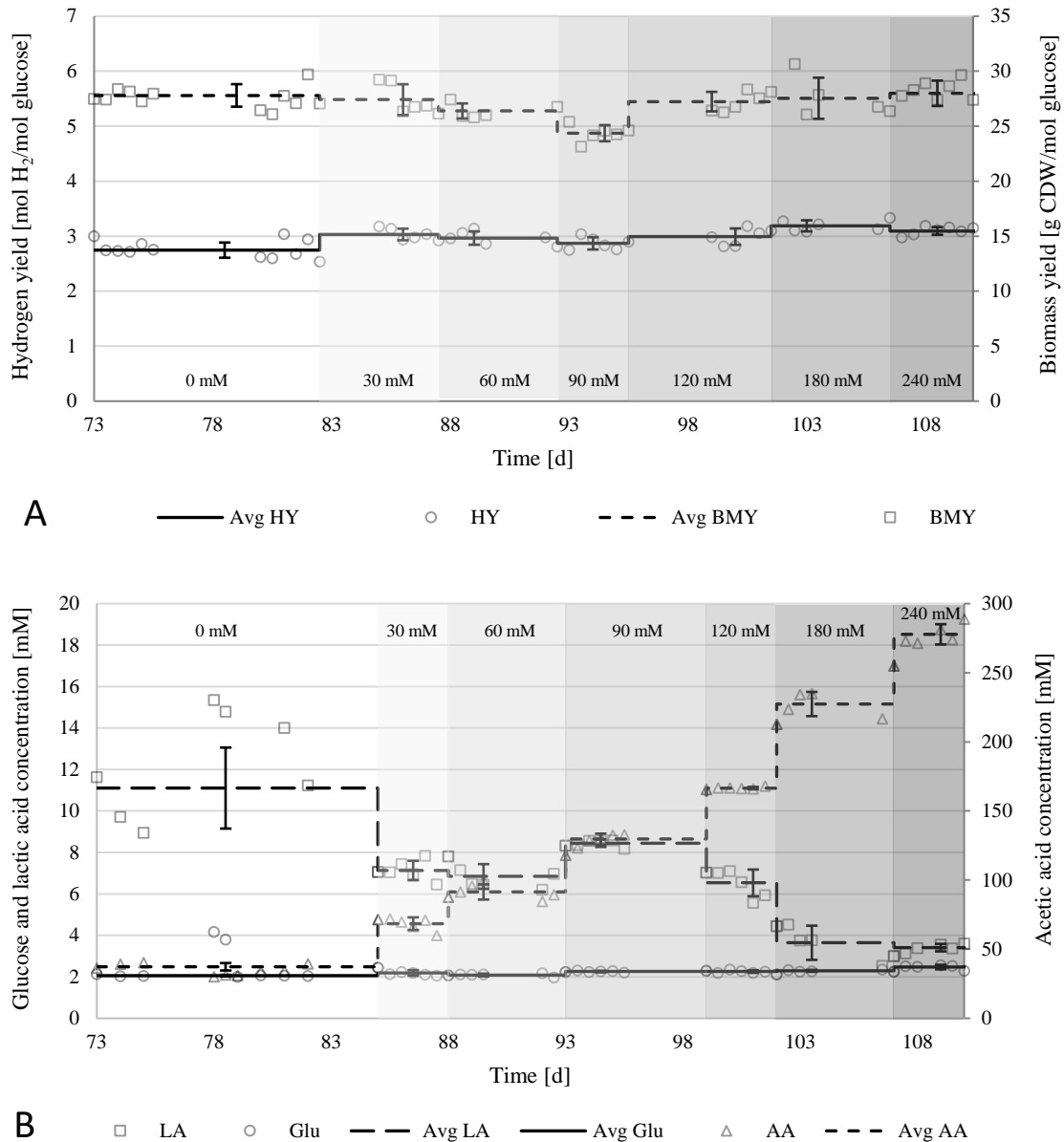


Fig. 6.5: Continuous hydrogen production by *T. neapolitana* at varying feed acetic acid (AA) concentrations (0 – 240 mM) at 27.8 mM of feed glucose (Glu) concentration and an HRT of 24 h. Yields of biomass (BMY) and hydrogen (HY) (A) as well as AA, lactic acid (LA) and residual Glu concentration in the effluent (B). Error bars depict the standard deviation.

6.3.4 Mechanisms for end product inhibition

Several effects have been discussed in the literature to explain the inhibition of high end product concentrations on dark fermentation (Elbeshbishy et al., 2017). The most common is the decrease of the extracellular pH until the normal functions of the cell cannot be kept active (Elbeshbishy et al., 2017; Srikanth and Venkata Mohan, 2014). This mechanism can be excluded in the present study, as the pH was continuously controlled at 7. At acidic pH,

inhibition may also occur due to the presence of undissociated organic acids (Elbeshbishy et al., 2017; Srikanth and Venkata Mohan, 2014), which penetrate the cells and disrupt the cell functions by changing the intracellular pH and osmolarity (Akutsu et al., 2009). In this study, a concentration of 270 mM AA (240 mM fed + approximately 30 mM produced) was observed in the reactor at the highest AA feed. At pH 7, this results in an undissociated AA concentration below 2 mM, calculated via the equation presented by Akutsu et al. (2009), and its inhibitory effect on the process was therefore considered negligible. Ciranna et al. (2014) identified the increase of ionic strength to be responsible for the feedback inhibition. However, Pradhan et al. (2017) reported no effect of salinity on HY and biomass growth of *T. neapolitana* up to 855 mM of NaCl. Jones et al. (2017) ascribed dark fermentation inhibition by VFAs to be a thermodynamic limitation.

6.3.5 Improved culture performance due to acclimatization at prolonged cultivation

Within the course of the continuous-flow operation, an initial glucose concentration of 27.8 mM was repeatedly used in phases G1, G3 and G7 to confirm the reproducibility and investigate the acclimatization of the culture. After 26 days of cultivation, the reactor performance in phase G3 remained comparable to that previously observed in phase G1, as demonstrated by the similar values of hydrogen and biomass yield (Fig. 6.1A) as well as fermentation end products (Fig. 6.1B). However, when using 27.8 mM as feed glucose again in phase G7 after 65 days of operation, an HY of 2.7 (± 0.1) mol H₂/mol glucose (Fig. 6.1A) was obtained, which was 29% higher than that observed in phases G1 and G3. Concomitantly, the AA/LA ratio increased to 3.5 (± 0.8) (Fig. 6.2A), while the biomass yield remained constant at 27.8 (± 1.0) g CDW/mol glucose (Fig. 6.1A). The same trend continued when raising the feed AA concentration from 0 to 240 mM with the HY increasing by a further 12% from 2.8 (± 0.2) to 3.1 (± 0.1) mol H₂/mol glucose (Fig. 6.2B and Fig. 6.5A) and a simultaneous reduction of the LA concentration from 11.1 (± 2.0) to 3.4 (± 0.2) mM (Fig. 6.5B). Hence, the HY increased by a total of 47% throughout the 110 days of continuous-flow operation (Fig. 6.1A and Fig. 6.4A). We presume that this substantial improvement of the process efficiency was directly correlated to an acclimatization of *T. neapolitana*.

The importance of acclimatization has previously been demonstrated for mixed cultures (Haroun et al., 2016), where it is generally considered as a shift in the microbial community structure (Cisneros-Pérez et al., 2017; Dessì et al., 2017). However, this study demonstrates

that acclimatization also occurs in pure cultures in terms of a metabolic shift and represents a large potential to enhance the process performance in continuous fermentation. Acclimatization in pure cultures is still poorly understood and can require long operation times, which complicate the assessment of the culture potential.

6.3.6 Microbiological considerations

In the MiSeq, the bacterial community analysis after 102 days of continuous operation revealed 2 genera above 0.1% relative abundance, i.e. *Thermotoga* and *Enterococcus* with 98 and 2%, respectively. As *Enterococci* are mesophilic bacteria growing in the range from 10 to 45 °C (Sherman, 1937), their minor appearance in the community analysis was most likely caused by a contamination during sampling. Further evidence for an exclusive substrate degradation by *T. neapolitana* was provided by the composition of end products in the effluent. Throughout the 110 d of continuous operation the sum of AA and LA, constituted for 95 (± 10)% of the glucose consumed. Nguyen et al. (2010) reported that batch bioassays using *T. neapolitana* remained free of contamination due to the extreme growth temperature of 80 °C. Our results suggest that this finding also applied to a prolonged cultivation of 102 days in non-sterile continuous operation.

Conclusions

- Increasing the feed glucose concentration from 11.1 to 27.8 mM simultaneously increased the HPR and decreased the HY. When further increased to 41.6 mM the additional glucose was metabolized to LA without producing extra hydrogen, thus resulting in a stable HPR.
- AA decreased the HPR by 50%, when increased from 30 to 240 mM in batch bioassays.
- In contrast, the process performance was unaffected by increasing feed AA concentrations under continuous operation. The HPR and HY remained constant at 77.9 (± 4.5) mL/L/h and 3.0 (± 0.2) mol H₂/mol glucose, respectively, indicating an increased resistance against end product inhibition.
- Throughout 110 days of continuous fermentation, the HY increased by 47%, likely due to an acclimatization of the culture.

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Declarations of interest: none

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7 High rate continuous biohydrogen production by hyperthermophilic *Thermotoga neapolitana*

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This chapter presents unpublished results.

Abstract

This study focused on the continuous-flow hydrogen production by *Thermotoga neapolitana* in a continuously stirred tank reactor at a hydraulic retention time (HRT) decreasing from 24 to 5 h. At each HRT reduction, an immediate drop of the hydrogen yield (HY) was initially observed. However, a prolonged cultivation time allowed the culture to recover, as the HY increased during the operation at constant HRT. The final HY observed at the end of each operating period gradually decreased from 3.4 (\pm 0.1) to 2.0 (\pm 0.0) mol H₂/mol glucose when reducing the HRT from 24 to 7 h, accompanied by a pathway shift from acetic to lactic acid production. Simultaneously, the hydrogen production rate and the hydrogen concentration in the liquid phase (H_{2aq}) increased from 82 (\pm 1) to 192 (\pm 4) mL/L/h and from 9.1 (\pm 0.3) to 15.6 (\pm 0.7) mL/L, respectively. At an HRT of 7 h, the effluent glucose concentration remained above 10 mM compared to the concentration of 27.8 mM in the feed. At the lowest HRTs, the H₂-rich biogas was recirculated to prevent inhibition by supersaturated H_{2aq}. The biogas recirculation efficiently maintained H_{2aq} at 9.3 (\pm 0.7) mL/L and resulted in a complete glucose consumption and the highest hydrogen production rate of 277 mL/L/h at a HRT of 5 h.

Key words: *Thermotoga neapolitana*, hydrogen, continuous-flow dark fermentation, acetic acid, hydraulic retention time, gas recirculation

Abbreviations

AA	Acetic acid
CDW	Cell dry weight
CSTR	Continuously stirred tank reactor
GaR	Biogas recirculation
HPR	Hydrogen production rate
HY	Hydrogen yield
LA	Lactic acid

7.1 Introduction

Dark fermentation is a sustainable process capable of converting organic matter to the clean and environmentally friendly energy carrier hydrogen (Lee et al., 2011; Ntaikou et al., 2010; Sivagurunathan et al., 2016). While being considered the most promising amongst the biological processes due to the independence from light and the simple reactor operation (Arimi et al., 2015; Balachandar et al., 2013), dark fermentation still faces major limitations. For instance, low hydrogen production rates (HPR) and hydrogen yields (HY) are the main drawbacks in order to obtain an economically viable process (de Vrije et al., 2007; Gupta et al., 2013; Lee et al., 2011).

In dark fermentation, the HY is closely connected to the culture used (Balachandar et al., 2013; Ghimire et al., 2015), with high yields being achieved by selecting a suitable production organism (O-Thong et al., 2008). Thermophilic strains are advantageous over mesophilic strains providing the highest conversion efficiencies (Elsharnouby et al., 2013; Gupta et al., 2016; Lee et al., 2011). Moreover, most other non-H₂ producing microorganisms competing for substrate or consuming the produced hydrogen are inhibited by elevated temperatures (Hawkes et al., 2007; Yasin et al., 2013).

Thermotoga neapolitana (briefly *T. neapolitana*) is a hyperthermophilic bacterium which has been extensively studied for the production of hydrogen (Pawar and van Niel, 2013; Pradhan et al., 2015). Besides achieving exceptional yields approaching the theoretical value of 4 mol H₂/mol glucose (d'Ippolito et al., 2010; Munro et al., 2009), this bacterium is capable to simultaneously metabolize (Eriksen et al., 2008) a wide range of substrates (Huber and Hannig, 2006; Pradhan et al., 2015). So far, *T. neapolitana* has exclusively been investigated in batch and fed batch operation (Pradhan et al., 2015). However, continuous-flow conditions are generally preferred for an industrial application (Kumar et al., 2014; Ntaikou et al., 2010) due to the more energy efficient reactor operation (Lin et al., 2012; Show et al., 2011). Furthermore, continuous mode allows the culture to reach an acclimatized steady state which has shown to provide better process stability and higher hydrogen yields (Elsharnouby et al., 2013; Hawkes et al., 2007).

In continuous operation, the hydraulic retention time (HRT) is a major factor affecting the reactor performance of dark fermentation (Arimi et al., 2015; Sivagurunathan et al., 2016). At a constant reactor volume and substrate removal efficiency, a decrease of the HRT

represents an acceleration of the process. Consequently, the same amount of organic waste can be treated in a shorter period of time, which considerably reduces the bioreactor size and capital costs (Hawkes et al., 2007). Furthermore, decreasing the HRT has shown to increase the HPR (Palomo-Briones et al., 2017; Whang et al., 2011; Zhang et al., 2013), additionally improving the economic viability of the process. Low HRTs are also advantageous as they selectively wash out unwanted microorganisms such as hydrogen consumers from the system, which exhibit lower growth rates compared to the hydrogen producing bacteria (Ghimire et al., 2015; Hawkes et al., 2007). The minimum accomplishable HRT is thereby determined by the growth rate of the hydrogen producing culture. An excessive shortening of the HRT generally leads to an incomplete substrate consumption or the complete washout of the culture (Ghimire et al., 2015; Lin et al., 2012). Hence, the optimization of the HRT, i.e. the proper bioreactor sizing, is essential for the establishment of a continuous production process.

Another crucial factor in dark fermentation is end product inhibition by hydrogen, which hampers the efficiency of the process (Balachandar et al., 2013; Verhaart et al., 2010). The concentration of liquid phase hydrogen (H_{2aq}), which directly acts on the microbial culture, is often wrongly considered to be in equilibrium with the easily measurable hydrogen partial pressure in the gas phase (Ghimire et al., 2015; Ntaikou et al., 2010). However, an increasing amount of studies have reported the supersaturation of H_{2aq} and demonstrated its considerable impact on dark fermentation (Gupta et al., 2016; Kraemer and Bagley, 2006; Ljunggren et al., 2011). Especially, the positive correlation between H_{2aq} and HPR (Pauss et al., 1990; Paper II; Paper III) highlights the importance to prevent H_{2aq} accumulation in order to achieve high H_2 productivities in dark fermentation.

In the present study, *T. neapolitana* was used in a continuous-flow biohydrogen production process. We investigated the effect of a decreasing HRT on the dark fermentation performance and H_{2aq} build-up. Furthermore, the use of H_2 -rich biogas recirculation was tested for its potential to counteract the supersaturation of H_{2aq} at the low HRTs.

7.2 Material and methods

7.2.1 Bacterial culture and medium

A pure culture of *T. neapolitana* was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The medium composition was based on a modified ATCC 1977 medium described by Dreschke et al. (2018) containing 27.8 mM of glucose. The pH adjusted medium (pH 7.5) was prepared in 10 L Schott Duran bottles before autoclaving at 110°C for 5 min. Subsequently, the headspace of the Schott Duran bottles was sparged with N₂ for 10 min to remove oxygen and subsequently stored anaerobically at 4 °C.

7.2.2 Experimental conditions

The experiment was conducted in a 3-L fully controlled continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The reactor was kept at a constant temperature of 80 °C and maintained at pH 7 by automatic addition of 5M NaOH, while a 500 rpm stirring was applied. The produced biogas was continuously released from the headspace of the reactor to prevent pressure build-up. To grow and acclimatize *T. neapolitana*, the reactor was operated in batch mode for approximately 16 h after the inoculation with 6% (v/v) of storage culture. Subsequently, the feeding was initiated at a flow rate of 83.3 mL/h resulting in an HRT of 24 h. The working volume was controlled using a level probe.

To investigate the effect of the HRT on dark fermentation by *T. neapolitana*, different operating conditions were used as described in Table 7.1. The HRT was gradually decreased from 24 to 5 h, whereas H₂-rich biogas recirculation (GaR) was added at the lowest HRTs (i.e. 7 and 5 h) to evaluate the impact of H_{2aq} on the process performance. GaR refers to the recirculation of the produced biogas from the headspace to a distribution device at the base of the reactor at a flow-rate of 350 mL/h via a peristaltic pump (Watson-Marlow, United Kingdom).

Table 7.1: Biohydrogen production by *T. neapolitana* in continuous dark fermentation of 27.8 mM feed glucose at decreasing HRT excluding or including H₂-rich biogas recirculation (GaR). Hydrogen yield (HY) and hydrogen production rate (HPR) are provided at the start and the end of each operating condition, calculated via the linear regression of each phase, as depicted in Fig. 7.1A and B as well as Fig. 7.2A and B.

HRT [h]	GaR	Operating period [d]	HY [mol H ₂ /mol glucose]		HPR [mL/L/h]	
			start	end	start	end
24	-	0 – 6	3.4	3.4	81	82
20	-	7 – 21	2.0	2.8	70	96
16	-	22 – 44	2.0	2.8	87	120
13	-	45 – 73	2.2	2.3	114	118
10	-	74 – 87	1.9	2.5	132	171
7	-	88 – 103	1.5	2.1	146	207
7	+	104 – 117	1.7	2.3	158	216
7	-	118 – 121	1.9	0.2	184	15
7	+	122 – 123	1.5	2.4	142	235
5	+	124 – 129	1.8	2.0	243	277

7.2.3 Sampling and analytical methods

To determine the concentration of glucose, acetic acid (AA) and lactic acid (LA), 2 mL of liquid sample was taken twice a day. Furthermore, 20 mL samples were withdrawn from the reactor for the determination of H_{2aq}, while 200 mL of effluent was used for the analysis of cell dry weight (CDW) as described by Dreschke et al. (Paper II). The biogas production was quantified by measuring the time to fill a 500 mL water displacement system. The procedures for liquid sample processing (glucose, AA and LA concentration) and the determination of the hydrogen concentration in the biogas were as described previously (Paper II and III). The conversion from volumetric to molar hydrogen production was performed by applying the ideal gas law (O-Thong et al., 2008).

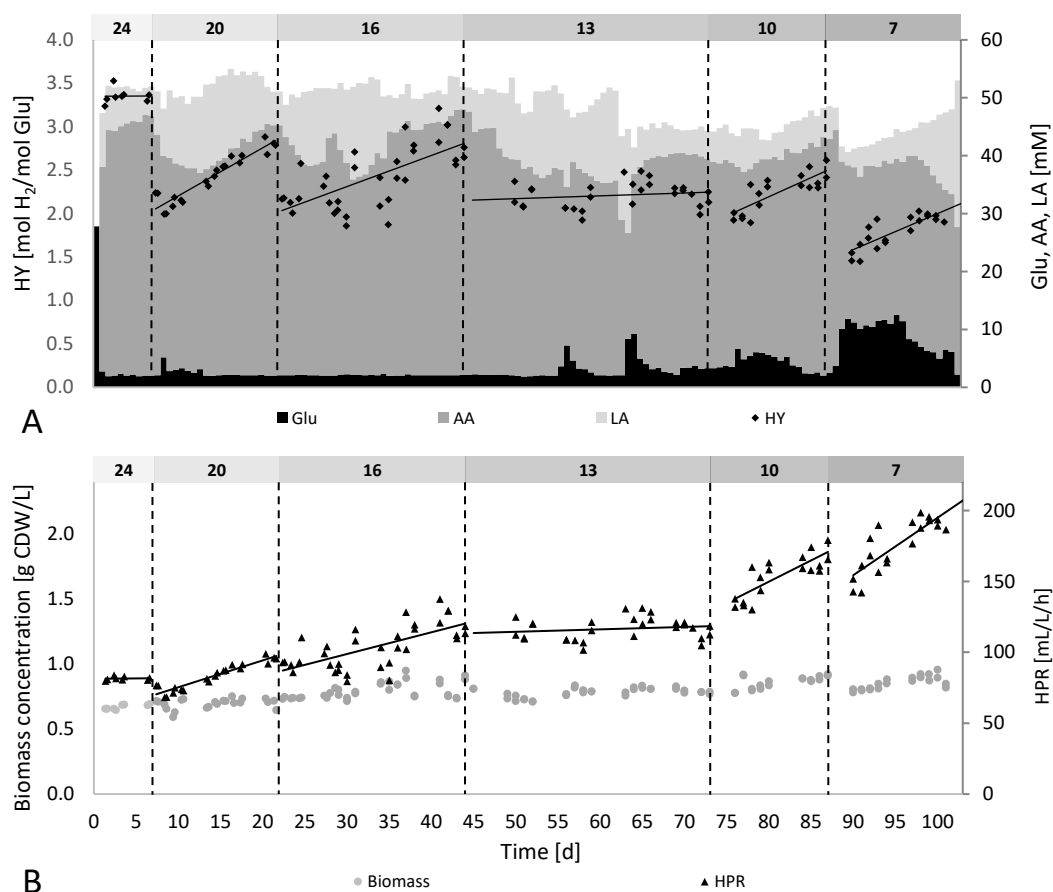


Fig. 7.1: Continuous dark fermentation of glucose (feed concentration 27.8 mM) by *T. neapolitana* at decreasing HRT from 24 to 7 h. Hydrogen yield (HY) and composition of the liquid phase, i.e. residual glucose (Glu), acetic acid (AA) and lactic acid (LA) (A) as well as biomass concentration and hydrogen production rate (HPR) (B).

7.3 Results and Discussion

7.3.1 Response of *T. neapolitana* to the HRT decrease

Fig. 7.1 shows the reactor performance at a decreasing HRT from 24 to 7 h. In 6 days of operation at an HRT of 24 h, we obtained a HY of 3.4 (\pm 0.1) mol H₂/mol glucose, a biomass yield (BMY) of 28.6 (\pm 0.7) mg CDW/mol glucose and a HPR of 82 (\pm 1) mL/L/h which induced a H_{2aq} of 9.1 (\pm 0.3) mL/L (Fig. 7.2A and B). Besides H₂, glucose was metabolized to AA (i.e. 44.0 (\pm 0.8) mM) and LA (i.e. 5.6 (\pm 0.8) mM) at an HRT of 24 h. A residual glucose concentration of 2.1 (\pm 0.1) mM remained in the effluent.

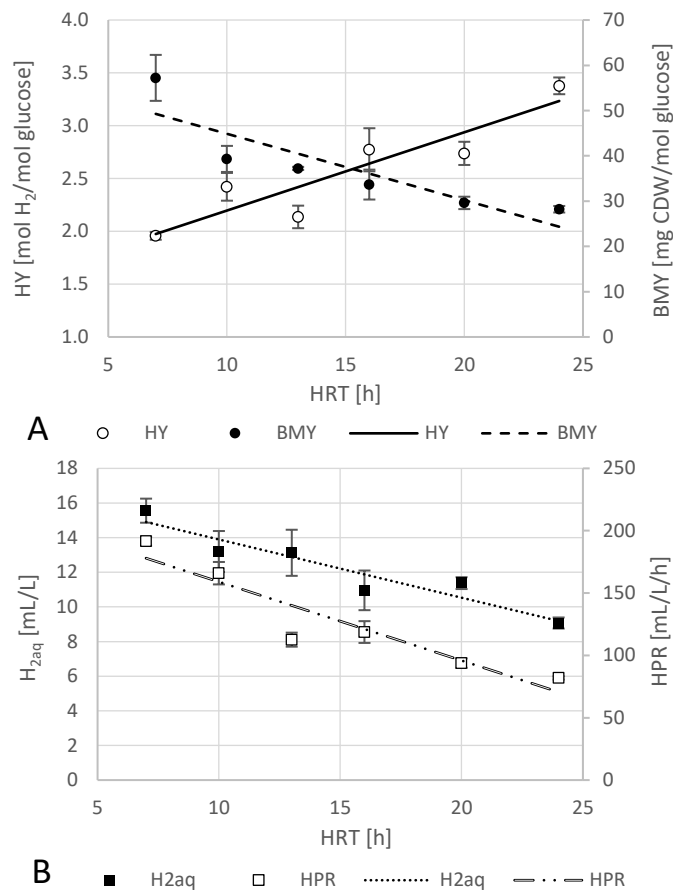


Fig. 7.2: Acclimatized values (average of the 3 final days of each operational phase) of the hydrogen yield (HY) and the biomass yield (BMY) (A) and hydrogen concentration in the liquid phase (H_{2aq}) and hydrogen production rate (HPR) (B) at decreasing HRT from 24 to 7 h during the continuous dark fermentation of 27.8 mM Glu by *T. neapolitana*. Error bars depict the standard deviation.

The reduction of HRT from 24 to 20 h induced an immediate decrease of the HY from approximately 3.4 mol H_2 /mol glucose on day 6 to 2.0 mol H_2 /mol glucose on day 7 (Table 7.1). A concomitant shift of the end product formation from AA to LA and a temporary increase of the residual glucose concentration to 5.2 mM (Fig. 7.1A) were observed. At the same time, the HPR declined from approximately 82 to 70 mL/L/h (Table 7.1), while the BMY remained relatively unaffected reaching 30.0 (\pm 1.4) mg CDW/mol glucose (Fig. 7.2A). Subsequent to the change of HRT from 24 to 20 h, the process recovered from day 7 to 21, as depicted by the HY increasing to approximately 2.8 mol H_2 /mol glucose (Fig. 7.1A, Table 7.1), the shift of end products back from LA to AA (Fig. 7.1A) and the increase of HPR to 96 mL/L/h (Fig. 7.1B, Table 7.1). Complete glucose consumption was observed from day 13 onwards (Fig. 7.1A).

A similar response to a decreasing HRT was observed by Kim et al. (2012) using anaerobic digester sludge as inoculum in a CSTR at a constant organic loading rate of 40 g glucose/L/day. Decreasing the HRT from 24 to 12 h temporarily decreased the glucose consumption and HY from approximately 95 to 40% and from 0.8 to 0.5 mol H₂/mol glucose, respectively. After 5 and 7 days of cultivation, the process recovered reaching 90% of glucose consumption and a HY of 1.2 mol H₂/mol glucose. Peintner et al. (2010) investigated the use of a pure *Caldicellulosiruptor owensensis* culture in a trickling bed bioreactor. They observed a drastic shift from AA to LA formation and cessation of hydrogen production in the first day after reducing the HRT from 7.5 to 5 h. In the subsequent days, the process recovered resulting in a stable hydrogen production and an increase of the AA/LA ratio.

The above described response, i.e. a drop of process efficiency and the ensuing recovery of the process performance, was subsequently observed at each stepwise HRT reduction (Fig. 7.1A). To allow a more detailed analysis, the recovery at each individual operating condition was described using a linear regression (Fig. 7.1A and B, Fig. 7.2A and B). This allowed the calculation of the initial and end value of the HY and HPR at each HRT, as reported in Table 7.1. The sum of LA, AA and residual glucose in the effluent constituted for 95 (\pm 5)% of the initial glucose feed throughout the entire experiment (Fig. 7.1A). The hydrogen concentration, i.e. 67.2 (\pm 2.4)%, in the produced biogas remained constant along the 129 days of operation (data not shown) and hence, unaffected by the change of operating condition.

7.3.2 *T. neapolitana* metabolism at decreasing HRT

The decrease of HY described in section 3.1 strongly indicates that the reduction of the HRT induced a shock response. The glucose degradation by *T. neapolitana* is dominated by 2 pathways defined by their final products, either AA or LA (Pradhan et al., 2015). Only the AA pathway leads to the formation of hydrogen, as demonstrated by the correlation between HY and AA production (Fig. 7.1A and 3A) and results in an additional energy gain of two mol ATP/mol glucose. However, the reaction to hydrogen requires a high redox potential ($E^{\circ} = -414$ mV), rendering the AA pathway energetically more challenging than the LA pathway (Balachandar et al., 2013). The metabolism of *T. neapolitana* is shifted towards the LA pathway as a response to unfavorable or changeable conditions to ensure a lower but more secure energy yield, as observed at a change of HRT in this study (Fig. 7.1A) or previously at

elevated hydrogen (d'Ippolito et al., 2010; Paper II; Paper III) and AA (Paper IV) concentrations. The subsequent recovery during each operating phase is assumed to be an acclimatization of *T. neapolitana* at stable environmental conditions, driven by the higher energy yield of the AA pathway. Accordingly, Dreschke et al. (Paper IV) observed a 47% increase of the HY over 130 days of continuous flow cultivation increasing the feed glucose (i.e. 11.1-41.6 mM) and AA (i.e. 0-240 mM) concentrations at a constant HRT. The described change in metabolism implies the synthesis of new enzymes, indicating why acclimatization is a slow process occurring exclusively during a prolonged cultivation at stable conditions.

7.3.3 Impact of HRT on hydrogen yield and production rate

As mentioned in section 3.1, the efficiency of the process considerably improved throughout each operating phase. For a better comparison of the reactor performance at different HRTs, an average value of HY and HPR in the final 3 days of each operating condition is given in Fig. 7.2. The HY gradually decreased from 3.4 (± 0.1) to 2.0 (± 0.0) mol H₂/mol glucose when the HRT was reduced from 24 to 7 h (Fig. 7.2A). At the same time, the HPR increased from 82 (± 1) to 192 (± 4) mL/L/h, despite the decline of the HY (Fig. 7.2B). An increasing HPR is generally observed when lowering the HRT and considered to be caused by the higher loading rate (Barca et al., 2015).

de Vrije et al. (2007) used *Caldicellulosiruptor saccharolyticus* in a CSTR at 72.5 (± 0.5) °C using 10.7 mM as feed glucose. In their study, lowering the HRT from 11.1 to 3.3 h decreased the HY from 4.0 (± 0.1) to 3.3 (± 0.1) mol H₂/mol glucose while increasing the HPR from 4.0 (± 0.3) to 9.9 (± 0.5) mmol/L/h. Similarly, Xing et al. (2008) reported an increase of HPR and a decrease of HY when reducing the HRT from 10 to 1.7 h using *Ethanoligenens harbinense* YUAN-3 in a CSTR with 1 g/L of feed glucose concentration. Jo et al. (2008) used *Clostridium tyrobutyricum* JM1 in a fixed bed bioreactor at 37°C. Reducing the HRT from 24 to 2 h increased the HPR approximately 7 times to a maximum of 7.2 L H₂/L/d with a glucose conversion efficiency of 97%. The further decrease to an HRT of 1 h induced a sharp drop of conversion efficiency to 41% and a HPR of approximately 2.2 L/L/d. The HY of *C. tyrobutyricum* JM1 was not discussed in detail by Jo et al. (2008).

7.3.4 Correlation of HPR and H_{2aq} at decreasing HRT

Similar to the HPR, also the H_{2aq} increased with decreasing HRT (Fig. 7.2B). At an HRT of 24 h, the H_{2aq} was $9.1 (\pm 0.3)$ mL/L (Fig. 7.2B), i.e. lower than 9.7 mL/L which is the liquid phase concentration in thermodynamic equilibrium with a gas phase containing 65% H_2 at 80 °C, as suggested by Henry's law (Paper II). The applied 500 rpm agitation provided sufficient gas-liquid mass transfer to efficiently remove hydrogen from the liquid phase as previously reported (Paper II and III). However, when the HRT was reduced to 7 h and the HPR increased to $192 (\pm 4)$ mL/L/h (Fig. 7.2B), the same agitation could not maintain the gas-liquid equilibrium leading to a supersaturated H_{2aq} of $15.6 (\pm 0.7)$ mL/L. The H_{2aq} was directly correlated to the HPR (Fig. 7.2B) under all operating conditions until day 103, i.e. prior to applying GaR.

The importance of the gas-liquid mass transfer on the process has been demonstrated in previous studies (Pauss et al., 1990; Beckers et al., 2015; Kraemer and Bagley, 2006; Paper II; Paper III). When adequate gas-liquid mass transfer is provided, H_{2aq} remains in equilibrium with the gas phase preventing the supersaturation of H_{2aq} (Pauss et al., 1990; Paper II; Paper III). If, however, the gas-liquid mass transfer is limited, hydrogen accumulates in the liquid phase depending on the HPR as theoretically and experimentally demonstrated by Pauss et al. (1990) using mixed cultures and observed by Dreschke et al. (Paper II and III) using *T. neapolitana*. Hydrogen is a well-known inhibitor of dark fermentation which acts on the yield as well as the dark fermentation rate (Paper II and III). Due to this inhibition of HPR by H_{2aq} , both parameters reciprocally impact each other, resulting in a process performance which is primarily determined by the mass transfer of the system.

In this study, the response of *T. neapolitana* at each stepwise HRT decrease might have been induced by a rapid increase of H_{2aq} , caused by the increase of the HPR. We assume that *T. neapolitana* reduced the hydrogen yield to prevent high H_{2aq} concentrations. This hypothesis is supported by the low impact of an HRT change on HPR which is directly correlated to H_{2aq} .

7.3.5 Application of GaR

At low HRTs, the glucose consumption efficiency was impaired. In particular, the residual glucose concentration remained above 5 mM and 10 mM for approximately 6 and 10 days when the HRT was reduced from 13 to 10 h and from 10 to 7 h (Fig. 7.1A), respectively.

Incomplete substrate consumption is commonly observed when decreasing the HRT below a certain threshold value (Kumar et al., 2014; Palomo-Briones et al., 2017; Whang et al., 2011). At an HRT of 7 h, glucose consumption improved from day 98 onwards. The additionally consumed fraction of glucose was primarily metabolized via the non-hydrogen-producing LA pathway, as demonstrated by the sharp LA increase in the reactor (Fig. 7.1A). The higher H_{2aq} concentrations, observed at an HRT of 7 h (Fig. 7.3B) likely hampered the dark fermentation yield and rate.

Therefore, GaR was initiated on day 104 to improve the gas-liquid mass transfer and discern whether the reduced performance was due to the inhibition by accumulated H_{2aq} , or a kinetic limitation of the culture. The use of GaR immediately decreased the H_{2aq} , maintaining it at $9.3 (\pm 0.7)$ mL/L independent from the HPR (Fig. 7.3B). GaR initially induced a slight decrease of HY and HPR from approximately 2.1 to 1.7 mol H_2 /mol glucose and 207 to 158 mL/L/h, respectively (Table 7.1). This is assumed to be caused by the response of *T. neapolitana* to the change of environmental conditions discussed in section 3.2. As previously observed, the process recovered, reaching a HY of 2.3 mol/mol glucose and a HPR of 216 mL/L/h (Table 7.1) after 13 days of operation, i.e. 7% higher than the values obtained at an HRT of 7 h in the absence of GaR. Furthermore, glucose was completely consumed throughout the operating period, while the AA concentration increased from approximately 28 to 33 mM and the LA concentration decreased from approximately 24 to 19 mM from day 104 to 117 (Fig. 7.3A).

To confirm that this higher process performance was due to a low H_{2aq} and not the continuing acclimatization at HRT 7 h, the GaR was stopped on day 118. Cessation of GaR drastically decreased the HY from 1.9 to 0.2 mol H_2 /mol glucose, simultaneously shifting from AA to LA production (Fig. 7.3A) and reducing the HPR from 184 to 15 mL/L/h (Table 7.1). It is not entirely clear why returning to an HRT of 7 h in the absence of GaR induced such a substantial difference in the process performance. The primary difference of the two phases was the velocity at which the environmental conditions were changed. Until day 103, *T. neapolitana* slowly acclimatized to increasing levels of H_{2aq} , whereas the deactivation of GaR immediately changed the gas-liquid mass transfer after cultivation at low H_{2aq} for 13 days. We assume that the considerable reduction of HPR was a shock response by *T. neapolitana* triggered by elevated levels of H_{2aq} , which subsequently decreased again to 11.9 mL/L on day 118 when H_{2aq} was first measured after the GaR stop (Fig. 7.3B). Despite the

absence of GaR, H_{2aq} declined even further until day 121 (Fig. 7.3B and C), due to the collapse of the hydrogen production with the HPR decreasing to 15 ml/L/h in this phase (Table 7.1).

On day 121, GaR was applied again, to continue investigating the impact of the HRT on *T. neapolitana* at low H_{2aq} concentrations at an HRT of 7 h. The process immediately recovered, as depicted by the increase of HPR and HY (Fig. 7.3A and C, Table 7.1). On day 124, the HRT was reduced to 5 h in the presence of GaR to determine whether a low H_{2aq} would permit a further increase of the process velocity. In contrast to the previous HRT reductions to 10 and 7 h, glucose continued to be completely degraded to $2.1 (\pm 0.6)$ mM in the presence of GaR (Fig. 7.3A) and the process continued to recover with the HY increasing from 1.8 to 2.0 mol H_2 /mol glucose in 5 days of cultivation (Table 7.1). This resulted in a HPR of 277 mL/L/h at the end of the operating period (Table 7.1), i.e. the highest obtained under all process conditions tested.

We demonstrate that the increase of HPR leads to an increase of H_{2aq} and inevitably inhibition. GaR is a successful technique preventing H_{2aq} supersaturation, allowing high glucose consumption and HPR even at an HRT of 5 h. However, the mechanisms acting on the culture are not entirely clear, as the initiation and stopping of GaR seem to induce a response of *T. neapolitana* similar to that observed at changes of H_{2aq} (Paper II and III) or HRT (Fig. 7.1). Further long-term investigations are necessary to determine the real potential of this technique.

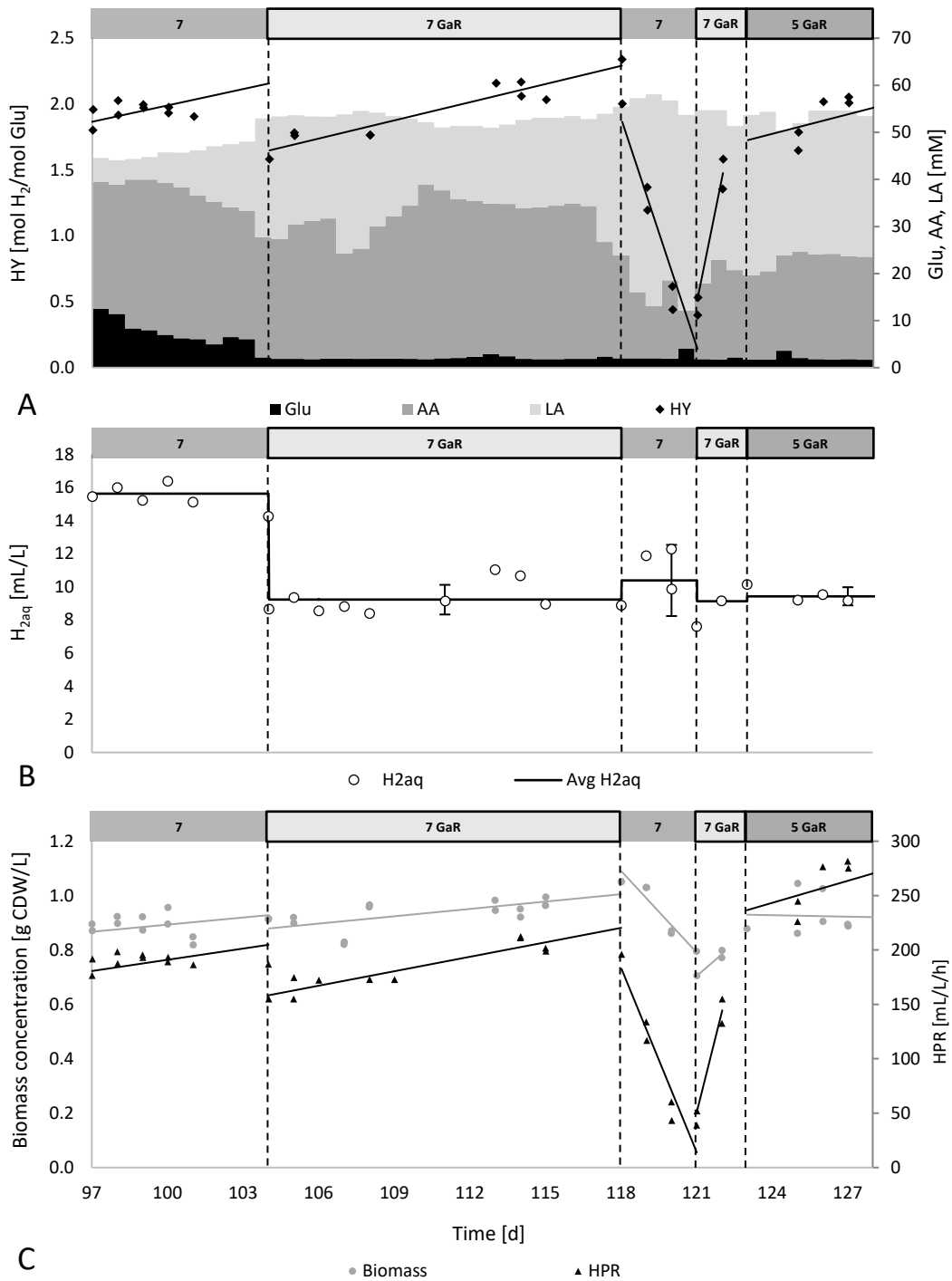


Fig. 7.3: Continuous dark fermentation of 27.8 mM of glucose (Glu) by *T. neapolitana* at an HRT of 7 and 5 h, including or excluding recirculation of the H₂-rich biogas (GaR). Hydrogen yield and composition of the digestate, i.e. residual Glu, acetic acid (AA) and lactic acid (LA) concentration (A), concentration of hydrogen in the liquid phase (H_{2aq}) (B) as well as hydrogen production rate (HPR) and biomass concentration (C). Error bars depict the standard deviation.

7.3.6 Effect of HRT on biomass yield, concentration and agglomerates

Contrary to the HY, the biomass concentration was not negatively affected by the HRT decrease, but gradually increased throughout the initial 101 days of operation from $0.67 (\pm 0.02)$ to $0.89 (\pm 0.05)$ g CDW/L (Fig. 7.1B). Interestingly, the biomass concentration remained in the same range (Fig. 7.1B), despite the considerably lower glucose consumption when decreasing the HRT from 10 to 7 h (Fig. 7.1A). This explains the steady increase of BMY from $28.6 (\pm 0.7)$ to $39.7 (\pm 2.9)$ mg CDW/mol glucose between the HRT 24 and 10 h, followed by the sharp increase to $57.6 (\pm 5.1)$ mg CDW/mol glucose at an HRT of 7 h (Fig. 7.2A). The results suggest that the biomass concentrations in a *T. neapolitana* cultivation is only marginally influenced by the HRT or the glucose consumption but increases slightly with acclimatization. Contrary to a change in HRT, the shock applied by the deactivation of GaR on day 118 induced a notable decrease of the biomass concentration, however exhibiting a considerably lower impact than on the HY and the HPR.

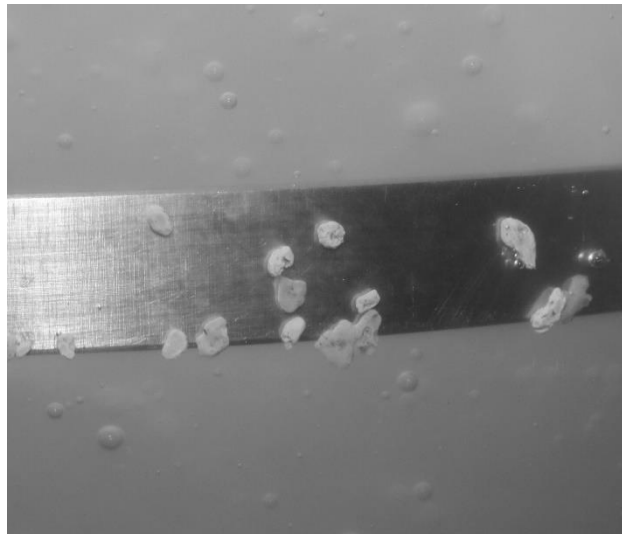


Fig. 7.4: Photograph of a stainless-steel baffle inside the reactor after 111 days of operation showing the formation of attached biomass agglomerates of up to approximately 4 mm in diameter.

A restrained growth by *T. neapolitana* to approximately 0.7 g CDW/L has previously been observed, when the biomass concentration remained unaffected by an increase of feed glucose concentration from 22.2 to 41.6 mM in continuous operation at an HRT of 24 h (Paper IV).

Such growth limitation is common for hyperthermophilic suspended cultures (Lee et al., 2011) and considered a major obstacle for their application in large scale hydrogen production (Gupta et al., 2016). However, in the present study, we noticed the formation of biomass agglomerates (Fig. 7.4) attached to the stainless-steel baffles inside the reactor. *T. neapolitana* has previously been reported to form aggregates in batch (Eriksen et al., 2011) or grow attached to solid surfaces in repeated fed-batch (Basile et al., 2012) cultivation. This strongly suggests the application of *T. neapolitana* in an advanced bioreactor system exploiting self-aggregation or biofilm formation to counteract low biomass concentrations. Such systems not only increase the biomass concentration, but generally allow lower HRTs resulting in higher HPRs (Cheng et al., 2010; Ghimire et al., 2015; Show and Lee, 2013), while being considered more stable and resistant against unfavorable environmental conditions (Cheng et al., 2010).

Conclusion

- HY decreased from 3.4 (\pm 0.1) to 2.0 (\pm 0.0) mol H₂/mol glucose when decreasing the HRT from 24 to 7 h. In contrast, the HPR increased, reaching a maximum of 277 mL/L/h at an HRT of 5 h including GaR.
- Each HRT reduction induced a shift from the AA to the LA pathway, a drop of the HY and an impaired glucose consumption at an HRT of 10 and 7 h. However, a prolonged cultivation at constant HRT allowed *T. neapolitana* to acclimatize, as indicated by an increase of HY.
- The H_{2aq} positively correlated with the HPR reaching 15.6 (\pm 0.7) mL/L at 192 (\pm 4) mL/L/h.
- The use of GaR effectively prevented the supersaturation of H_{2aq}, allowing a complete glucose consumption by *T. neapolitana* at a HRT as low as 5 h.

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Declarations of interest: none

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8 Conclusion and future perspectives

In the present work, hydrogen production by *T. neapolitana* was enhanced by using different approaches to counteract current process limitations and prevent the most relevant inhibitions.

8.1 Effect of biomass concentration

The hydrogen production rate of a reactor is directly correlated to its biomass concentration. Hence, the characteristic growth of thermophilic cultures at low cell concentration is often mentioned as a major obstacle preventing high production rates. As a counteracting measure, we recycled *T. neapolitana* biomass to establish 4 different elevated initial concentrations in batch cultivation (Paper I). Raising the biomass concentrations from 0.46 to 1.74 g CDW/L successfully accelerated dark fermentation and increased the HPR from 323 (± 11) up to 654 (± 30) mL/L/h, compared to 51 mL/L/h, which is the maximum HPR of similar experiments in literature. Due to the higher process velocity the fermentation time of 5 g/L of glucose was reduced from 7 to 3 h, while the HY of 2.45 (± 0.09) mol H₂/ mol glucose and the hydrogen concentration in the produced gas, i.e. 68%, remained unaffected. The results confirm, that the biomass concentration is a crucial factor to accelerate dark fermentation and improve the HPR of the process.

However, limited growth of *T. neapolitana* at higher cell densities was observed in paper I. The biomass yield at initial biomass concentrations between 0.46 and 1.33 g CDW/L was considerably higher (i.e. from 0.12 to 0.14 g CDW/mol glucose) compared to that obtained at the highest initial biomass concentration (i.e. 0.08 g CDW/mol glucose). In continuous operation (paper IV), the biomass concentration of *T. neapolitana* remained stable at 687 (± 21) mg CDW/L despite a variation in feed glucose concentration between 22.2 and 41.6 mM but decreased significantly at feed glucose concentrations below 16.7 mM. Similarly, the variation of HRT from 24 to 7 h had little effect on the biomass concentration (paper V), which remained between 0.67 (± 0.02) and 0.89 (± 0.05) g CDW/L. Hence, the cell concentration of *T. neapolitana* in suspended growth seems to be intrinsically limited.

To exploit the promising findings of paper I, follow-up research should address the use of more advanced bioreactors implying installations to artificially increase the biomass retention. The formation of agglomerates observed in paper V indicates that *T. neapolitana*

is much likely capable to grow in biofilm form, which is an extraordinary property potentially exploitable in attached-cell bioreactors such as moving bed, packed bed or fluidized-bed bioreactors. Another option is the application of membranes to prevent the washout of biomass in continuous operation. The characteristics of *T. neapolitana* regarding the formation of agglomerates and biofilms will determine which reactor system is most suitable for an efficient hydrogen production system.

8.2 Supersaturation of liquid phase hydrogen

In paper I the 4-fold increase of the biomass concentration merely resulted in the doubling of the volumetric HPR. Hence, the specific HPR was inversely correlated to the biomass concentration and the volumetric HPR. Increasing the HPR has shown to cause the supersaturation of hydrogen in the liquid phase when the gas-liquid mass transfer is insufficient.

To gain a more profound understanding we studied the accumulation of H_{2aq} in detail (Fig. 8.1). In paper II, we directly measured H_{2aq} for the first time in a *T. neapolitana* application. We confirmed H_{2aq} supersaturation at low cell concentrations up to an agitation speed of 500 rpm, with a consequent negative impact on the HPR and the glucose consumption rate. Investigating the effect of agitation at low cell concentrations in paper III, we observed an accumulation of H_{2aq} up to approximately 30 mL/L at 100 rpm agitation, which is 3-times the equilibrium concentration suggested by Henry 's law. Increasing the agitation speed to 200, 300 and 400 rpm improved the gas-liquid mass transfer by enhancing the turbulence and decreased the H_{2aq} accordingly. Above 500 rpm, the liquid phase hydrogen was maintained in equilibrium with the gas phase. The decrease of H_{2aq} resulted in an increase of the dark fermentation rate, i.e. the HPR and the BGR. The influence of H_{2aq} is thereby considerable, with an 196% enhancement of the HPR when the H_{2aq} was reduced from 21.9 (\pm 2.2) to 8.5 (\pm 0.1) mL/L by increasing the agitation speed from 200 to 600 rpm (paper III).

To prevent an accumulation of H_{2aq} , it is highly important to understand the relationship between H_{2aq} , HPR and the gas-liquid mass transfer. If the gas-liquid mass transfer is high, H_{2aq} is maintained in equilibrium with the gas phase, allowing the microorganism to grow unaffected by hydrogen inhibition. However, when the gas-liquid mass transfer is limited, H_{2aq} accumulates and inhibits the activity of the culture and consequently the HPR. A lower HPR subsequently reduces the accumulation of H_{2aq} and its inhibitory effect. As a result of

this reciprocal interaction of the 2 parameters, the H_{2aq} and HPR of the process are primarily determined by the gas-liquid mass transfer rather than the kinetic potential of the culture.

The above described interaction between HPR and H_{2aq} was directly observed in paper III when the agitation speed was omitted after 15 h of cultivation at 100 rpm. H_{2aq} increased from 29.7 (± 1.4) to 40.6 (± 0.6) mL/L at 17 h inducing a drastic drop of HPR, which in turn reduced the H_{2aq} again to 30.1 (± 1.3) mL/L after 23 h of cultivation at 0 rpm.

Besides agitation speed, H_2 -rich biogas recirculation and bubble induction by addition of K1 carriers were investigated to counteract H_{2aq} supersaturation. Solid materials, such as K1 carriers increase the gas-liquid mass transfer by enhancing bubble formation through heterogeneous nucleation. As a result, the mere addition of K1 carriers caused a 50 % lower H_{2aq} at 100 rpm (paper III). Furthermore, in the presence of K1 carriers an agitation speed of 200 rpm was sufficient to maintain the gas-liquid equilibrium. Similarly, GaR in combination with 200 rpm agitation (paper II) and GaR without additional agitation (paper III) effectively kept the H_{2aq} below the equilibrium concentration. The reduction of H_{2aq} by both counteracting measures induced a similar improvement of the process performance as previously observed with agitation speed.

As described above, the supersaturation of H_{2aq} is aggravated by an increase of HPR. Hence, different measures (agitation at 300 and 500 rpm, including and excluding GaR) to prevent H_{2aq} accumulation were used together with elevated biomass concentrations (to 0.79 g CDW/L) in batch bioassays. In the studied range, the H_{2aq} primarily affected the rate of dark fermentation. At 300 rpm, the metabolization of 5 g glucose/L required more than 10 h (paper II) compared to approximately 5 h at the similar biomass concentration in paper I. We assume this substantial difference to be caused by the upscale of the reactor from 0.2 to 2 L working volume and the concomitant impact on the systems mass transfer. The increase of agitation to 500 rpm allowed the completion of the fermentation in approximately 6 h. However, the process was further accelerated by the addition of GaR leading to a fermentation time of 4 h independent from the agitation speed. This demonstrates that, while 500 rpm was adequate to prevent hydrogen supersaturation at low biomass concentration, an increase of the HPR through high biomass concentration requires a more efficient gas-liquid mass transfer. Hence, at 0.79 g CDW/L the volumetric HPR was improved by 262% up to 850 ± 71 mL H_2 /h/L by adding GaR compared to a sole agitation at 300 rpm agitation. The BGR and the total biomass production were simultaneously increased by the

improvement of the mass transfer as previously observed at low cell concentrations. The HY under all conditions was similarly high between 3.0 and 3.5 mol H₂/mol glucose, with the slightly higher values when GaR was applied.

Besides the current environmental conditions, a cultures performance is highly impacted by its growth history. The condition of the inoculum is therefore crucial in batch bioassays. To produce comparable results in paper II and III, the inoculum was prepared according to the same protocol. However, the environmental conditions such as substrate and end product concentration constantly change in batch operation, especially when the fermentation time is low. *T. neapolitana* has also shown to acclimatize to prevalent conditions (paper IV and V), considerably improving the process performance. Therefore, we investigated the impact of an increasing HPR on the H_{2aq} in continuous-flow operation (paper V). At an HRT of 24 h and a low HPR, 500 rpm was sufficient to remove hydrogen from the liquid phase. However, the reduction of HRT increased the HPR which showed to be directly correlated to H_{2aq} and accumulated up to 15.6 mL/L at an HRT of 7 h. Concomitantly, the H_{2aq} was negatively correlated to the HY. However, we could not determine, whether the decrease of HY was induced by high levels of H_{2aq}. Nevertheless, the application of GaR at low HRTs led improved the glucose consumption and allowed a further reduction of the HRT to 5 h resulted in a high HPR of 277 mL/L/h.

Our results demonstrate that supersaturation of H_{2aq} is a major factor limiting the performance of dark fermentation. While this project exclusively examined the effect on *T. neapolitana*, supersaturation of H_{2aq} is a general phenomenon occurring in dark fermentation. Therefore, we conclude that an adequate gas-liquid mass transfer is essential for an industrial hydrogen production process, which becomes even more crucial at high productivity.

GaR and the addition of biomass carriers efficiently improve the gas-liquid mass transfer. GaR is superior to the often-used inert gas sparging as it does not involve the undesired dilution of the produced gas. Further research should focus on the optimization of this technique, fine tuning the recirculation flow to match the mass transfer requirements of the process and keep the energy consumption low. Additionally, sole GaR might suffice for efficient mixing, eliminating the need for cost-intensive stirring. Large scale, long term experiments are required to evaluate the costs of GaR and compare them with economic revenues from process enhancement and savings by reducing agitation. When using

heterogeneous nucleation to increase the gas-liquid mass transfer, the surface area and characteristics are most significant influencing factors. A range of materials could potentially be better suited and reduce the capital investment by replacing high priced K1 carriers. Solid objects are most commonly used to provide a surface for biofilm formation in biological processes. More detailed investigations need to be conducted combining biomass retention and bubble induction by applying solid materials inside the reactor.

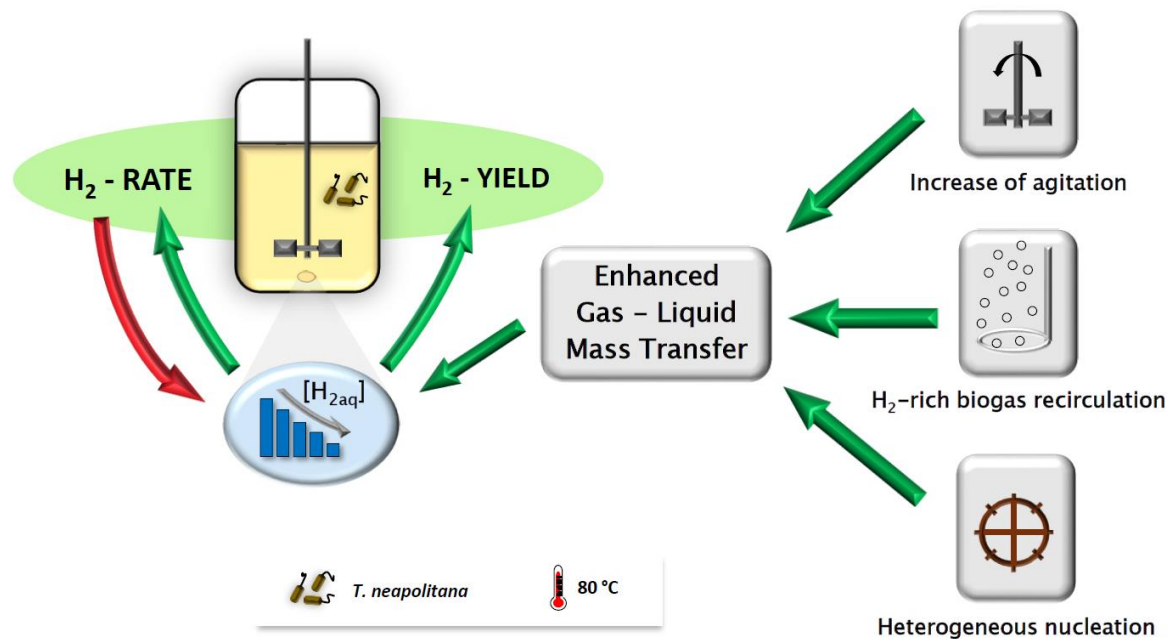


Fig. 8.1.: Impact of agitation speed, H₂-rich biogas recirculation and heterogeneous nucleation on the gas-liquid mass transfer and the subsequent effect the hydrogen yield and rate of dark fermentation by *T. neapolitana*.

8.3 *T. neapolitana* in continuous-flow operation

The final phase of the project addressed the establishment of a continuous-flow operation using *T. neapolitana*. Pure culture systems allow the selection of highly-productive strains and optimization of the environmental conditions towards their preference. Under mesophilic conditions, these systems are highly susceptible to contamination, whereas under hyperthermophilic conditions high temperatures preclude the establishment of most undesired organisms inside the reactor. We confirmed this hypothesis by a microbial community analysis after 102 days of unsterile continuous-flow cultivation revealing *Thermotogaea* as the only relevant genus inside the reactor (paper IV).

A continuous-flow operation is primarily determined by the HRT and the OLR, i.e. the combination of the substrate concentration and the HRT. The optimum substrate range was investigated in paper IV by varying the glucose concentration of the feed. The HY of the process gradually decreased with increasing feed glucose in the range of 11.1 to 41.6 mM. Concomitantly, the HPR increased until 27.8 mM of glucose and remained constant at 41.6 mM glucose, due to the degradation of additional glucose via the non-hydrogen producing LA pathway. This suggests that the HPR can be increased up to a certain point by increasing the feed substrate concentration, however at the expense of the conversion efficiency. As reported in literature, we suspected the accumulation of AA to cause the shift towards the LA pathway at higher glucose concentrations.

To verify this hypothesis, different AA concentrations, i.e. from 0 to 240 mM, were tested in batch and continuous operation. In batch bioassays, the increase of AA concentration to 240 mM primarily decreased the rate of dark fermentation, depicted by the 130% drop of HPR. Similarly, but to a minor extent, the HY decreased from 2.8 to a minimum of 2.0 mol H₂/mol glucose. Surprisingly, this reduction of HY and HPR was absent in continuous-flow operation, where both parameters remained stable up to 240 mM of AA. This resistance against end product inhibition by AA is likely caused by an acclimatization of the culture. *T. neapolitana* gains more energy when fermenting via the AA pathway, whereas the LA pathway provides a robust and more secure supply of energy. Hence, we observed the shift towards the LA pathway if the environmental conditions are unfavorable, e.g. due to end product inhibition, or changeable. In line, the culture adapts its metabolism, degrading via the AA pathway when the culture conditions are maintained stable. The cultures potential to enhance the process performance via acclimatization is considerable as indicated by a 47% increase of the HY throughout 110 days of continuous fermentation (paper IV).

When the HRT was decreased in the range from 24 to 7 h, each individual reduction step induced a shock response from *T. neapolitana* temporarily lowering the HY. However, the prolonged cultivation at invariant conditions allowed the process and the HY to recover and reach a stable phase. The HY after acclimatization decreased with decreasing HRT whereas the HPR and the H_{2aq} concomitantly increased as discussed in section 8.2. A low glucose consumption efficiency obtained at HRTs of 7 and 10 h suggests that *T. neapolitana* reaches its kinetic limit and is not capable to fully degrade the substrate at this process rate.

In summary, we demonstrated a contamination-free, continuous-flow operation using pure culture *T. neapolitana*. Superiority of the continuous operation was confirmed by an increase of HY and resistance against end product inhibition due to acclimatization of the culture. However, in suspended continuous growth the biomass concentration, which had considerably enhanced the rate of hydrogen production when artificially increased in batch cultivation, is limited. The formation of agglomerates by *T. neapolitana* suggests the potential increase of the biomass concentration via advanced bioreactor systems exploiting attached growth for biomass retention. Furthermore, we showed that an increase of hydrogen productivity demands an efficient gas-liquid mass transfer, which is efficiently provided by GaR and heterogenous nucleation, to prevent inhibition by hydrogen supersaturation.