Towards enhanced second-generation n-butanol production from sugarcane

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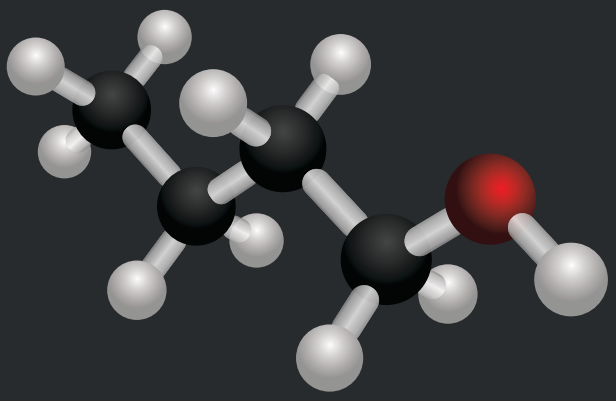

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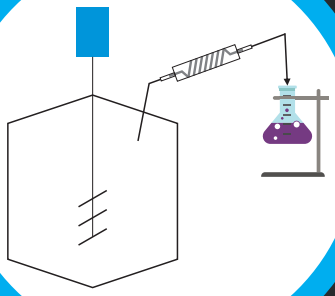

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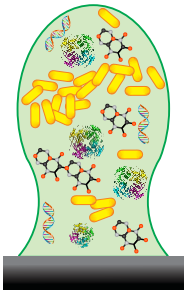

Biofilm

Towards enhanced second-generation *n*-butanol production from sugarcane

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J. van der Hagen,
chair of the Board for Doctorates
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by

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Dedicated to the memory of my beloved father Porfirio Zetty,
and to my most valuable gem, my mother Luz Mary Arenas,
for their unconditional love, support, and eternal teachings
about what really matters in life.

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Summary

Summary

Nowadays, the biotechnology industry is facing the challenge of producing suitable equivalents for petroleum-based products from renewable resources in a sustainable and economically feasible way. Finding cleaner alternatives for gasoline, fuels, and chemicals, has been the subject of research worldwide, whether for economic, geopolitical, or environmental reasons. Among these alternatives, liquid fuels derived from biomass stand out for their eco-friendly production.

In the context of second-generation (2G) ethanol production from lignocellulosic feedstocks, a significant part of the sugars, namely the pentoses (C5), cannot be metabolized by conventional *Saccharomyces cerevisiae* yeasts. Amongst several possibilities of using C5 as feedstock, the conversion to butanol is attracting interest because it is a valuable chemical building block and at the same time, an advanced biofuel with closer physical properties to gasoline. Butanol can be produced either from petroleum derivatives (oxo process from propylene) or from renewable feedstocks in the acetone, butanol, and ethanol (ABE) fermentation process by solvent-producing *Clostridium* spp., which are capable of metabolizing C5 sugars and a wide variety of other substrates. However, bio-based butanol production is more challenging compared to ethanol production and its economic feasibility on an industrial scale faces obstacles such as butanol inhibition, low process energy efficiency, greater separation difficulties compared to ethanol, low yield and low productivity, as well as relatively high substrate cost, representing up to two-thirds of the costs of the whole butanol production process. Hence, efficient recovery of butanol from dilute fermentation broth (~12 g butanol/L) determines, to a large extent, the production process efficiency. Furthermore, in a 2G process, the ABE fermentation is sensitive to inhibitory compounds present in the hemicellulosic hydrolysate generated in the biomass pre-treatment.

Facing these challenges and considering the high impact that the usage of low-cost lignocellulosic feedstocks could represent, this work aims to explore different strategies for contributing towards the development of sugarcane-based biorefinery systems, with a primary focus on C5 sugars valorization. Therefore, in

Chapter 2, a systematic fermentation study is described of four wild-type Clostridia strains, namely *C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864, and *C. saccharoperbutylacetonicum* DSM 14923, as potential candidates for ABE biosynthesis using xylose or glucose as the primary carbon source. Here, the ability of *C. saccharobutylicum* DSM 13864 and *C. saccharoperbutylacetonicum* DSM 14923 is highlighted, as well as the remarkable ability of the latter strain to reach a relatively high ABE titer (>7.0 g/L) from the non-detoxified hemicellulosic hydrolysate. The results indicated the potential of *C. saccharoperbutylacetonicum* DSM 14923 as a promising microbial platform for second-generation butanol production. New insights regarding the performance, synergistic effect of inhibitors, robustness, and butanol tolerance of *C. sacharoperbutylacetonicum* DSM 14923 are presented.

Subsequently, **Chapter 3**, addresses a study on the applicability of mixtures of sugarcane bagasse hemicellulosic hydrolysate (HH) and sugarcane molasses (SCM) as substrates for second-generation ABE production. The co-fermentation of these two substrates was investigated as a strategy to lower the concentrations of the inhibitors present in HH, thus avoiding the costs of detoxification steps. The best results in terms of ABE titer (8.22 g/L) and yield (0.34 g/g) were obtained when 75% of the sugars were from SCM. When HH was concentrated (from 15 to 52 g/L), both the ABE titer and yield increased to 9.79 g/L and 0.36 g/g, respectively, most likely as a result of a positive synergistic effect between low concentrations of 5-hydroxymethylfurfural (HMF) and the media compounds. The sugar preference of the strain on these mixed media was as follows: glucose>fructose>sucrose>xylose>arabinose. The obtained results demonstrated that the addition of SCM at high ratios promoted the effective bioconversion of concentrated C5 hydrolysates into butanol at high yields and productivities. Thus, new insights towards a closed-loop path of butanol in a circular economy for the valorization of a sugarcane biorefinery were obtained.

In addition, two strategies for increasing the butanol productivity of the process were approached by alleviating product inhibition to the cells and allowing the processing of concentrated sugar solutions: i) a fermentation technology with integrated product recovery by means of intermittent vacuum

(Chapter 4), and ii) an in-depth research of biofilm formation by *C. sacharoperbutylacetonicum* DSM 14923. Hereby the composition and properties of the extracellular polymeric substances present in the biofilm were compared to those of planktonic cells to obtain a better understanding of how the biofilm lifestyle can protect the cells against harsh environments **(Chapter 5)**.

Thus, extractive ABE batch fermentations by *C. saccharoperbutylacetonicum* DSMZ 14923 were carried out in which intermittent vacuum cycles were applied for *in-situ* butanol removal (**Chapter 4)**. Firstly, an ABE standard solution at 56 mmHg and 25 mmHg at 30 °C was used to characterize the vacuum recovery process. Subsequently, the lowest absolute vacuum was chosen to assess the impact of the presence of acetic acid, 5-hydroxymethylfurfural (HMF), and furfural on evaporation from the standard ABE solution during vacuum recovery. Finally, ABE fermentations were carried out whereby the optimum medium composition obtained in **Chapter 3** (i.e., 75% of SCM and 25% of HH) was used. During the fermentations, *in-situ* recovery by cycles of 2-hours low pressure and 4-hours of atmospheric pressure was performed. Vacuum fermentation resulted in a decrease in the fermentation time, 97% conversion of total sugars, and improved cell growth and ABE production by *C. saccharoperbutylacetonicum*. As a result, this approach increased ABE productivity and ABE yield.

In **Chapter 5,** entirely novel results are presented about the ability of the wild-type *C. saccharoperbutylacetonicum* DSM 14923 to grow as a biofilm, as well as the first analysis of biofilm composition in terms of extracellular polymeric substances (EPS). To this end, a comparative study was conducted to investigate the functional mechanisms in biofilms compared to planktonic cells. Hereby we applied cutting-edge analytical techniques such as spectroscopy, chromatography, morphological, and colorimetric analysis, along with quantitative proteomics of sessile and planktonic cells to obtain an in-depth characterization of these biofilms. The fastest biofilm growth was observed at a high dilution rate ($D = 0.28\ h^{-1}$) during chemostat cultivation under acidogenic conditions. Autofluorescence revealed the likely presence of tryptophan. It was found that planktonic cells, aggregates, and biofilm contained glycerol, galactosamine, rhamnose, glucosamine, glucose, and ribose. Xylose and mannose were only identified in the biofilm

sample. Biofilm, aggregates, and planktonic cells contained respectively 4 wt%, 7 wt%, and 11 wt% sugar mix equivalents, and respectively 99 wt%, 84 wt%, and 53 wt% BSA equivalents. Therefore, it was concluded that the biofilm formed by *C. saccharoperbutylacetonicum* is dominated by polypeptides/proteins. A total number of 164 proteins were enriched in the biofilm samples when compared with the planktonic cells, of which 124 were identified, either based on homology (47%) or sequence similarity (53%), and 40 could not be characterized. Most remarkably, a β-lactamase homolog was identified in the biofilm sample, indicating possible antibiotic resistance of biofilm grown cells.

In **Chapter 6**, the potential and robustness of *C. saccharoperbutylacetonicum* are evidenced in this thesis. The impact of butanol production using biofilms and vacuum product recovery on the bioenergy sector as well as the importance of using sugarcane bagasse hemicellulosic hydrolysate (an abundant and inexpensive substrate) are highlighted as strategies to partly solve the problem of the economic viability of second-generation ABE fermentation. Nevertheless, challenges such as low yield and energy-intensity of the recovery process must be overcome to successfully apply butanol as an alternative fuel, thus showing the need for future investigations to make second-generation butanol production on industrial scale becomes a reality.

Keywords: Second-generation butanol, Clostridia biofilms, Extracellular polymeric substances, Sugarcane industry by-products, *In-sit*u product recovery, ABE fermentation.

Resumo

Atualmente, a indústria da biotecnologia encara o desafio de produzir produtos equivalentes aos derivados do petróleo a partir de recursos renováveis, de maneira sustentável e economicamente viável. Encontrar alternativas mais limpas para substituir combustíveis e produtos químicos de origem fóssil têm sido objeto de pesquisas em todo o mundo, seja por razões econômicas, geopolíticas ou ambientais. Entre essas alternativas, merecem destaque os combustíveis líquidos derivados da conversão de biomassa lignocelulósica, conhecidos como combustíveis de segunda geração ou 2G.

No contexto da produção de etanol de segunda geração a partir de matérias-primas lignocelulósicas, o principal açúcar utilizado na fermentação, a glicose, provém da hidrólise da celulose sendo que uma parte significativa de açúcares, nomeadamente pentoses (C5), não são metabolizados pelas cepas selvagens industriais de *Saccharomyces cerevisiae,* largamente utilizadas nas usinas de etanol. Dentre várias possibilidades de uso do C5 como matéria-prima, a conversão em butanol tem atraído o interesse por suas extensas possibilidades de aplicação como produto químico, intermediário químico e/ou combustível avançado com propriedades físicas próximas à gasolina. O butanol pode ser produzido a partir de derivados de petróleo (processo oxo de propileno) ou a partir de matérias-primas renováveis (processo acetona, butanol e etanol (ABE)). A fermentação ABE é realizada por bactérias solventogênicas do gênero *Clostridium* spp., capazes de metabolizar açúcares C5 e uma grande variedade de outros substratos.

Entretanto, a rota biológica de produção de butanol é desafiadora e sua viabilidade econômica em escala industrial enfrenta obstáculos como: inibição do butanol, baixa eficiência energética do processo, baixo rendimento e baixa produtividade, além do custo relativamente alto do substrato, representando até dois terços dos custos do processo de produção de butanol. Portanto, a recuperação eficiente de butanol do caldo de fermentação diluído (~12 g de butanol/L) determina em grande parte a eficiência do processo de produção. Além disso, em um processo 2G, a fermentação ABE é sensível a compostos inibitórios presentes no hidrolisado hemicelulósico gerados no pré-tratamento da biomassa.

Diante desses desafios e considerando o alto impacto que o uso de matérias-primas lignocelulósicas de baixo custo poderia representar, este trabalho tem como objetivo explorar diferentes estratégias para contribuir com o desenvolvimento de configurações de biorrefinaria com base na cana-de-açúcar focadas na valorização de açúcares C5.

Portanto, no **Capítulo 2**, é descrito um estudo sistemático da fermentação de quatro cepas de Clostridia do tipo selvagem, *C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864 e *C. saccharoperbutylacetonicum* DSM 14923, como potenciais candidatos para a biossíntese de ABE usando xilose ou glicose como fonte primária de carbono. Destaca-se a capacidade de *C. saccharobutylicum* DSM 13864 e *C. saccharoperbutilylacetonicum* DSM 14923, bem como a notável capacidade dessa última cepa em atingir um título de ABE relativamente alto (>7,0 g/L) do hidrolisado hemicelulósico não detoxificado. Os resultados indicaram o potencial de *C. saccharoperbutilacetonicum* DSM 14923 como uma plataforma microbiana promissora para a produção de butanol de segunda geração. Por fim, são apresentados novos insights de *C. sacharoperbutilacetonicum* DSM 14923 sobre seu desempenho fermentativo, efeito sinérgico dos inibidores, robustez e tolerância ao butanol.

Posteriormente, o **Capítulo 3** aborda um estudo sobre a aplicabilidade de misturas de hidrolisado hemicelulósico (HH) e melaço de cana (SCM) como substratos para a produção de ABE de segunda geração. A co-fermentação desses dois substratos foi avaliada como uma estratégia para diminuir as concentrações dos inibidores presentes na HH, evitando assim os custos das etapas de detoxificação. Os melhores resultados em termos de concentração de ABE (8,22 g/L) e rendimento (0,34 g/g) foram obtidos no meio contendo 75% dos açúcares provenientes de SCM. Quando o HH foi concentrado (de 15 a 52 g/L), tanto o título quanto o rendimento de ABE aumentaram para 9,79 g/L e 0,36 g/g, respectivamente, provavelmente como resultado de um efeito sinérgico positivo entre baixas concentrações de 5-hidroximetilfurfural (HMF) e os compostos dos meios. A preferência de açúcar da cepa nesses meios mistos foi glicose> frutose> sacarose> xilose> arabinose. Os resultados obtidos demonstraram que a adição de

SCM em altas proporções promoveu a bioconversão efetiva de hidrolisados C5 concentrados em butanol com altos rendimentos e produtividades. Assim, novos insights foram obtidos sobre a potencialidade da produção de butanol contribuindo para uma biorrefinaria de cana-de-açúcar.

Além disso, duas estratégias para aumentar a produtividade de butanol do processo foram abordadas com o objetivo de aliviar a inibição do produto nas células e permitir o processamento de soluções concentradas de açúcar: i) uma tecnologia de fermentação com recuperação integrada do produto por meio de vácuo intermitente (**Capítulo 4**), e ii) uma pesquisa aprofundada da formação de biofilme por *C. sacharoperbutilylacetonicum* DSM 14923. Nesta última estratégia, a composição e as propriedades das substâncias poliméricas extracelulares (EPS) presentes no biofilme foram comparadas com as das células planctônicas para obter uma melhor compreensão de como o biofilme pode proteger as células contra ambientes adversos (**Capítulo 5**).

No **Capítulo 4**, fermentações extrativas por batelada de ABE foram realizadas por *C. saccharoperbutilacetonicum* DSMZ 14923, nas quais ciclos de vácuo intermitentes foram aplicados para remoção *in-situ* de butanol (**Capítulo 4**). Primeiramente, uma solução padrão de ABE a 56 mmHg e 25 mmHg a 30 °C foi usada para caracterizar o processo de recuperação a vácuo. Posteriormente, o menor vácuo absoluto foi escolhido para avaliar o impacto da presença de ácido acético, 5-hidroximetilfurfural (HMF) e furfural na evaporação da solução ABE padrão durante a recuperação a vácuo. Durante as fermentações, foi realizada a recuperação *in-situ* por ciclos de 2 horas a vácuo e 4 horas a pressão atmosférica. A fermentação a vácuo resultou na redução do tempo de fermentação, na conversão de 97% dos açúcares totais, melhoria no crescimento celular e produção de ABE por *C. saccharoperbutilylacetonicum*. Como resultado, essa abordagem aumentou a produtividade e o rendimento dos solventes ABE.

No **Capítulo 5**, são apresentados inéditos resultados sobre a capacidade do *C. saccharoperbutilylacetonicum* DSM 14923 de crescer como biofilme, bem como a primeira análise da composição do biofilme em termos de substâncias poliméricas extracelulares (EPS). Para tanto, foram aplicadas técnicas analíticas de ponta, como espectroscopia, cromatografia, análise morfológica e colorimétrica, juntamente

com proteômica quantitativa para obter uma caracterização aprofundada desses biofilmes. O crescimento mais rápido do biofilme foi observado em uma alta taxa de diluição ($D = 0,28\ h^{-1}$) durante o cultivo em quimiostato, sob condições acidogênicas. Verificou-se que as células planctônicas, agregados e biofilme continham glicerol, galactosamina, ramnose, glucosamina, glicose e ribose. Xilose e manose foram identificadas apenas na amostra de biofilme. As células de biofilme, agregados e planctônicas continham 4%, 7% e 11% (em peso) respectivamente de equivalentes da mistura de açúcar e 99%, 84% e 53% (em peso) respectivamente de equivalentes de BSA. Portanto, concluiu-se que o biofilme formado por *C. saccharoperbutilacetonicum* é dominado por polipeptídeos/proteínas. Um número total de 164 proteínas foi enriquecido nas amostras de biofilme quando comparado às células planctônicas das quais 124 foram identificadas, com base na homologia (47%) ou na similaridade de sequência (53%), sendo que 40 não puderam ser caracterizadas. Notavelmente, um homólogo de β-lactamase foi identificado na amostra de biofilme, indicando possível resistência a antibióticos de células cultivadas em biofilme.

No **Capítulo 6**, o potencial e a robustez do *C. saccharoperbutilacetonicum* são evidenciados nesta tese. O impacto da produção de butanol usando biofilmes e recuperação de produtos a vácuo no setor da bioenergia, bem como a importância do uso de hidrolisado hemicelulósico de bagaço de cana (um substrato abundante e barato) são destacados como estratégias para resolver parcialmente o problema da viabilidade econômica do processo de fermentação ABE de segunda geração. No entanto, desafios como baixo rendimento e intensidade energética do processo de recuperação devem ser superados para usar com sucesso o butanol como combustível alternativo, mostrando assim a necessidade de futuras investigações para tornar a produção de butanol de segunda geração em escala industrial uma realidade.

Palavras-chave: Butanol de segunda geração, Biofilmes de Clostridia, Substâncias poliméricas extracelulares, Subprodutos da indústria da cana-de-açúcar, Recuperação *in-situ* do produto, Fermentação ABE.

Samenvatting

Een van de uitdagingen van de moderne biotechnologie is om producten die nu nog worden gemaakt op basis van aardolie op een duurzame en economisch haalbare manier te produceren uit hernieuwbare grondstoffen. Het vinden van schonere alternatieven voor benzine, brandstoffen en chemicaliën is wereldwijd het onderwerp van onderzoek, zowel om economische, geopolitieke als milieuredenen Op dit moment is eerste generatie (1G) bio-ethanol, geproduceerd uit (riet)suiker de belangrijkste biobrandstof.

Bij de productie van tweede generatie (2G) bio-ethanol uit lignocellulose houdende grondstoffen is het probleem dat een aanzienlijk deel van de suikers, namelijk de pentosen (C5 suikers), niet kunnen worden gemetaboliseerd door conventionele *Saccharomyces cerevisiae* gisten. Er zijn verschillende mogelijkheden om C5 suikers als grondstof te gebruiken, waarbij er grote belangstelling is voor de vergisting naar butanol in plaats van ethanol vanwege het feit dat butanol een waardevolle chemische bouwsteen is en tevens een geavanceerde biobrandstof met betere fysische eigenschappen dan ethanol.

Butanol kan zowel worden geproduceerd uit aardoliederivaten (oxosynthese uit propeen) als uit hernieuwbare grondstoffen via het aceton-, butanol- en ethanol (ABE) fermentatieproces door *Clostridium* spp. die zowel C5-suikers als een grote verscheidenheid aan andere substraten kunnen metaboliseren. De productie van bio-butanol is echter een grotere uitdaging in vergelijking met de productie van ethanol. De economische haalbaarheid op industriële schaal wordt beïnvloed door de toxiciteit van butanol voor het producerend organisme, de lage energetische efficiëntie van het productieproces, de minder eenvoudige opwerking in vergelijking met ethanol, de lage opbrengst en lage productiviteit. Verder zijn de substraatkosten hoog en kunnen tot wel twee derde van de kosten van het gehele productieproces van butanol uitmaken. Vandaar dat een efficiënte winning van de butanol die in lage concentratie in het fermentatiebeslag aanwezig is (~12 g butanol/L) in hoge mate de efficiëntie van het productieproces bepaalt. Bovendien is in een 2G-proces de ABE-fermentatie

gevoelig voor toxische verbindingen die aanwezig zijn in het hemicellulose hydrolysaat en ontstaan zijn tijdens de (thermische) voorbehandeling van de biomassa.

Gezien de bovengenoemde uitdagingen en de grote impact die het gebruik van goedkope lignocellulose houdende grondstoffen zou kunnen hebben, beoogt dit werk verschillende strategieën te onderzoeken om bij te dragen aan de ontwikkeling van bioraffinage-systemen op basis van suikerriet, met de nadruk op valorisatie van de aanwezige C5-suikers.

In **Hoofdstuk 2** worden vier wildtype *Clostridium*-stammen, namelijk *C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864 en *C. saccharoperbutylacetonicum* DSM 14923, als potentiële kandidaten voor ABE-biosynthese uit xylose of glucose als primaire koolstofbron met elkaar vergeleken. *C. saccharobutylicum* DSM 13864 en *C. saccharoperbutylacetonicum* DSM 14923 bleken de best presterende stammen waarbij de laatste de hoogste ABE-titer (>7,0 g/L) werd behaald uit niet-ontgift hemicellulose hydrolysaat. Deze resultaten wijzen op het potentieel van *C. saccharoperbutylacetonicum* DSM 14923 als microbieel platform voor de productie van tweede generatie butanol, aangezien de fermentatie van hemicellulose hydrolysaat met succes werd uitgevoerd. Nieuwe inzichten met betrekking tot de prestaties, het synergetische effect van remmers, robuustheid en butanol tolerantie van *C. sacharoperbutylacetonicum* DSM 14923 worden gepresenteerd.

Vervolgens wordt in **Hoofdstuk 3** verder onderzoek naar de toepassing van mengsels van suikerrietbagasse hemicellulose hydrolysaat (HH) en suikerrietmelasse (SCM) als substraten voor tweede generatie ABE-productie. De co-vergisting van deze twee substraten werd toegepast als strategie om de concentraties van de in HH aanwezige remmers te verlagen waardoor de kosten van ontgifting werden vermeden.

De beste resultaten in termen van butanol titer (8,22 g/L) en opbrengst (0,34 g/g) werden verkregen wanneer 75% van de suikers afkomstig waren van SCM. Wanneer HH werd geconcentreerd (van 15 tot 52 g/L), namen zowel de butanoltiter als de opbrengst toe tot respectievelijk 9,79 g/L en 0,36 g/g, waarschijnlijk als gevolg van een positief synergetisch effect tussen lage

concentraties van 5- hydroxymethylfurfural (HMF) en de mediasamenstellingen. De suikervoorkeur van de stam op deze gemengde media was glucose> fructose> sucrose> xylose> arabinose. Uit de resultaten bleek dat de toevoeging van SCM de effectieve bioconversie van geconcentreerde C5-hydrolysaten de butanol opbrengst en productiviteit verhoogde. Aldus werden nieuwe inzichten verkregen hoe een gesloten kringloop van butanol in een circulaire economie voor de valorisatie van een bioraffinage van suikerriet kan worden gerealiseerd.

Vervolgens werden twee strategieën voor het verhogen van de butanol productiviteit van het proces toegepast waarbij productremming werd vermeden en de verwerking van geconcentreerde suikeroplossingen mogelijk werd gemaakt:

i) toepassen van een fermentatietechnologie met geïntegreerde productterugwinning door middel van intermitterend vacuüm (**Hoofdstuk 4**), en

ii) een diepgaand onderzoek naar biofilm vorming door *C. sacharoperbutylacetonicum* DSM 14923, waarbij de samenstelling en eigenschappen van de extracellulaire polymere stoffen (EPS) aanwezig in de biofilm werden vergeleken met die in vrije cellen om beter te begrijpen hoe biofilm vorming de cellen kan beschermen tegen agressieve milieu omstandigheden (**Hoofdstuk 5**).

Zo werden extractieve ABE-batchfermentaties met *C. saccharoperbutylacetonicum* DSMZ 14923 uitgevoerd waarin intermitterende vacuümcycli werden toegepast voor in-situ-butanolverwijdering (**Hoofdstuk 4**). Als eerste werd een ABE-standaardoplossing bij 56 mmHg en 25 mmHg bij 30 °C gebruikt om het vacuüm proces te karakteriseren. Vervolgens werd het laagste absolute vacuüm gekozen om de impact van de aanwezigheid van azijnzuur, furfural en 5-hydroxymethylfurfural (HMF) op de verdamping van de standaard ABE-oplossing tijdens vacuümterugwinning te beoordelen. Ten slotte werden ABE-fermentaties uitgevoerd waarbij de optimale mediumsamenstelling, zoals beschreven in **Hoofdstuk 3** (dwz 75% van SCM en 25% van HH) werd gebruikt. Tijdens de fermentaties werden cycli van 2 uur lage druk en 4 uur atmosferische druk uitgevoerd. Vacuümverdamping tijdens de ABE-fermentatie leidde tot een reductie van de fermentatietijd, 97% uitputting van totale suikers en verbeterde celgroei en ABE-productie door *C. saccharoperbutylacetonicum*. Al met al leidde

deze in-situ productverwijderings techniek tot een verhoging van de ABE-productiviteit en ABE-opbrengst.

In **Hoofdstuk 5** presenteren we geheel nieuwe resultaten over het vermogen van het wildtype *C. saccharoperbutylacetonicum* DSM 14923 om te groeien als een biofilm. Tevens wordt een eerste analyse van de biofilm samenstelling in termen van extracellulaire polymere stoffen (EPS) uitgevoerd. Hiertoe werd een vergelijkende studie uitgevoerd om de functionele mechanismen in biofilms te vergelijken met die in vrije cellen. Hierbij werden geavanceerde technieken zoals spectroscopie, chromatografie, morfologische en colorimetrische analyse gecombineerd met kwantitatieve proteoom analyse van sessiele en vrije cellen voor een diepgaande moleculaire karakterisering van deze biofilms. De snelste biofilm aangroei werd waargenomen bij een hoge verdunningssnelheid ($D=0{,}28\ h^{-1}$) tijdens chemostaat cultivatie onder acidogene condities. Autofluorescentie onthulde de waarschijnlijke aanwezigheid van tryptofaan. Zowel vrije cellen, cel aggregaten en biofilm bevatten glycerol, galactosamine, rhamanose, glucosamine, glucose en ribose. Xylose en mannose werden alleen geïdentificeerd in het biofilmmonster. Biofilm, aggregaten en planktoncellen bevatten respectievelijk ongeveer 4 gew.%, 7 gew.% En 11 gew.% suikermixequivalenten en respectievelijk 99 gew.%, 84 gew.% En 53 gew.% BSA-equivalenten. Hieruit bleek dat de biofilm gevormd door *C. saccharoperbutylacetonicum* wordt gedomineerd door polypeptiden / eiwitten. Een totaal aantal van 164 eiwitten bleek in grotere mate aanwezig in de biofilmmonsters in vergelijking met de vrije cellen, waarvan 124 konden worden geïdentificeerd op basis van homologie (47%) of sequentiegelijkenis (53%) en 40 met onbekende functie. Het meest opvallend was dat in het biofilmmonster een β-lactamase-homoloog werd geïdentificeerd, hetgeen een mogelijke antibioticaresistentie van in biofilm gekweekte cellen aangeeft.

In **Hoofdstuk 6** wordt het potentieel en de robuustheid van *C. saccharoperbutylacetonicum* verder toegelicht. De impact van butanolproductie met behulp van biofilms en in-situ product terugwinning met behulp van vacuüm fermentatie, evenals het gebruik van suikerriet bagasse hemicellulosic hydrolysaat (een overvloedig en goedkoop substraat), worden benadrukt als strategieën om

het probleem van de economische levensvatbaarheid van tweede generatie ABE gisting op te lossen. Desalniettemin moeten uitdagingen zoals een lage opbrengst en energie-intensiteit van het terugwinningsproces worden overwonnen om butanol met succes als alternatieve brandstof toe te passen. Daarmee wordt de behoefte aan verder onderzoek aangetoond om tweede generatie butanol productie op industriële schaal te realiseren.

Trefwoorden: tweede generatie butanol, *Clostridium*-biofilms, extracellulaire polymere stoffen (EPS), bijproducten van de suikerrietindustrie, *in-situ* product terugwinning, ABE-fermentatie.

Chapter

1

General Introduction

1.1 *n*-Butanol production

The increasing global interest for cleaner energy sources, the depletion of fossil fuels, and the oil price instability have led to a growing demand for renewable alternatives whether for environmental, economic, and geopolitical reasons. Efforts have been addressed worldwide on attaining environmentally friendly fuels (as bioethanol and biobutanol) from renewable feedstocks, thereby contributing to lower greenhouse gas (GHG) emissions (Maiti et al., 2016b). Although bioethanol is the major biofuel currently utilized in the world, being produced in Brazil on a large scale by processing sugarcane juice and/or molasses in a process known as first-generation (1G) ethanol (Della-Bianca et al., 2014), other biofuels produced by microbial fermentation as for example butanol and isobutanol have attracted attention and large investments in research with butanol as one of its highlights (Patakova et al., 2018).

n-Butanol, also known as butyl alcohol or 1-butanol (hereafter referred to as butanol) is a four-carbon primary alcohol that has stood out as an alternative superior biofuel and also as an important chemical building block for many industries (Rathour et al., 2018). Butanol and its derivatives have been used in several industrial applications, such as surface coatings, plasticizers, rubbers, and diluents. It is also used in the manufacture of glycolic ethers, antibiotics, vitamins, and hormones, corroborating the versatility and market importance of this product (Green 2011).

Butanol (C_4H_9OH) is considered to be a superior biofuel in comparison with ethanol (C_2H_5OH) due to several advantageous properties, such as i) higher miscibility with gasoline or diesel, ii) lower water solubility making it less corrosive, iii) higher energy density, lower octane value, and iv) higher flash point and lower vapor pressure (Isomäki et al., 2017). A comparison of the butanol properties to those of other liquid fuels is presented in **Table 1**.

Table 1. Comparison of the physical properties of butanol with ethanol and gasoline.

Fuel properties	Butanol	Ethanol	Gasoline
Energy content (MJ/L)	29.2	21.2	32.0
Boiling point (°C)	118	78	25-215
Density (g/mL)	0.81	0.79	0.7-0.8
Air-fuel ratio	11.1	9.0	14.6
Motor octane number	78	89	81-89

Adapted from Rathour et al. (2018) and Wang et al. (2014)

Moreover, the butanol ''drop-in'' characteristics make it a more attractive product as gasoline, diesel, and ethanol-blending fuel. Moreover, butanol may be catalytically upgraded to jet fuel, and can be produced using the existing ethanol infrastructure, and can be transported via existing pipelines (Mariano et al., 2016).

Butanol can be produced either by renewable feedstocks via ABE fermentation using solventogenic *Clostridium* species or from petroleum derivatives (oxo process from propylene). Apart from solventogenic *Clostridium* spp., it is believed that no other genera of bacteria, archaea, or eukaryotes are sufficiently efficient to naturally produce butanol (Qureshi and Ezeji, 2008).

1.1.1 Global market and techno-economic aspects of butanol

Most of the currently marketed butanol is still produced by the petrochemical route using the oxo-process. Main global producers are Dow, BASF, Celanese, and Eastman, as well as Sasol in South Africa, KH Neochem in Japan, and Elekeiroz in Brazil (Natalense and Zouain, 2013). The average butanol price from January 2010 to February 2014 was 1.10±0.07 €/kg (Straathof and Bampouli, 2017).

Therefore, biobased butanol has the potential to substitute both ethanol and biodiesel in the biofuel market, estimated to be worth $247 billion by 2020 (Green, 2011). The butanol market is projected to grow from USD 4.2 billion in 2017 to USD 5.6 billion by 2022, at a compound annual growth rate (CAGR) of 5.9% during the forecast period. Increasing demand from a wide range of applications such as latex

paint formulations in industrial and architectural activities, enamels, textiles, and paper finishes is driving the market.

In 2008, biobased butanol production was re-established in China to supply its growing market. Cathay Industrial Biotech began supplying butanol under their Biosol brand, production capacity is known 100,000 tonnes. There are six major plants that produce about 30 000 tonnes of butanol pa from corn starch. Most plants operate in a semi continuous mode with each fermentation lasting up to 21 days. The plants typically house several trains of up to eight fermentation tanks (300–400 m^3 volumes) linked together in series (Green, 2011).

Companies seeking to commercialize biobased butanol include Butamax Advanced biofuels, the UK companies Green Biologics and Solvert, US technology companies Cobalt Technologies, and Gevo development. Butamax and Gevo are mostly focused on isobutanol technology (Jong et al., 2011).

1.2 ABE fermentation

Bio-based butanol is produced by solvent-producing *Clostridium* spp. that present the desired capability of metabolizing a huge range of simple and complex sugars such as pentoses and aldohexoses which are converted into acetone, butanol, and ethanol in an anaerobic fermentation process, known as ABE fermentation. Historically, ABE fermentation was already taking place on a large scale up to the late 1950's. This fermentation is mainly performed by *Clostridium acetobutylicum* and *Clostridium beijerinckii* since they have been the most studied strains (Lütke-Eversloh, 2014; Yoo et al., 2015).

ABE fermentation is generally a bi-phasic process consisting of an acidogenesis and solventogenesis phase. From the solventogenic Clostridia metabolism, it is known that during acidogenesis, carbohydrates are metabolized to acetic and butyric acids along with ethanol, hydrogen, and carbon dioxide. Subsequently, as a result of acid production, the pH drops, which triggers the Clostridia cells to switch its metabolism to solventogenesis during which the acids are converted into acetone, butanol, and ethanol.

Clostridia metabolize hexoses by the Embden-Meyerhof-Parnas (EMP) pathway thereby generating 2 mol of pyruvate, 2 mol of adenosine triphosphate (ATP) and 2 reduced nicotinamide adenine dinucleotide (NADH) per mol hexose, whereas pentoses are metabolized via the pentose phosphate pathway (PPP) and are converted to pentose-5-phosphate and dissimilated by transketolases and transaldolases through the non-oxidative branch of the pentose phosphate pathway, resulting in the production of glyceraldehyde 3-phosphate and fructose-6-phosphate, that finally enter the EMP pathway for further conversion. During the dissimilation through the PPP, carbon dioxide (CO_2), ATP and NADPH are produced (Dürre, 2007; Ranjan and Moholkar, 2012; Shinto et al., 2008), as shown in **Figs. 1 and 2**. However, *Clostridium saccharoperbutylacetonicum* (the primary strain used in this study) does not contain the phosphoketolase pathway; and thus, pentoses can only be metabolized via the PPP and whereby 3 mol of pentoses are converted to 5 mol of pyruvate, 5 mol of ATP, and 5 mol of NADH (Shinto et al., 2008).

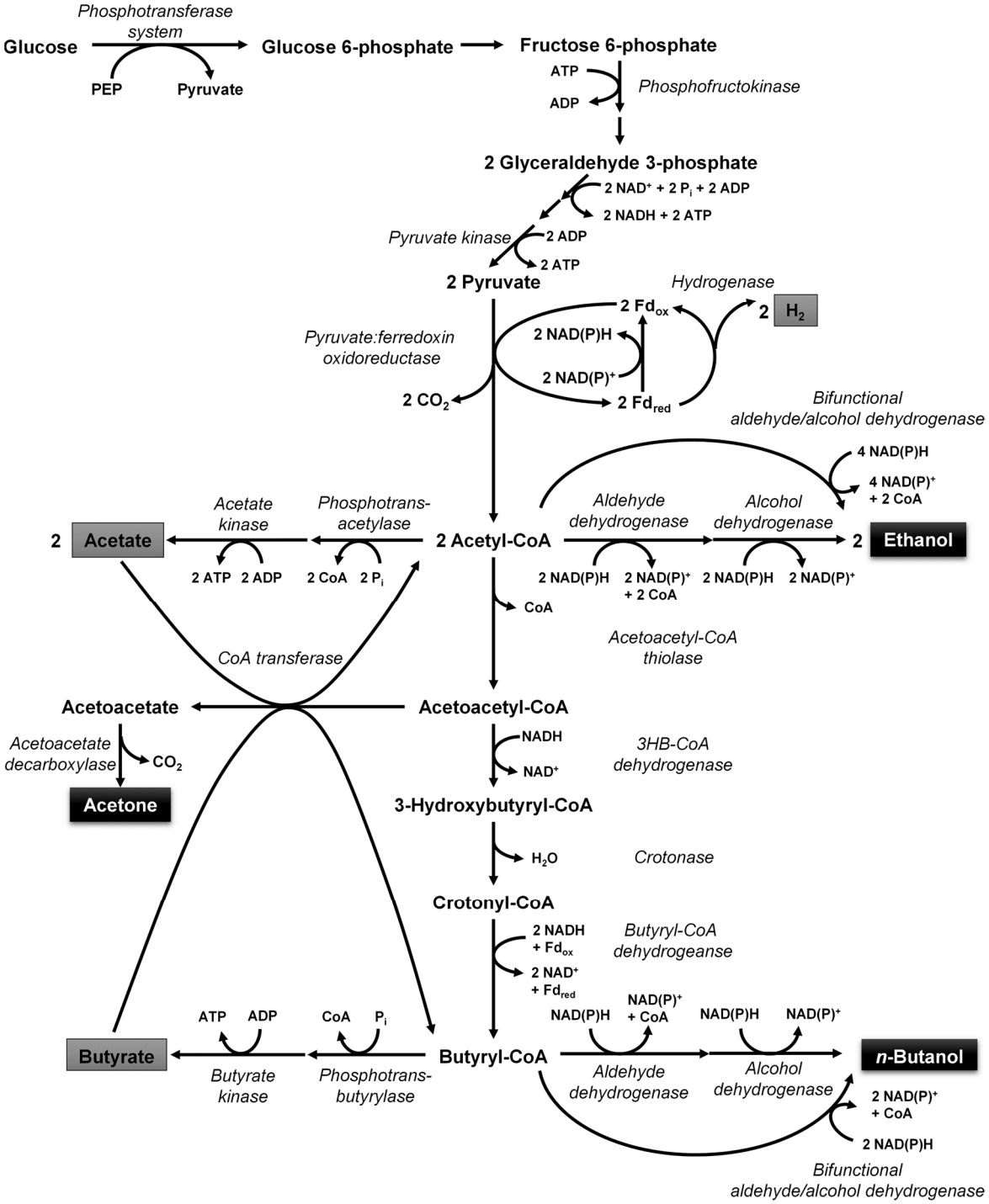

Figure 1. Metabolic pathways and enzymes in Clostridial ABE fermentation using glucose as carbon source. Reproduced from Moon et al. (2016).

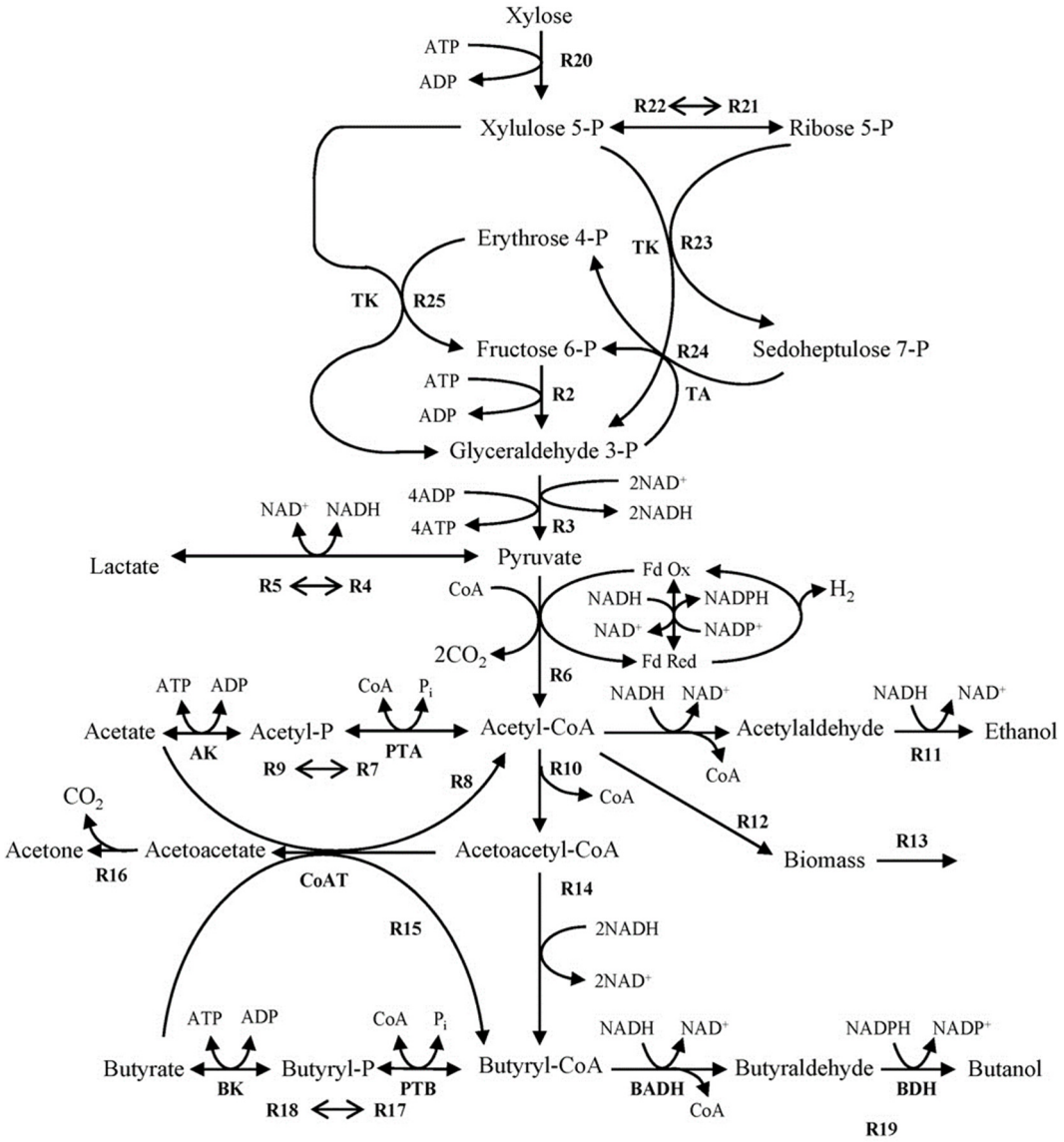

Figure 2. Metabolic pathways in *Clostridium acetobutylicum* ATCC 824T using xylose as carbon source. Enzymes are written in bold and abbreviated as shown: transketolase (TK); transaldolase (TA); phosphotransacetylase (PTA); acetate kinase (AK); CoA transferase (CoAT); phosphotransbutyrylase (PTB); butyrate kinase (BK); butyraldehyde dehydrogenase (BADH); butanol dehydrogenase (BDH). Reproduced from Shinto et al. (2008).

The genus *Clostridium* is one of the biggest bacterial genera, consisting of more than 150 described species (Goldman and Green 2008). Bacteria belonging to this genus share some characteristics, such as a gram-positive cell wall, spore formation, obligate anaerobic metabolism, inability to reduce sulfate to sulfite, and a low percentage of guanines and cytosines in their genomic DNA. Although Clostridia are obligate anaerobic bacteria, their oxygen sensitivity varies within the genus, ranging from aerotolerant to strictly obligate anaerobic species (Dworkin et al. 2006).

The principal factors hampering the commercial production of conventional ABE batch fermentation processes include the high raw material price which is ~66% of the whole process (Jiang et al., 2015), the low butanol titers and yields achieved in batch fermentation due to the toxicity of the produced solvents (specially butanol), and the low butanol selectivity which results in high downstream processing costs (Jiang et al., 2015; Patakova et al., 2018).

Systematic studies comparing the performance of different wild-type strains in ABE fermentation using pentose-rich media are currently limited. Magalhães et al. (2018) assessed the fermenting capability to produce butanol of twelve solventogenic *Clostridium* spp. from a glucose-rich straw hydrolysate. Their work highlights the notable capability of *C. saccharoperbutylacetonicum* to preferentially synthesize butanol compared to other solvents, *i.e.,* ethanol and acetone, and the ability of *C. saccharobutylicum* to efficiently metabolize glucose and xylose.

The ability of *C. saccharoperbutylacetonicum* to ferment a broad range of carbohydrates and to tolerate some inhibitory compounds that are found in lignocellulosic carbon sources has been reported recently by Yao et al. (2017) using synthetic hemicellulosic hydrolysates. Based on this work, individual sugars can be efficiently consumed, with the following preference:
glucose>cellobiose>xylose>arabinose>mannose. It was observed that sugar consumption is sugar-type and concentration-dependent when mixed sugars are used in the medium. In terms of inhibitors, the authors found that phenolic compounds (*p*-coumaric, syringaldehyde, and ferulic acid) were more toxic than furfural and 5-hydroxymethylfurfural (HMF) (Yao et al., 2017).

Recent reports have addressed the development of both the microorganisms and the ABE fermentation processes using different metabolic engineering approaches to overcome such inherent process limitations (Moon et al., 2016). Therefore, the chosen strain, operating conditions, and reactor design play a crucial role in fermentation performance (Schügerl, 1997).

Commercial solvent titers peak at about 20 g/L from 55 to 60 g/L of substrate giving solvent yields of around 0.35 g/g sugar. The butanol: solvent molar ratio is typically 0.6 with an A:B:E ratio of 3:6:1. Butanol is the preferred solvent since it attracts the highest price in the chemical market (Green, 2011).

1.3 First- and second-generation butanol production in a sugarcane biorefinery

The use of biomass presents significant benefits over oil-based energy sources. Biomass is all organic matter, whether, of animal or vegetable origin, that can be utilized as feedstock for fuel production, and therefore, viewed as a potential source of energy (Kelman, 2008). It is a renewable and abundant source for energy production on a scale large enough to play a significant role in the development of vital renewable energy programs and in creating a more ecologically conscious society. Thus, the biorefinery concept appears as the facility that integrate biomass conversion processes to manufacture a portfolio of bio-based products such as fuels (e.g. bioethanol, biobutanol, biodiesel), bioenergy (heat and/or electricity), chemicals and materials, among others, analogous to fossil refineries (Hasunuma et al., 2013).

Biorefineries are classified according to the raw material used as first- (1G), second- (2G), or third-generation (3G). 1G biorefineries utilize food crop resources (e.g., sugar and vegetable oil), whereas 2G biorefineries use non-food raw materials which are usually lignocellulose-rich, i.e., agricultural waste, wood, and energy crops; and finally, 3G biorefineries use algal biomass (Palmeros Parada et al., 2017).

Fuels generated from biomass emit lower levels of greenhouse gases (GHG) through time and encourage reduced utilization of oil. Since lignocellulosic raw materials are the source of the most abundant sugars in the world, 2G biorefineries using this kind of feedstock appear to be extremely promising for butanol production. In fact, more than 40 million tons of inedible plant material are produced each year, many of which are discarded (Aslanzadeh et al., 2014; Kolesinska et al., 2019). Thus, the generation of biochemicals and biofuels from lignocellulosic biomass is one of the possible mitigations for climate change issues and energy crisis (Jang et al., 2012a). These materials include agricultural wastes, such as corn stover, switchgrass, wheat straw, and sugarcane bagasse (Kumar et al., 2012).

1.3.1 Sugarcane

Recently, sugarcane has been studied as a suitable feedstock for economically feasible production of butanol compared to other raw materials as it is an abundant source of soluble carbohydrates, which is ready to be used in fermentations (Villela Filho et al., 2011). Sugarcane (*Saccharum officinarum*) is a grass belonging to the Poaceae, originating from the tropical region of Southeast Asia and was introduced in Brazil (the world's largest producer of sugarcane) in the early 16th century by Portugueses (Rosillo-Calle, F., Bajay, S. V., Rothman, 2005).

The main features of this plant family are the shape of the inflorescence (spike) stem growth in stalks, leaves with silica blades at their edges, and open sheath. From its origin to the present day, it has been undergoing modifications, resulting in diverse species, which mainly differ in fiber and sugar content. Nowadays, most of the cultivated sugarcane is a multispecific hybrid, named *Saccharum* spp. (Joaquim, 1997). Sugarcane is composed of roots and rhizomes (underground part), and of stem, leaves, and flowers (aerial part), as shown in **Fig. 3**.

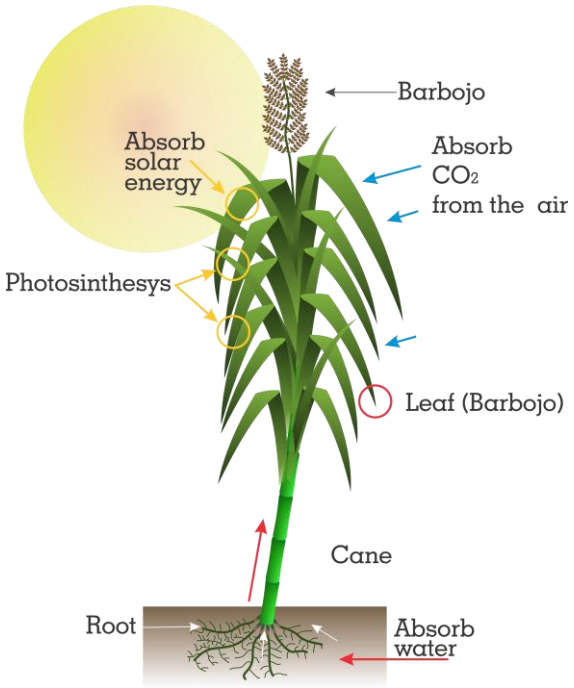

Figure 3. Parts of sugarcane. Adapted from Bocci et al. (2009).

1.3.2 Lignocellulosic materials from sugarcane

The transition towards renewable lignocellulosic raw materials can improve process sustainability and considerably decrease the cost of biofuel production once this biomass is energetically efficient, abundant, and not food competitive (Bharathiraja et al., 2017; Qureshi and Ezeji, 2008). Lignocellulosic biomass is basically composed of cellulose, hemicellulose, lignin, and minor amounts of other components, which are arranged as depicted in **Fig. 4.**

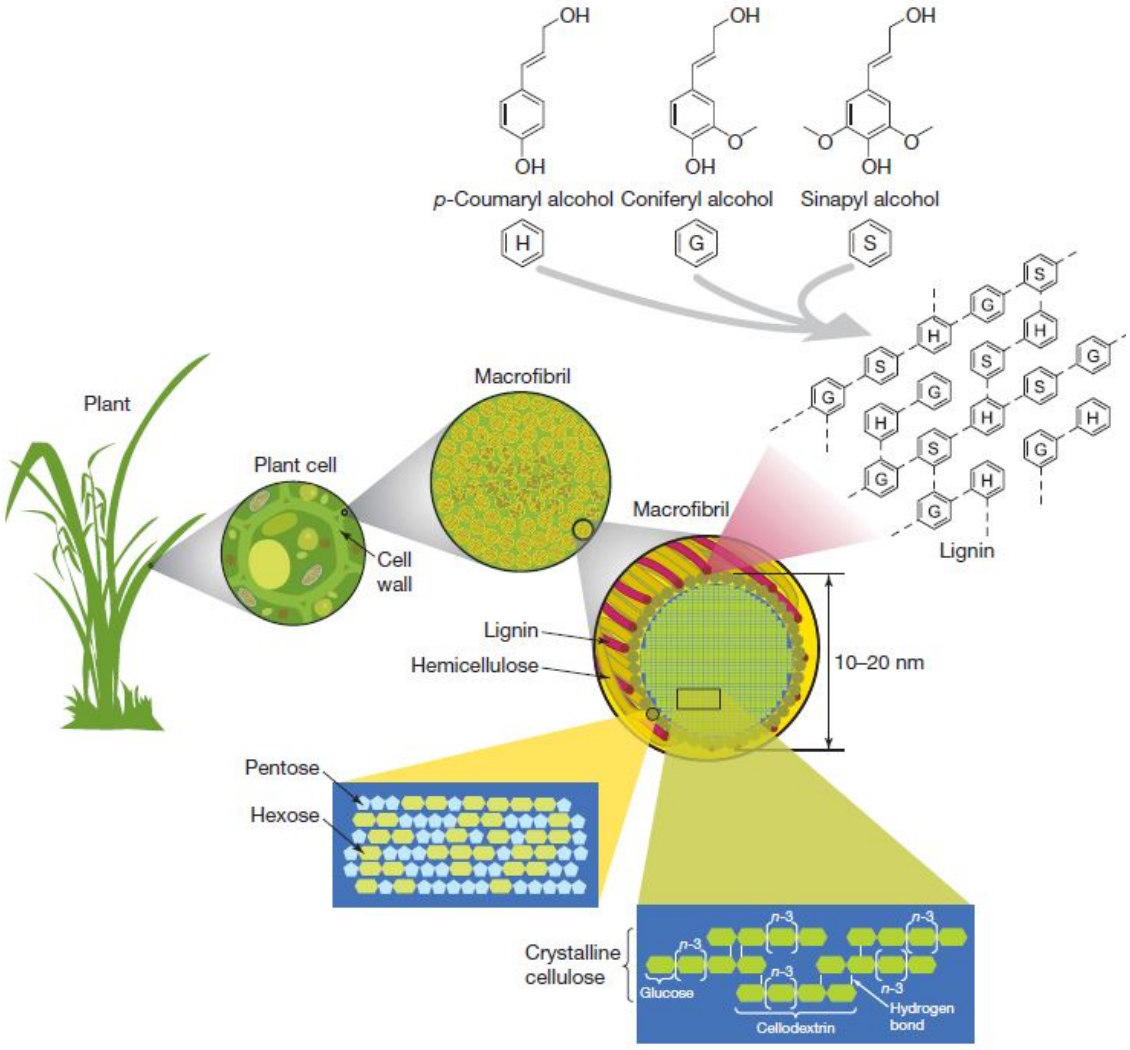

Figure 4. Lignocellulose structure. Reproduced from Rubin (2008).

Further, the raw material used for fermentation depends on the geographic location of the industry, for reasons of availability and logistics of transportation and utilization. In this context, Brazil has a great potential to produce butanol from lignocellulosic material since it stands out as the second-largest producer of first-generation (1G) biofuel ethanol in the world after the United States. The sugarcane mills in Brazil are based on sugarcane juice extraction to produce sugar and ethanol, which generates a large amount of sugarcane bagasse. The on-site sugarcane bagasse is primarily used for energy cogeneration and could be used for cost-competitive renewable production of chemicals and fuels in a sugarcane biorefinery (Tovar et al., 2017), as depicted in **Fig. 5**. Nevertheless, the use of lignocellulosic biomass (such as sugarcane bagasse) requires pre-treatment to break down its tight structure for increasing the accessibility of the material and facilitating the hydrolysis of hemicelluloses and cellulose sugars into oligomers and monomers which can be subsequently fermented into biofuels. Hemicellulose hydrolysis has been achieved usually using weak acid treatment and generates a pentose sugars-rich mixture containing mainly xylose and arabinose. The breakdown of cellulignin has been addressed by enzymatic hydrolysis using a fungal cellulolytic enzyme cocktail, generating a glucose-rich stream. The acid and enzymatic processes for hydrolysis are being intensely researched in order to allow the use of large amounts of non-food lignocellulosic feedstocks (Kumar et al., 2012).

However, besides sugars, inhibitory compounds are also formed during the pre-treatment step and have a negative impact on ABE fermentation. These inhibitory compounds are classified as organic acids: acetic, levulinic, and formic acids; furan derivatives: furfural and 5-hydroxymethylfurfural; and phenolic compounds: 4-hydroxybenzaldehyde, vanillin, syringaldehyde, catechol, and caffeic, *p*-coumaric, ferulic, glucuronic, syringic, and vanillic acids (Galbe and Zacchi, 2012; Zha et al., 2014). The group of phenolic compounds has been reported to severely impact the ABE fermentation, even at low concentrations (Baral and Shah, 2014; Yao et al., 2017). For instance, ferulic and *p*-coumaric acids have been shown to be inhibitory at concentrations as low as 0.3 g L^{-1} (Ezeji et al., 2007; Jang et al., 2012b; Magalhães et al., 2018). Some important features of butanol production from lignocellulosic feedstocks are summarized in **Table 2.**

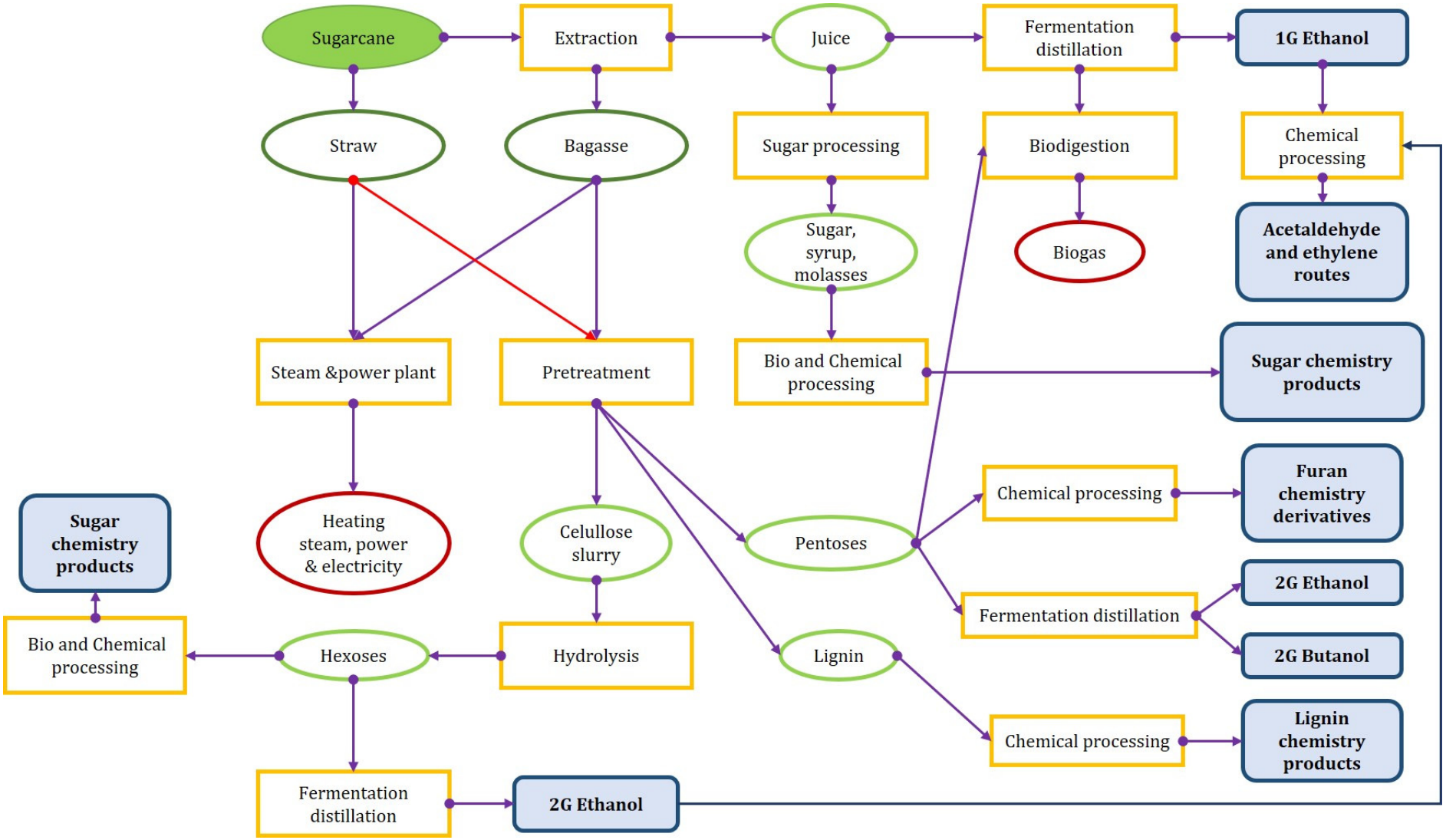

Figure 5. Sugarcane biorefinery (main products in bold).

Table 2. Butanol production from lignocelluloses.

Products (typical yields, kg/ton)	Butanol (80) Acetone (40) Ethanol (10) Hydrogen (7) Acetate and Butyrate Distiller's sludge, for biogas and Vitamin B12		
Typical yield (g/g glucose)	0.2–0.38		
Typical productivity	0.3–0.5 g/L·h		
Sugars preference by microorganisms	*C. acetobutylicum 824*	*C. acetobutylicum 260*	*C. beijerinckii*
	Glucose	Glucose	Cellobiose
	Arabinose	Cellobiose	Glucose
	Xylose	Mannose	Xylose
	Cellobiose	Arabinose	Arabinose
	Galactose	Galactose	Mannose
	Mannose	Xylose	Galactose
Typical fermentation conditions	T: 37 °C, pH: 6.8 Strictly anaerobic		
Main inhibitors in lignocellulosic hydrolysates	Lignin-derived: Syringaldehyde, ferulic, and *p*-coumaric acids Neutralization salts		
Main objective of pretreatment	Improvement of cellulose digestibility Facilitating recovery of hemicellulose Lignin removal		
Main objective of hydrolysis	Hydrolysis of cellulose to glucose Hydrolysis of hemicellulose to fermentable sugars		

* DDGD: dried distillers' grains and solubles; HMF: 5-hydroxymethylfurfural.
Reproduced from Amiri and Karimi, (2018).

Unlike *Saccharomyces cerevisiae,* traditionally used in 1G ethanol mills, which is unable to naturally metabolize pentose sugars (arabinose and xylose) (Amiri and Karimi, 2018; Mariano et al., 2013), solventogenic *Clostridium* spp. strains can metabolize a broad range of sugars, including xylose, the primary sugar that is available in the hemicellulosic fraction of the sugarcane bagasse hydrolysate. Since pentose sugars constitute a significant part of sugarcane bagasse, butanol would become an option for the biorefinery if this fraction were made available for fermentation.

General research in this field has attempted to address these problems by using metabolic engineering to improve yield and butanol tolerance (Green, 2011; Jang et al., 2012a; Jiang et al., 2009; Lee et al. , 2009b) as well as developing in-situ product recovery technologies to address butanol toxicity problems in microorganisms (Ezeji et al., 2003; Li et al., 2011; Lu et al., 2012 ; Mariano et al., 2011; Nielsen and Prather, 2009; Roffler et al., 1988; Xue et al., 2012). Several strategies to increase cell density (Tashiro et al., 2005), butanol titers, yield, and productivity (Ezeji et al., 2003) have also been explored using immobilized cells in biofilm reactors.

1.4 Clostridia biofilms

A biofilm is a community of microorganisms with the ability to settle on a surface or aggregate together by a self-produced complex three-dimensional matrix of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, lipids, and extracellular DNA (Limoli et al., 2015). Usually, there are five steps involved in biofilm formation on a surface: i) initial binding of planktonic cells to a hydrated surface; ii) initiation of the production of extracellular polymeric substances and formation of small aggregates; iii) irreversible binding to the surface by producing EPS; iv) development of a mature biofilm; and v) detachment of cells from the biofilm to colonize new regions and form new microcolonies (**Fig. 6**).

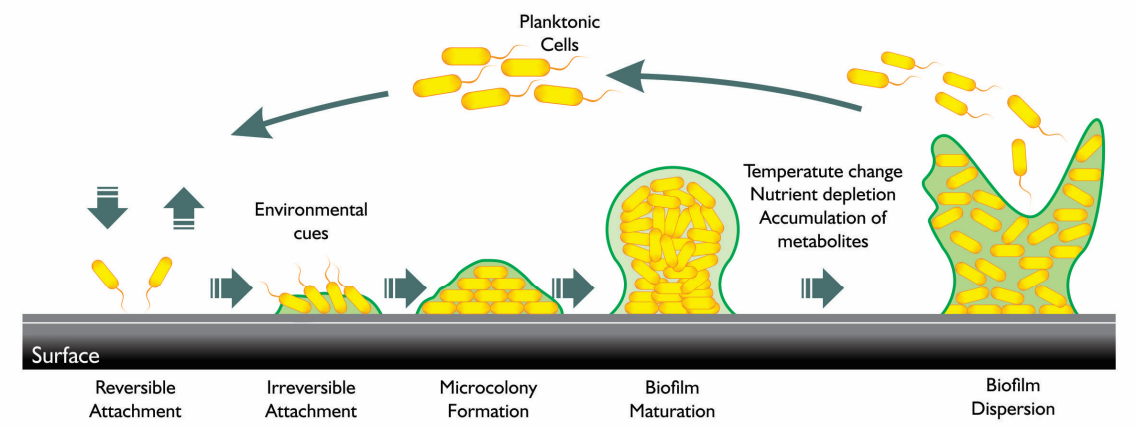

Figure 6. Schematic representation of biofilm formation on a surface.

Biofilms provide benefits to cells such as easy uptake of nutrients, and protection against environmental stressors by acting against antibiotics and disinfectants (Garrett et al., 2008). Therefore, microbial biofilms can be both beneficial or harmful to several emerging societal problems such as antimicrobial tolerance, industrial processes, water sanitation, and pollution. The understanding of the composition and function of the extracellular polymeric substances (EPS) of the sessile microbial lifestyle are poorly known, limiting the potential to design biofilm processes and hindering the progress within the water and wastewater fields (Seviour et al., 2019). Despite that fact, few research works have approached biofilms of *Clostridium* spp. in comparison with biofilms formed by other bacteria such as *Staphylococcus* spp. or *Bacillus* spp. raising the question of whether *Clostridium* spp. could develop a proper biofilm or only be recruited into a pre-existing biofilm (Pantaléon et al., 2015).

Remarkably, most structural studies using *Clostridium* spp. have been carried out exclusively with pathogens or with a non-pathogens present in the gut (Pantaléon et al., 2014). Researchers have exclusively approached the biofilm formation of solventogenic *Clostridium* spp. for industrial application of immobilized cells for enhanced ABE production and butanol tolerance (Qureshi et al., (2005). Immobilized solventogenic Clostridia spp. in biofilm reactors have shown improved performance in ABE fermentation, and are considered one of the most useful strategies for continuous large-scale ABE fermentation **(Table 3).**

Notably, the only study of EPS production by solventogenic *Clostridium* spp. conducted until now was carried out by Zhuang et al. (2016), who reported an increased butanol production by biofilm grown *C. acetobutylicum* CGMCC 5234, in addition, to improved tolerance to butanol and acetic acid in biofilms, when compared to planktonic cells through cellular morphology and fermentative performance. Thus, the application of cellular immobilization technologies would be greatly facilitated by understanding the mechanism through such biofilm protection occurs.

Table 3. Comparison of ABE fermentation using continuous biofilm bioreactors.

Strain	Dilution rate (h^{-1})	Substrate	Initial sugar concentration (g/L)	Sugar Conversion	Yield (g/g)	Butanol Productivity (g/L/h)	ABE Productivity (g/L/h)	Butanol Concentration (g/L)	ABE Concentration (g/L)	Support/ carrier	Ref.
C.acetobutylicum	1	Whey permeate	60 (45-50 lactose)	0.3	0.32 gABE/g	n.a.	5.8	n.a.	5.8	bone char	(Qureshi and Maddox, 1988)
C.beijerinckii BA101	2	Glucose	60	0.35	0.38g B/g	n.a.	15.8	n.a.	7.9	clay brick	(Qureshi et al., 2000)
C.acetobutylicum ATCC 824	0.97	Lactose	30	0.62	0.28 gABE/g	4.4	5	4.6	5.2	tygon rings	(Napoli et al., 2010)
C.acetobutylicum DSM 792	1.9	Glucose	60	0.3	0.4 gABE/g	8.6	13.7	4.5	7.2	wood pulp	(Bankar et al., 2012)
C.acetobutylicum ATCC 824	0.54	Cheese whey	28 (19.3 lactose)	0.68	0.32 gABE/g	2.7	3.2	4.9	6	tygon rings	(Raganati et al., 2013)
C.acetobutylicum DSM 792-ADH	0.75	Glucose	58	0.39	0.3 gABE /g	n.a.	5.5	n.a.	7.2	wood pulp	(Bankar et al., 2014)
C.acetobutylicum	0.05	Glucose	60	0.8	0.24 gABE/g	0.39	0.59	7.8	11.8	porous matrix of PVA	(Dolejs et al., 2014)
C.acetobutylicum ABE 1201	0.04	Corn stalk juice	73 (13 sucrose, 30 glucose, 30 fructose)	0.84	0.32 gABE/g	0.5	0.8	12.4	19.9	corn stalk bagasse	(Chang et al., 2016)
C.acetobutylicum DSM 792	0.85	Lactose	100	0.61	0.25 gABE/g	9.2	12.8	10.8	15	tygon rings	(Raganati et al., 2016)

Adapted from Raganati et al. (2016)

1.5 Product recovery processes for ABE fermentation

A high butanol production during ABE fermentation is limited by the fact that butanol can inhibit the metabolism of the cells. Consequently, low ABE yield (0.28-0.35 g/g), and low productivity (0.30-0.50 g/L/h) are achieved, as well as low concentration of sugars in the media is required (Maiti et al., 2016a; Nanda et al., 2014). As a result, the toxicity of the products does not allow that the fermentation continues once the product concentration has reached about 10-15 g butanol/L. Thus, the recovery of a dilute fermentation stream results in high downstream processing costs due to the conventional distillation demands a high energy consumption (Staggs and Nielsen, 2015).

New alternatives of fermentation processes to increase solvent titers and thus decrease process steam consumption have been studied in the past few years, advancing to fermentation with *in-situ* product recovery, such as liquid-liquid extraction, adsorption, pervaporation, perstraction, gas stripping, and vacuum fermentation (Mariano et al., 2016; Outram et al., 2017). Nevertheless, each of these techniques offers advantages and disadvantages as compared in **Table 4**. The desired characteristics for these techniques are high selectivity and removal rate, process stability, ease of operation and installation, economical, resource-efficient, and harmless to the cells. Preferably, the method should be coupled to the fermentation process in order to not reach the inhibitory product levels to the cells (Aslanzadeh et al., 2014).

Since adsorbent-based processes introduce the problem of biofouling, and solvent-based processes can compromise cellular integrity due to potential toxicity, vapor-based processes such as gas stripping and vacuum evaporation are gaining attention because they evade these problems and are east to be implemented (Mariano et al., 2016; Staggs and Nielsen, 2015).

Vacuum evaporation for *in-situ* butanol fermentation recovery, it is one of the processes under development and evaluation in Brazil. The vacuum fermentation technology was developed in the late 1970s and was first applied to ABE fermentation many decades later (Mariano et al., 2011). This process provides the removal of solvents from the broth during the fermentation by applying vacuum.

Therefore, the solvent concentration and thus the inhibition is reduced, improving both the cell growth and the concurrent consumption of concentrated sugar solutions which results in greater solvent productivity (Dias et al., 2012). However, the condensation system efficiency must be improved to avoid losses during the ABE recovery.

Table 4. Comparison of the main advantages and disadvantages of the different separation techniques for butanol recovery.

Technique	Advantages	Disadvantages	Selectivity	Energy requirements (MJ/kg of butanol)
Pervaporation	High selectivity	Fouling problem, membrane material cost	2–209	2–145
Liquid-liquid extraction	High selectivity	Emulsion, extractant cost, toxic to culture, extractant recovery and loss	1.2–4100	7.7 or 26[a]
Gas stripping	No fouling, easy to operate, no harm to the culture	Low selectivity, low efficiency	4–22	14-31
Vacuum fermentation	No fouling, easy to operate, no harm to the culture	Low selectivity, low efficiency	15.5–33.8	10.2[b]-16.8[c]
Perstraction	High selectivity, low toxicity to the culture	Fouling problem, emulsion, and material cost	1.2–4100	7.7
Adsorption	Easy to operate, low energy requirement	High material cost, low selectivity, adsorbent regeneration	130–630	1.3–33

Adapted from Xue et al. (2014). Data for selectivity and energy requirements are from: (Groot et al., 1992; Mariano et al., 2011; Mariano et al., 2012; Oudshoorn et al., 2009; Roffler et al., 1987). [a]The energy requirement of 26 MJ/kg is for ABE solvents, [b]intermittent vacuum, and [c]continuous vacuum.

In a typical intermittent vacuum evaporation process, the ABE fermentation is initially performed without applying vacuum during the first 18-22 h. Thereafter, the produced solvents are recovered by applying 2-h vacuum, alternating with 4-h fermentation at atmospheric pressure, and repeating this process for the remainder of the fermentation. This also prevents the increase of the solvent concentrations to reach toxic levels. In terms of energy demand, intermittent vacuum fermentations are considerably more competitive than traditional distillation (Outram et al., 2017).

Additionally, Mariano et al. (2012) evidenced that the utilization of vacuum in ABE fermentation decreased the energy requirement of downstream distillation by 11.8 MJ/kg and 15.6 MJ/kg for intermittent and continuous vacuum application, respectively. By combining them with the energy required for vacuum fermentation, the total energy requirement resulted in 32.4 and 22.0 MJ/kg for continuous and intermittent vacuum, respectively. When comparing these results with a control batch process without in-situ product recovery, the energy requirement was 26.8 MJ/kg. Therefore, the use of an intermittent vacuum showed an 18% reduction in energy and appears promising to be used as a simple or hybrid in-situ product recovery technique in ABE fermentation processes.

1.6 Thesis objective and outline

In view of the challenges of biobased second-generation butanol production to become economically feasible, this thesis aims to investigate integrated approaches for Acetone-Butanol-Ethanol (ABE) production using C5 and C6 carbohydrates from low-cost sugarcane feedstocks (hemicellulosic hydrolysate and molasses) as carbon sources. Firstly, screening of different solventogenic Clostridia strains was performed under various fermentation conditions, in which promising candidates for butanol production using pentose-rich media were identified. Thereafter, ABE production on culture medium based on mixtures of sugarcane hemicellulosic hydrolysate and sugarcane molasses was evaluated. Subsequently, integrated product recovery by vacuum fermentation was studied as a strategy to alleviate product inhibition. Finally, the ability of *Clostridium saccharoperbutylacetonicum* to form biofilms was assessed for the first time. Afterward, the biofilm was characterized in terms of its extracellular polymeric substances (EPS) and proteome composition to obtain more understanding about the potential benefits of the biofilm over the planktonic lifestyle, and its application in continuous biofilm reactors.

Chapter 2 describes a systematic fermentation study of four wild-type solventogenic Clostridia strains as potential candidates for ABE biosynthesis using xylose or glucose as the primary carbon source. Here, the remarkable ability of *C. saccharoperbutylacetonicum* DSM 14923 to biosynthesize butanol from pure xylose and sugarcane bagasse hemicellulosic hydrolysate without any detoxification process is highlighted, indicating the potential of this strain as a promising microbial platform for second-generation butanol production. New insights regarding the performance, robustness, and butanol tolerance of *C. sacharoperbutylacetonicum* DSM 14923 are presented.

In **Chapter 3**, the use of mixtures of sugarcane molasses and sugarcane bagasse hemicellulosic hydrolysate as low-cost carbon sources for butanol production is evaluated. Growth characteristics, substrate consumption, the impact of inhibitory compounds, and fermentation performance were investigated. Here, the addition of sugarcane molasses to the hemicellulosic hydrolysate is considered

as advantageous for ABE fermentation, as it results in the reduction of inhibitor concentrations and the toxicity effect on the bacterial cells.

Chapter 4 describes the development of an integrated batch fermentation process for ABE production with *C. saccharoperbutylacetonicum* DSM 14923, using molasses as feedstock, wherein cyclic vacuum extraction was applied for *in-situ* product removal. Identical batch fermentations without applying vacuum were carried out as control. Additionally, *in situ* butanol recoveries by vacuum extraction (at 25 and 56 mmHg) were tested with an ABE model solution at 30 °C. Yields and productivities are presented, and the advantages and disadvantages of the integrated process are discussed.

Chapter 5 explores entirely novel results about the ability of the wild-type *Clostridium saccharoperbutylacetonicum* DSM 14923 to grow as a biofilm. To this end, a comparative study was conducted combining proteomic and spectroscopic approaches of its extracellular polymeric substances (EPS) with the aim to gain more insight into the functional mechanisms in biofilms compared to planktonic cells.

Finally, in **Chapter 6,** the main findings of this research are integrated, and an overview of the opportunities and bottlenecks of the different strategies is given to explore how second-generation butanol production could become a reality. Furthermore, some remaining issues and approaches for future research are highlighted.

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Chapter

2

Towards enhanced *n*-butanol production from sugarcane bagasse hemicellulosic hydrolysate: Strain screening, and the effects of sugar concentration and butanol tolerance

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Abstract

Robust strains are essential towards success of *n*-butanol production from lignocellulosic feedstock. To find a suitable strain to convert a non-detoxified hemicellulosic hydrolysate of sugarcane bagasse, we first assessed the performance of four wild-type butanol-producing *Clostridium* strains (*C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864, and *C. saccharoperbutylacetonicum* DSM 14923) in batch fermentations containing either xylose or glucose at 30 g/L as sole carbon sources. *C. saccharoperbutylacetonicum* was selected after achieving butanol yields as high as 0.31 g/g on glucose and 0.25 g/g on xylose. In a 48-h fermentation containing a mixture of sugars (93% xylose and 7% glucose) that mimicked the hydrolysate, *C. saccharoperbutylacetonicum* delivered the highest butanol concentration (14.5 g/L) when the initial sugar concentration was 50 g/L. Moreover, the selected strain achieved the highest butanol yield (0.29 g/g) on xylose-rich media reported so far. Meanwhile, *C. saccharoperbutylacetonicum* produced 5.8 g/L butanol (0.22 g/g butanol yield) when fermenting a non-detoxified sugarcane bagasse hemicellulosic hydrolysate enriched with xylose (30 g/L total sugars). Although sugars were not exhausted (4.7 g/L residual sugars) even after 72 h because of the presence of lignocellulose-derived microbial inhibitors, these results show that *C. saccharoperbutylacetonicum* is a robust wild-type strain. This microorganism with high butanol tolerance and yield on xylose can, therefore, serve as the basis for the development of improved biocatalysts for production of butanol from non-detoxified sugarcane bagasse hemicellulosic hydrolysate.

Keywords: Hemicellulosic hydrolysate; Xylose; Wild-type strain; *Clostridium saccharoperbutylacetonicum*; Butanol tolerance.

2.1 Introduction

The increasing global interest in biofuels, especially in those with fuel properties similar to gasoline, has created a market pull for advanced biofuels such as *n*-butanol (hereafter referred to as butanol). It has several advantages in relation to ethanol, such as higher miscibility with gasoline, higher energy density, lower volatility, and better biodegradability. However, technical difficulties still limit its production in large scale. Conventionally, bio-based butanol is produced by solventogenic *Clostridium* strains in a strictly anaerobic process known as ABE (acetone-butanol-ethanol) fermentation. The primary challenges of this process are the high feedstock cost (60 – 70% of the production cost), the low butanol yield (~0.2 g/g), and the low productivity (<0.2 g butanol/L/h) and titer (10 – 12 g butanol/L) due to the toxicity of butanol (Ndaba et al., 2015). To overcome such limitations, recent studies have focused on the optimization of the ABE fermentation process and strain development using several metabolic engineering strategies (Moon et al., 2016). In addition, substantial progress has been made in the use of low-cost agricultural wastes as feedstock to improve sustainability and reduce costs of butanol production (Qureshi and Ezeji, 2008).

The economics of butanol production can certainly benefit from existing sugarcane ethanol mills in countries such as Brazil, Colombia, India, and China because these facilities produce large amounts of bagasse. This lignocellulosic material is currently mainly used for energy cogeneration, but it could also be used to produce chemicals and fuels. Butanol is an interesting option because butanol-producing *Clostridium* strains can convert sugars derived from hemicellulose (arabinose and xylose). These sugars, on the other hand, cannot be metabolized by industrial *Saccharomyces cerevisiae* strains, thereby hampering their use for ethanol production. Since xylose is the primary sugar available in the hemicellulosic portion of bagasse, butanol can thus be an interesting alternative to add value to sugarcane bagasse (Mariano et al., 2013).

However, the processing of lignocellulosic biomass, such as sugarcane straw and sugarcane bagasse, generates by-products that are inhibitory to microorganisms. The inhibitory compounds are organic acids (acetic, levulinic, and formic acids), furan derivatives [5-hydroxymethylfurfural (HMF) and furfural], and phenolic compounds (Baral and Shah, 2014; Yao et al., 2017). These compounds are mainly present in the hemicellulosic hydrolysate, and they impact negatively the ABE fermentation (Amiri and Karimi, 2018). As a result, studies have been searching for wild-type strains more efficient to convert sugars derived from the lignocellulosic fractions (straw and bagasse) of sugarcane. For example, Magalhães et al., (2018) assessed twelve *Clostridium* strains for their ability to produce butanol from sugarcane straw hydrolysate. They found that *C. saccharobutylicum* can consume all sugars available in that feedstock. They also highlighted the high butanol-to-acetone ratio delivered by *C. saccharoperbutylacetonicum*. More recently, Grassi et al., (2018) found that butanol production from sugarcane straw hydrolysate by *C. saccharoperbutylacetonicum* can improve when xylooligosaccharides are added to the fermentation. Other studies assessed ABE production from the overall hydrolysate (cellulosic + hemicellulosic) obtained from pretreatment and enzymatic hydrolysis of sugarcane bagasse (Pang et al., 2016; Su et al., 2015).

However, rather less attention has been paid to finding butanol-producing *Clostridium* strains able to use the hemicellulosic hydrolysate of sugarcane bagasse as the sole carbon source. To fill this gap, in the first step of this study we assessed the performance of four wild-type strains (*C. acetobutylicum*, *C. beijerinckii, C. saccharobutylicum,* and *C. saccharoperbutylacetonicum)* in producing butanol from xylose or glucose as sole carbon source. The strain with the highest butanol yield (*C. saccharoperbutylacetonicum)* was then further investigated to find the more suitable initial sugar concentration and to determine the tolerance of the strain to butanol. In the last step, we assessed the ability of the selected strain to produce butanol from a non-detoxified sugarcane bagasse hemicellulosic hydrolysate.

2.2 Material and methods

2.2.1 Microorganisms, culture maintenance, and inoculum preparation

The microorganisms used in this study (*C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864, and *C. saccharoperbutylacetonicum* DSM 14923) were obtained from the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ). The strains were activated and propagated following the supplier's recommendations. Stock cultures were routinely maintained in 2-mL aliquots of 20% glycerol aqueous solution at -80 °C. Inoculum was prepared in anoxic pre-sterilized Reinforced Clostridial Medium (RCM, Fluka, Sigma-Aldrich, Spain). Cells were cultivated anaerobically until the exponential growth phase (optical density, OD, at 600 nm = 1.0 – 1.5) in an anaerobic chamber (Whitley DG250 Workstation, Don Whitley Scientific Ltd., West Yorkshire, United Kingdom). Inoculum size was 20 vol% in all fermentations. Morphological changes of the microorganisms were analyzed using microscopic inspection throughout the fermentation studies to monitor possible contaminations.

2.2.2 Screening of the *Clostridium* strains

In the first step of this study, the *Clostridium* strains were screened based on their ability to convert xylose and glucose, and their product yields. Fermentations were conducted in 100-mL screw capped bottles (triplicate) incubated still under N_2-enriched conditions in the anaerobic chamber. Fermentation medium (50 mL) contained 30 g/L sugar (glucose or xylose) was supplemented with modified P2 medium (g/L): yeast extract, 5.0; KH_2PO_4, 0.75; K_2HPO_4, 0.75; NaCl, 1; $MgSO_4.7H_2O$, 0.4; $MnSO_4.H_2O$, 0.4; $FeSO_4.7H_2O$, 0.01; CH_3COONH_4, 4.3, para-aminobenzoic acid, 0.1, and biotin, 0.001. The medium was previously sterilized in autoclave at 121 °C for 20 min, while stock solutions containing $FeSO_4.7H_2O$, CH_3COONH_4, para-aminobenzoic acid, and biotin were filter-sterilized through a 0.22-µm nitrocellulose filter and subsequently added to the medium under sterile conditions

inside a laminar flow hood. The initial pH was 6.4 and the cells were cultivated for 48 h at 35 °C (*C. acetobutylicum*, *C. beijerinckii,* and *C. saccharobutylicum*), and 30 °C (*C. saccharoperbutylacetonicum*). The optimal temperatures were found in preliminary tests (data not shown) based on the cultivation temperature ranges recommended by the supplier. Culture samples (2 mL) were collected at intervals (0, 3, 6, 24, and 48 h) and analyzed for cell growth (OD_{600nm}), concentration of sugar (glucose or xylose) and fermentation products.

2.2.3 Effect of initial sugar concentration on the selected strain

To assess the effect of the initial sugar concentration on the performance of the selected strain (*C. saccharoperbutylacetonicum*), the fermentation medium used in the screening step (section 2.2.2) was modified to contain a mixture of sugars (93% xylose and 7% glucose) with different initial concentrations (30; 40; 50; and 60 g/L). The sugars ratio was defined based on the typical composition of hemicellulosic hydrolysates after post-hydrolysis in H_2SO_4 solution (0.4 wt%) (Santucci et al., 2015). Fermentations were conducted (triplicate) in 300-mL bioreactors (Dasgip Box, DASGIP, Germany) at 30 °C and 200 rpm for 48 h. Working volume was 100 mL. The initial pH was adjusted to 7.0 using sterile 2 M NaOH solution. Prior to inoculation, the bioreactors were flushed with N_2 (100 mL/L, i.e., 1 vvm) for 2 h to create anoxic conditions before the start of each fermentation. During gas flushing, agitation and temperature were kept at 200 rpm and 30 °C, respectively. Flushing was stopped upon inoculation, and the positive pressure created by fermentation gases (CO_2 + H_2) sufficed to keep the anaerobic condition (confirmed by on-line measurement of dissolved O_2 concentration). Culture samples (2 mL) were collected at intervals (0, 3, 6, 24, 28 and 48 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugar (glucose and xylose) and fermentation products.

2.2.4 Growth and production kinetics of the selected strain

Kinetic parameters [maximum specific growth rate (μ_{max}), cells yield ($Y_{x/s}$), butanol yield ($Y_{but/s}$), and maximum rate of substrate consumption (q_s)] of *C. saccharoperbutylacetonicum* were calculated considering the more suitable initial sugar concentration determined in the previous section. Fermentation was conducted in a 7-L bioreactor (New Brunswick Scientific Bioflo®/Celligen® 115, New Jersey, USA) at 30 °C and 200 rpm. Initial pH of the modified P2 medium was adjusted to 7.0 (using sterile 2 M NaOH solution), and it contained 50 g/L sugars (93% xylose and 7% glucose). Anaerobic conditions were maintained according to the procedure described in section 2.3. Culture samples (2 mL) were collected at intervals (0, 2, 4, 6, 8, 10, 12, 24, and 48 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugar (glucose and xylose) and fermentation products.

2.2.5 Tolerance of the selected strain to butanol

Fermentations to assess the tolerance of *C. saccharoperbutylacetonicum* to butanol were conducted in 100-mL screw capped bottles (duplicate) incubated still under N_2-enriched conditions in the anaerobic chamber. Cells were cultivated at 30 °C in RCM medium (30 mL) containing different initial butanol concentrations (3, 6, 12, 17, and 23 g/L). Cell growth (OD_{600nm}) was analyzed at different intervals (12, 24, 36 and 48 h) and was used to calculate the percentage of relative tolerance (RT) to butanol (Borden and Papoutsakis, 2007). RT in each sampling time (t) is given by Eq. 1, in which control refers to fermentation without butanol addition.

$$RT\ (\%) = 100 \times \left(OD_{600nm,t} - OD_{600nm,t=0}\right) \times \left(OD_{600nm,t}^{control} - OD_{600nm,t=0}^{control}\right)^{-1} \quad (1)$$

2.2.6 Fermentation of sugarcane bagasse hemicellulosic hydrolysate

In the last step of this study, we assessed the ability of *C. saccharoperbutylacetonicum* to ferment sugarcane bagasse hemicellulosic hydrolysate. The sugarcane bagasse (50 wt% moisture content) was kindly supplied by Usina da Pedra, a sugarcane mill located at Serrana, SP, Brazil. The bagasse was dried at room temperature and processed as received, i.e. the bagasse was not washed to remove ashes and residual sugars. The bagasse was hydrothermally pretreated in the Pilot Plant for Process Development (PPDP) at the Brazilian Bioethanol Science and Technology Laboratory (CTBE) (CNPEM, Campinas, Brazil). The pretreatment was conducted in a 350-L Hastelloy C-276 reactor (POPE Scientific Inc., Saukville, USA) under the following conditions: 160 °C, 60 min, and solid-to-liquid ratio of 1:10. Upon completion of the pretreatment time, the reactor was slowly depressurized and cooled. The pretreated liquor was collected and filtered (Nutsche filter, POPE Scientific, USA) and subsequently transferred to the acid-post-hydrolysis step (**Fig. 1**).

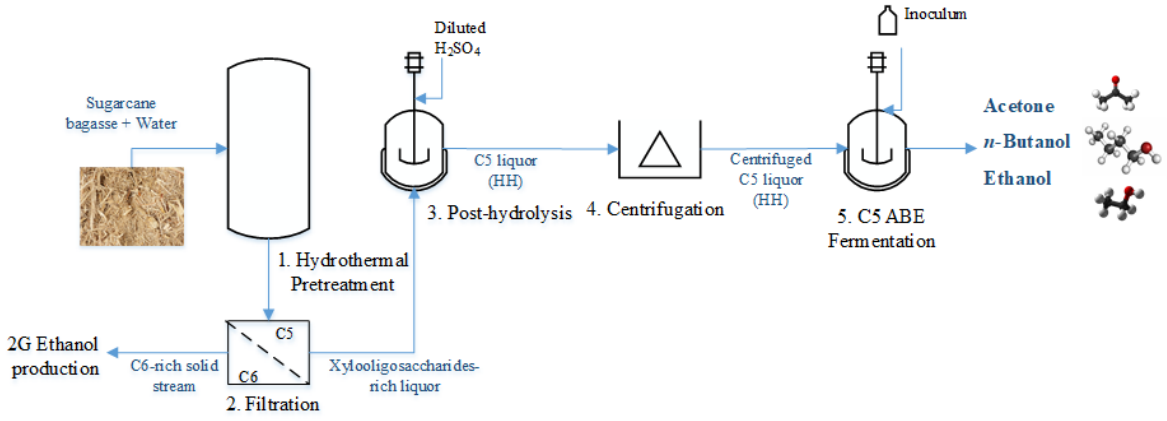

Figure 1. Schematic diagram of the production of sugarcane bagasse hemicellulosic hydrolysate (HH) and its use for ABE production.

The hydrolysis xylooligosaccharides was carried out in a 2-L stainless steel reactor (PARR Instrument Company, Moline, USA) using H_2SO_4 aqueous solution (0.4 wt%). This reactor was operated at 130 °C and 200 rpm for 30 min. These conditions were previously determined (Santucci et al., 2015) to complete the hydrolysis of the oligomers without increasing the amount of microbial inhibitory

compounds. Subsequently, the suspension was centrifuged (9000 rpm) at 10 °C for 20 min. The resulting hemicellulosic hydrolysate containing approximately 17 g/L of sugars was then filtered (0.22-µm polyethersulfone top filter; Nalgene, Rochester, NY, USA) for sterilization and removal of insoluble materials that would make it difficult to measure cell growth by absorbance. The filtered hydrolysate was stored in sterile glass bottles at -4 °C until use. All the procedures were carried out under sterile conditions in a laminar flow hood. The composition of the hemicellulosic hydrolysate is presented in **Table 1**.

Table 1. Composition of the sugarcane bagasse hemicellulosic hydrolysate obtained in the hydrothermal pretreatment, and its composition with xylose supplementation before inoculation.

Component	**Hemicellulosic hydrolysate** (g/L)	**Hemicellulosic hydrolysate + modified P2 medium + xylose (Fermentation medium)** (g/L)
Xylose	13.12	27.04
Arabinose	2.32	1.72
Cellobiose	0.63	0.47
Glucose	0.82	0.72
Total Reducing Sugars (TRS)	16.89	29.95
Acetic acid	4.17	3.36
Formic acid	0.18	0.11
HMF	0.12	0.10
Furfural	0.27	0.23
Syringaldehyde	0.07	0.06
p-Coumaric acid	0.21	0.18

Batch fermentation of the hemicellulosic hydrolysate was conducted in 300-mL bioreactors (Dasgip Box, DASGIP, Germany) (triplicate) at 30 °C and 200 rpm for 72 h. Anaerobic conditions were obtained according to the procedure described in section 2.3. The initial pH of the fermentation medium (240 mL) was adjusted to 7.0 using sterile 25% NH_4OH aqueous solution. Pre-sterilized hydrolysate was supplemented with modified P2 medium (described in section 2.2.2) and xylose to yield an initial xylose concentration of 30 g/L. Medium components were added to the hydrolysate under sterile conditions in a laminar flow hood. The composition of the resulting fermentation medium is presented in **Table 1**.

Culture samples (2 mL) were collected at intervals (0, 3, 6, 20, 24, 30, 48, and 72 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugars (glucose and xylose) and fermentation products.

2.2.7 Analytical procedures

Samples before chromatographic analysis were centrifuged (8000 rpm) at 4 °C for 10 min. The clean supernatant was transferred into 2-mL microtubes and stored at -10 °C until analysis. Before injection into the high-performance liquid chromatography (HPLC), samples were filtered using a 0.22-µm Millipore Millex-HV PVDF membrane filter. Solvents (acetone, butanol, and ethanol), sugars (glucose, xylose, and arabinose), and organic acids (acetic and butyric) were separated in a Bio-Rad Aminex® HPX-87H column (at 35 °C; 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min) and detected with refractive index detector (RID). Microbial inhibitory compounds (formic acid, HMF, furfural, syringaldehyde, and *p*-coumaric acid) were analyzed by reversed-phase HPLC, separated in a Thermo Scientific Acclaim® 120 C18 column (at 25 ^oC; 1:8 volume ratio of acetonitrile to water with 1 wt% acetic acid as the mobile phase at a flow rate of 0.8 mL/min), and detected with UV–Vis at 274 nm.

Culture growth was determined by measuring the optical density at 600 nm (OD_{600nm}) using a UV-Vis spectrophotometer (Thermo Scientific - Evolution 60S, Ann Arbor, Michigan, USA). In the kinetic studies (section 2.2.4), OD–dry cell weight relationships (Eq. 2 and 3) were used to convert OD_{600nm} values to dry cell weight (DCW) per volume of culture medium (g/L) during growth and death phases.

$$DCW_{growth} = 0.4065 \times OD_{600nm} \quad (r^2 = 0.98) \tag{2}$$

$$DCW_{death} = 0.325 \times \left(OD_{600nm} + 3.20\right) \quad (r^2 = 0.99) \tag{3}$$

2.3 Results and discussion

2.3.1 Screening of the *Clostridium* strains

Among the four wild-type *Clostridium* strains assessed in this study, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* exhibited marked better performance. The former exhausted glucose in the glucose fermentation, achieving the highest ABE concentration [16.8 g ABE/L or 1.2 (A) + 10.9 (B) + 4.7 (E) g/L] (**Fig. 2**).

This strain also exhausted xylose in the xylose fermentation and produced 13.3 g ABE/L [0.5 (A) + 8.3 (B) + 4.5 (E) g/L]. Consequently, in both glucose and xylose fermentations, *C. saccharoperbutylacetonicum* achieved the highest ABE yield (glucose: 0.42 g/g, and xylose: 0.35 g/g, **Table 2**). The latter (*C. saccharobutylicum*) also produced ABE in relatively large concentrations: 15.2 g/L of ABE (glucose fermentation) and 14.5 g/L (xylose fermentation). Notably, *C. saccharobutylicum* exhausted xylose in 24 h, while *C. saccharoperbutylacetonicum* required 48 h. However, *C. saccharobutylicum* was outperformed with respect to yields (0.29 g/g ABE on glucose and 0.28 g/g ABE on xylose). Interestingly, both strains delivered high ABE concentrations regardless of the carbon source (glucose or xylose).

Moreover, the alcohols accounted for more than 90% of the total mass of solvents. Another advantage is that both strains presented relatively lower production and re-assimilation of acids, especially butyric acid. It suggests that butanol was synthesized through a different pathway in which the synthesis occurs via a direct route from acetyl-coenzyme A (CoA) and butyryl-CoA. This route was designated as the hot pathway by Jang et al., (2012).

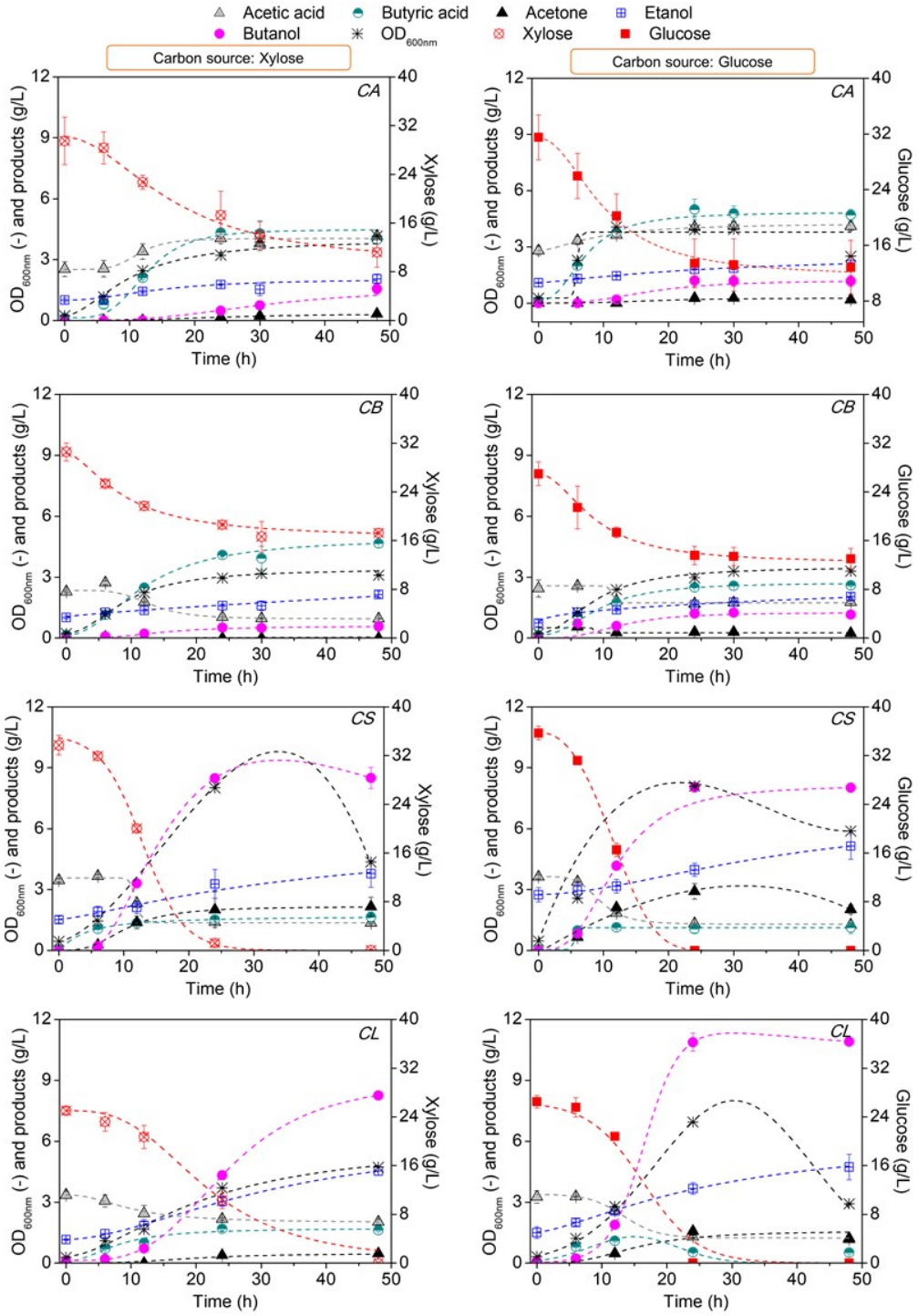

Figure 2. Production of ABE and acids, cell growth, and sugar consumption in ABE fermentations to screen the *Clostridium* strains. Xylose fermentation on the left column and glucose fermentation on the right column. CA: *C. acetobutylicum* DSM 622, CB: *C. beijerinckii* DSM 6422, CS: *C. saccharobutylicum* DSM 13864, and CL: *C. saccharoperbutylacetonicum* DSM 14923. Dashed lines represent a general tendency.

The other two strains (*C. acetobutylicum* and *C. beijerinckii*) were not able to exhaust either glucose or xylose. Furthermore, they had poor solvents production (<4 g/L of ABE L) (**Fig. 2**). As a result, yields were lower than 0.1 g/L of ABE g (**Table 2**).

One possible explanation for the poor performance is the fact that both strains produced relatively higher amounts of butyric acid during the growth phase up to 24 h. While this behavior is expected because acid production is coupled to the synthesis of one extra molecule of ATP to promote cell growth (Shinto et al., 2008), the strains were not able to re-assimilate the acids to produce the solvents. As a result, acid accumulation may have inactivated microbial growth because of a sudden drop in the pH, a phenomenon known as "acid crash" (Maddox et al., 2000). For instance, this phenomenon was observed in other studies on ABE fermentation by *C. acetobutylicum* ATCC 824 (Cornillot et al., 1997) and *C. acetobutylicum* ATCC 39236 (Lemmel et al., 1986). Nevertheless, further studies are needed to confirm our hypothesis and to elucidate the poor performance of *C. acetobutylicum* DSM 6228 and *C. beijerinckii* DSM 6422 observed in the present study.

For the next steps of this study, we selected *C. saccharoperbutylacetonicum* because this strain exhausted the sugars and presented the highest yields on both glucose and xylose fermentations. Yields are essential to the economics of commodity bioprocesses such as the ABE fermentation.

Table 2. Performance comparison of the *Clostridium* strains in glucose fermentation and xylose fermentation. Initial sugar concentration was approximately 30 g/L and fermentation time was 48 h.

Carbon source	Strain	OD_{600nm}[a]	Yield[b] (g/g)		Productivity (g/L/h)		Residual sugar (%)
			Butanol	*ABE*	*Butanol*	*ABE*	
Xylose	*C. acetobutylicum*	4.16±0.09	0.056±0.001	0.094±0.001	0.032±0.005	0.061±0.006	38.3±3.4
	C. saccharobutylicum	8.07±0.05	0.253±0.013	0.281±0.020	0.177±0.015	0.269±0.016	0.0±0.1
	C. beijerinckii	3.10±0.04	0.025±0.002	0.060±0.002	0.012±0.001	0.036±0.002	56.4±2.9
	C. saccharoperbutylacetonicum	4.73±0.01	0.247±0.017	0.351±0.012	0.169±0.003	0.250±0.003	0.0±0.0
Glucose	*C. acetobutylicum*	4.07±0.02	0.041±0.002	0.069±0.010	0.024±0.006	0.048±0.006	40.7±6.6
	C. saccharobutylicum	8.58±0.05	0.225±0.008	0.293±0.009	0.165±0.002	0.259±0.003	0.0±0.0
	C. beijerinckii	3.30±0.05	0.052±0.010	0.110±0.012	0.024±0.004	0.056±0.004	48.3±2.9
	C. saccharoperbutylacetonicum	6.95±0.02	0.310±0.012	0.422±0.012	0.225±0.003	0.317±0.003	0.0±0.0

[a] Maximum optical density in the fermentation.

[b] Yield was calculated as grams of butanol produced per grams of sugar consumed.

2.3.2 Effect of initial sugar concentration on *C. saccharoperbutylacetonicum*

The batch fermentations of *C. saccharoperbutylacetonicum* using a mixture of xylose (93%) and glucose (7%) at different initial sugar concentrations (30 to 60 g/L) demonstrated that the more adequate concentration is 50 g/L. While ABE concentration increased with sugar concentration, cell growth (maximum OD_{600nm} of 10.80) and ABE yield (0.35 g/g) were superior when the initial sugar concentration was 50 g/L (**Table 3**).

Table 3. Effect of initial sugar concentration on the performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923. Fermentation time was 48 h.

Initial sugar (g/L) (93% Xyl + 7% Glu)	**OD_{600nm} (-)**	**Yield (g/g)**		**Productivity (g/L/h)**	
		Butanol	*ABE*	*Butanol*	*ABE*
30	7.23±0.27	0.22±0.03	0.28±0.04	0.15±0.03	0.18±0.02
40	7.67±0.03	0.26±0.08	0.32±0.06	0.24±0.04	0.29±0.04
50	10.80±1.53	0.29±0.07	0.35±0.06	0.30±0.06	0.36±0.08
60	9.14±0.28	0.27±0.04	0.35±0.03	0.30±0.06	0.39±0.08

Moreover, sugars were not exhausted when the concentration was higher than 50 g/L of sugar (**Fig. 3**). Other important advantages were the improved solvents concentration (**Fig. 3**) and butanol yield. Concentration of butanol (14.5 g/L) and ABE (18.0 g/L), and butanol yield (0.29 g/g) were higher than the values found in the xylose fermentation presented in the previous section. Notably, when fermenting the sugar mixture at 50 g/L by *C. saccharoperbutylacetonicum,* the butanol yield achieved is, to the best of our knowledge, the highest value reported thus far for an ABE fermentation using xylose-rich media (**Table 4**). Consequently, the butanol-to-ABE ratio was as high as 0.80.

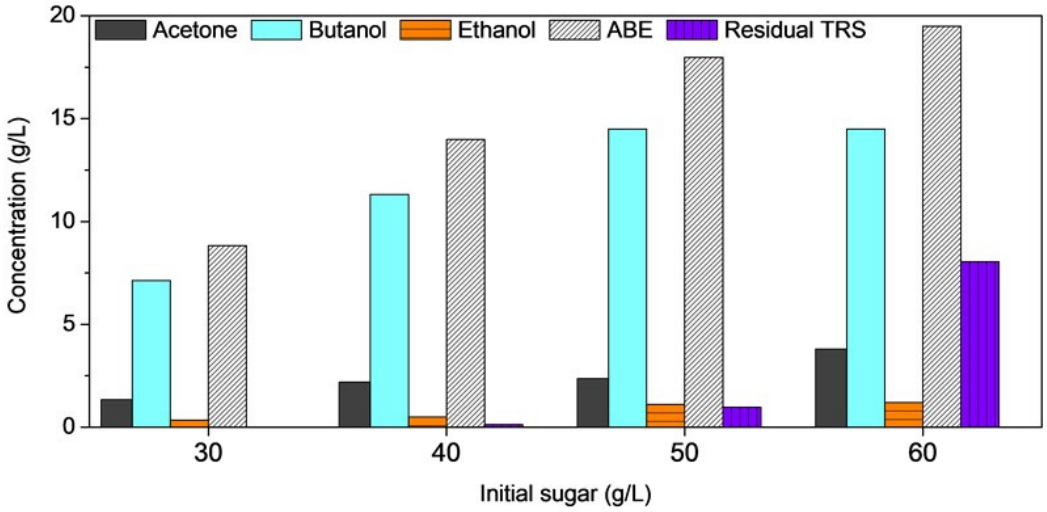

Figure 3. Effect of initial sugar concentration on production of ABE and sugar consumption by *C. saccharoperbutylacetonicum* DSM 14923. Synthetic fermentation medium contained mixed sugars (97% xylose and 7% glucose). Fermentation time was 48 h.

2.3.3 Growth and production kinetics of *C. saccharoperbutylacetonicum*

The 1-L fermentation to assess the kinetics of *C. saccharoperbutylacetonicum* confirmed the results obtained in the 100-mL fermentations (section 2.3.2), i.e. this strain can exhaust 50 g/L of a mixture of xylose and glucose in 48 h (**Fig. 4**). The maximum rate of substrate consumption (q_s) and μ_{max} were 2.57±0.33 g sugar/g DCW/h and 0.37±0.01 h^{-1}, respectively (both parameters were calculated during the exponential growth phase). Interestingly, xylose and glucose were exhausted simultaneously. It was probably because glucose was in much lower concentration. When these sugars are in equivalent concentrations, previous studies found that *C. saccharoperbutylacetonicum* preferentially consumes glucose due to carbon catabolite repression (Noguchi et al., 2013; Yao et al., 2017). Upon consumption of both sugars in our kinetic experiment, butanol was the major product ($Y_{but/s}$ = 0.29±0.04 g/g) and the cells yield ($Y_{x/s}$) was 0.14±0.05 g/g.

Table 4. Comparison of ABE production from xylose-rich media by *C. saccharoperbutylacetonicum* DSM 14923 with other wild-type *Clostridium* strains reported in various studies.

Strain	Carbon source	ABE (g/L)	ABE Yield (g/g)	Butanol (g/L)	Butanol yield (g/g)	Sugar consumption (g/L)	Butanol-to-ABE ratio	Ref.
C. saccharoperbutylacetonicum DSM 14923	93% Xylose + 7% Glucose	18.0	0.35	14.5	0.29	50.0	0.80	This work
	Non-detoxified sugarcane bagasse hemicellulosic hydrolysate + xylose	7.11	0.26	5.85	0.22	30.0	0.82	
C. saccharoperbutylacetonicum DSM 4923	Detoxified rice bagasse hydrolysate	18.2	0.28	14.8	0.27	54.0	0.81	(Soni et al., 1982)
C. acetobutyllicum ATCC 824	Xylose	6.7	0.28	4.2	0.18	23.3 (from 60 g/L)	0.62	(Gu et al., 2009)
C. beijeinckii NCIMB 8052	Xylose	7.9	0.24	6.8	0.22	32.7 (from 60 g/L)	0.86	(Xiao et al., 2012)
Clostridium sp. BOH3	Xylose	21.4	0.36	14.9	0.25	60.0	0.70	(Xin et al., 2014)
	Detoxified hemicellulosic hydrolysate	-	-	11.9	0.19	60.0	-	
C. acetobutylicum ATCC 824	Xylose	9.4	0.34	7.3	0.26	28.0	0.77	(Kudahettige-Nilsson et al., 2015)
	Detoxified kraft black liquor	2.8	0.12	2.3	0.10	22.8	0.82	
C. saccharoperbutylacetonicum	Xylose	17.5	0.35	12.2	0.24	50.0	0.69	(Yao et al., 2017)
C. beijerinckii NCIMB8052	Xylose-rich medium	-	-	5.0	0.28	24.4	-	(Birgen et al., 2018)
C. acetobutylicum DSM 1731	Xylose	5	0.25	3.6	0.18	20.1 (from 50 g/L)	0.72	(Yang et al., 2015)
	Xylose-rich medium	9.5	0.27	6.3	0.18	35.1 (from 50 g/L)	0.66	
	Mixture of barley straw hydrolysate and grain + xylose (89.9% xyl)	1.1	0.07	0.7	0.04	17.4 (from 49.8 g/L)	0.64	
	Mixture of barley straw hydrolysate and grain + xylose (67.5% xyl)	6.9	0.35	4.2	0.21	19.8 (from 45.9 g/L)	0.61	
C. acetobutylicum DSM 1731	Xylose (80% xyl) + starchy slurry	13.64	0.31	8.36	0.19	44.0 (from 50 g/L)	0.61	(Yang et al., 2017)
	Xylose (80% xyl) + starchy slurry	4.84	0.22	3.08	0.14	22.0 (from 50 g/L)	0.63	

(-) not reported

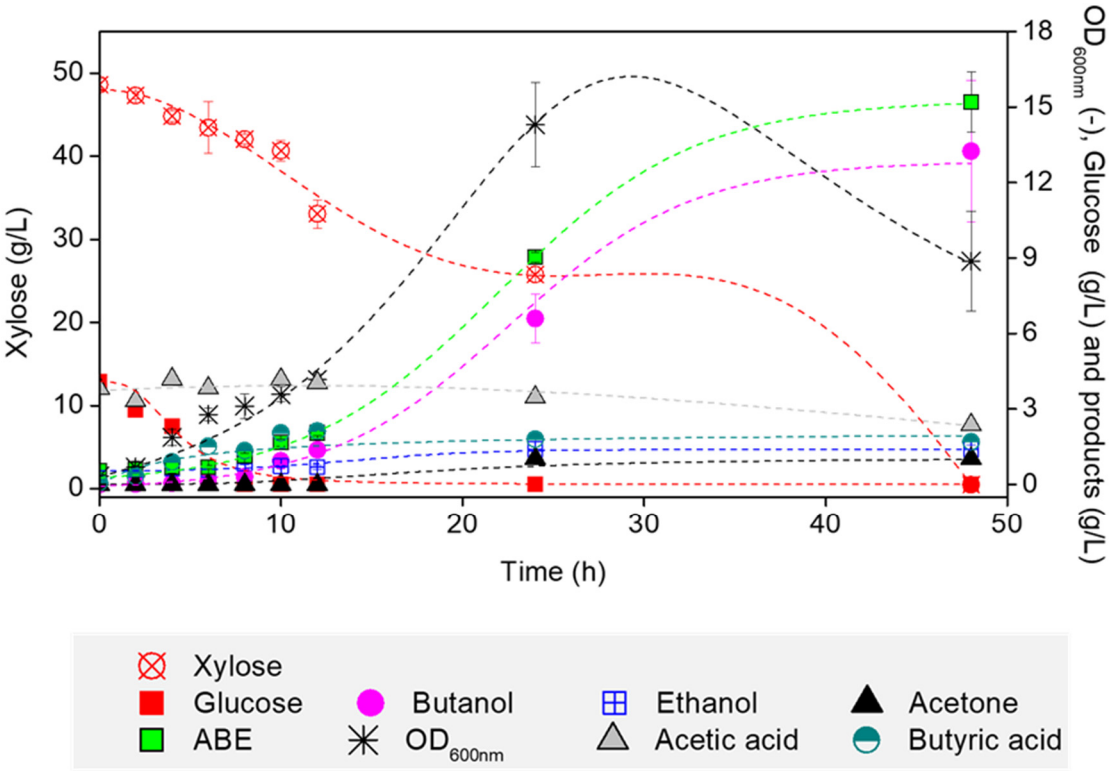

Figure 4. Kinetics (production of ABE and acids, sugar consumption, cell growth) of *C. saccharoperbutylacetonicum* DSM 14923 cultivated in a synthetic fermentation medium containing mixed sugars (97% xylose and 7% glucose) at 50 g/L.

2.3.4 Tolerance of *C. saccharoperbutylacetonicum* to butanol

The inhibitory effect of butanol on growth of *C. saccharoperbutylacetonicum* was more pronounced when the culture was challenged by initial butanol concentrations equal to or higher than 12 g/L. When exposed to lower concentrations (3 and 6 g/L of butanol) the cells needed 24 h to achieve a RT value of 100% (i.e. a cell growth equal to the control without butanol addition) (**Fig. 5**). In contrast, RT was 100% only after 48 h in the fermentation with 12 g/L. With respect to the concentrations of 17 and 23 g/L of butanol, the cells were severely affected, and RT did not exceed 10%. This result agrees with the maximum butanol concentration (14.5 g/L) achieved in the experiments presented in section 2.3.2.

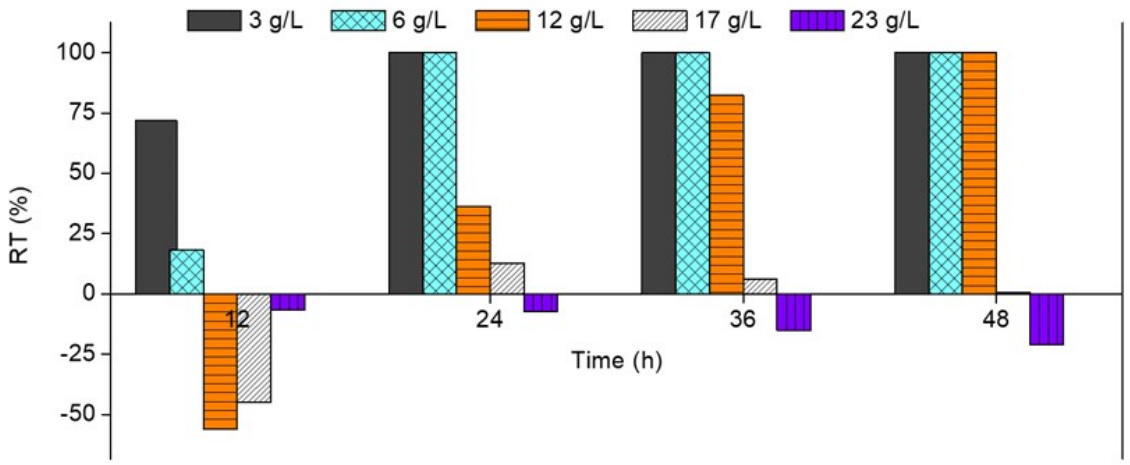

Figure 5. Inhibitory effect of different butanol concentrations on growth of *C. saccharoperbutylacetonicum* DSM 14923 during batch fermentation of 48 h. RT is the percentage of relative tolerance as defined in *Eq. 1*.

Additionally, previous studies found that *C. saccharoperbutylacetonicum* can produce 16 g/L of butanol from xylose (30 g/L) mixed with cellobiose (30 g/L) (Noguchi et al., 2013). Thus, the maximum tolerance of *C. saccharoperbutylacetonicum* to butanol certainly lies in the range of 15 to 17 g/L. These values are remarkably higher than the usual concentrations of 10 to 12 g/L obtained with wild-type strains (Mariano et al., 2016), and this advantage can result in important gains in terms of energy consumption to distillate ABE (Mariano and Maciel Filho, 2012).

2.3.5 Fermentation of sugarcane bagasse hemicellulosic hydrolysate

Microbial inhibitory compounds found in the hydrolysate had detrimental effects on growth of *C. saccharoperbutylacetonicum* DSM 14923, sugar consumption, and solvents production. If compared with the fermentation of synthetic medium containing 30 g/L (section 3.2), the maximum absorbance (OD_{600nm}) decreased from 7.23 (synthetic medium) to 3.63 (hydrolysate medium). With respect to sugar consumption, *C. saccharoperbutylacetonicum* took 48 h to exhaust the sugars in the synthetic medium. In the fermentation of the hydrolysate, xylose was not completely consumed (4.7 g/L of residual sugars) even after 72 h (**Table 5**).

Table 5. Performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate supplemented with xylose.

Parameter	Value	
Fermentation time (h)	48	72
OD_{600nm} (-)	3.63±0.02	3.63±0.02
Butanol yield (g/g)	0.21±0.02	0.22±0.02
ABE yield (g/g)	0.24±0.04	0.26±0.04
Butanol productivity (g/L/h)	0.07±0.02	0.08±0.03
ABE productivity (g/L/h)	0.08±0.03	0.09±0.05
Residual sugars (%)	46.3±2.4	15.5±2.1

Nonetheless, the low amounts of glucose (0.7 g/L) and arabinose (1.7 g/L) were exhausted in 3 and 20 h, respectively (**Fig. 6**). The lower consumption of sugars impacted the solvents concentration. Butanol concentration was 5.8 g/L and lower than that obtained with the synthetic medium (7.1 g/L).

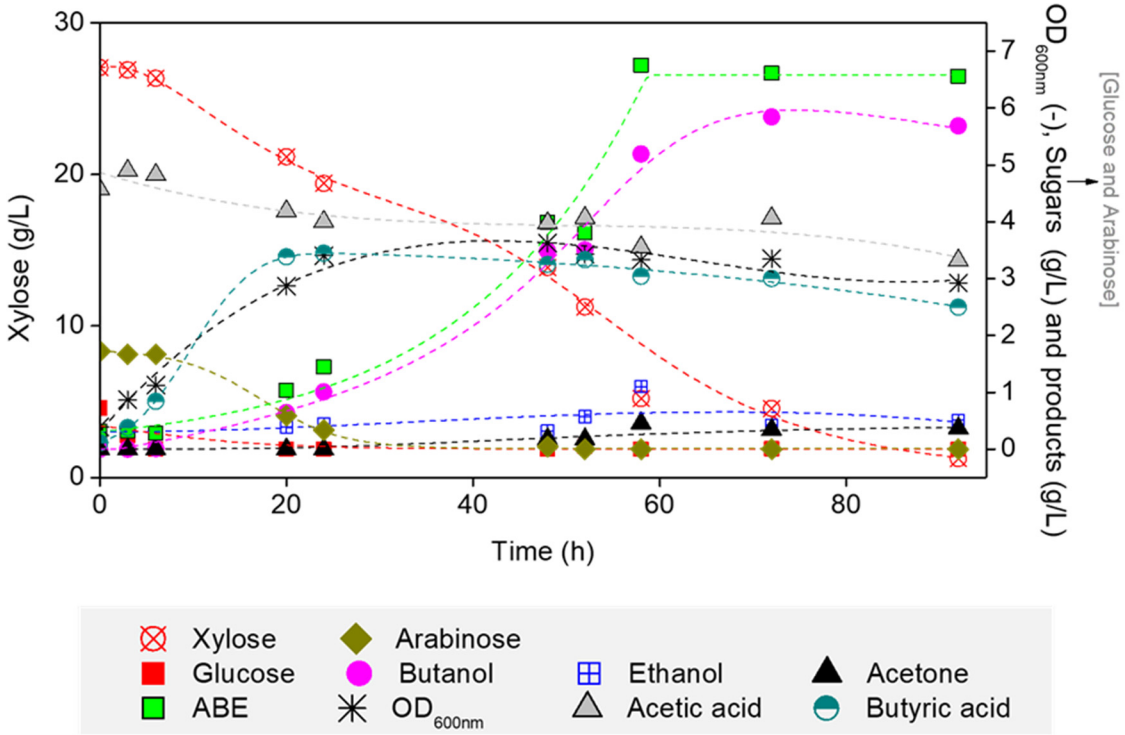

Figure 6. Production of ABE and acids, cell growth, and sugar consumption in the ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate supplemented with xylose.

Consequently, butanol productivity decreased from 0.15 (synthetic medium) to 0.08 (hydrolysate medium) g/L/h. Despite that, butanol yield was not affected (0.22 g/g in both synthetic and hydrolysate media) and the butanol-to-ABE ratio was also high (0.82). The lower performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate certainly resulted from synergistic effects of the inhibitory compounds. It means that their concentration (**Table 1**) would probably not be harmful if they were present individually. For example, acetic acid concentration in the hydrolysate medium (3.36 g/L) is similar to the initial concentration in the screening experiments presented in section 2.3.1 (**Fig. 2**).

Moreover, acetic acid concentration decreased throughout the fermentation with hydrolysate medium (**Fig. 6**), indicating its consumption. In the case of *p*-coumaric acid and syringaldehyde, their concentration in the hydrolysate medium (0.18 and 0.06 g/L, respectively) are lower than the concentrations (0.4 gL of *p*-coumaric acid and 0.8 g/L of syringaldehyde) that inhibited the growth of *C. saccharoperbutylacetonicum* in the studies conducted by Yao et al. (2017). They also reported that the strain tolerated concentrations of furfural and HMF of 2 g/L without having cell growth and ABE titer affected; moreover, the presence of HMF at concentrations between 1 and 3 g/L enhanced ABE titer. In the present study, furfural and HMF concentrations (0.23 and 0.10 g/L, respectively) were well below those thresholds.

However, if we had adjusted the xylose content in the hemicellulosic hydrolysate by evaporation (instead of adding synthetic xylose), this procedure would have increased the concentration of non-volatile inhibitors (mainly the phenolic compounds). This situation would certainly be even more aggravated if the hemicellulosic hydrolysate were concentrated by about three times to achieve the desired concentration of 50 g/L of sugars determined by the fermentations with synthetic medium (section 2.3.2).

On the one hand, the processing of a concentrated sugar stream would result in fewer fermentors and improved wastewater and energy footprints (Assumpção et al., 2018; Mariano and Filho, 2012).

On the other hand, these expected economic gains may not offset the costs related to evaporation and detoxification of the hemicellulosic hydrolysate. Thus, further technoeconomic studies with focus on this trade-off are needed.

2.4 Conclusions

The wild-type strains *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* presented a remarkable ability to ferment xylose-rich media. Notably, *C. saccharoperbutylacetonicum* attained the highest butanol yield (0.29 g/g) on xylose-rich media reported so far. This wild-type strain also presented high tolerance to butanol, achieving a maximum butanol concentration of 14.5 g/L. Our study also demonstrated that butanol production (5.8 g/L) by *C. saccharoperbutylacetonicum* using non-detoxified sugarcane hemicellulose hydrolysate is comparable to that (7.1 g/L) using synthetic medium with same sugar load (30 g/L). We conclude, therefore, that *C. saccharoperbutylacetonicum* can be used as the basis for the development of improved biocatalysts for production of butanol from sugarcane bagasse hemicellulosic hydrolysate.

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Chapter

3

Co-fermentation strategy of molasses and hemicellulosic hydrolysate from sugarcane for second-generation butanol production in a circular economy

This chapter has been submitted to a scientific journal

Abstract

Sugarcane bagasse hemicellulosic hydrolysate (HH) and sugarcane molasses (SCM) were investigated as abundant and inexpensive sugar-based substrates for second-generation ABE production. Fermentations were conducted with *Clostridium saccharoperbutylacetonicum* DSM 14923 using different amounts of SCM added to the HH. Sugars originating from SCM/HH mixtures varied from 0% to 25, 50, 75, and 100%. The total sugar concentration in all mixtures was 30 g/L. After 30 h of fermentation, the best results in terms of ABE titer (8.22 g/L) and yield (0.34 g/g) were obtained when 75% of the sugars were from the SCM. When HH was concentrated (from 15 to 52 g/L), both ABE titer and yield increased to 9.79 g/L and 0.36 g/g, respectively. The results demonstrated that the combination of these two carbon sources presented in the conventional sugarcane industry could be successfully used in ABE fermentation proving the potential to a biorefinery establishment.

Keywords: Hemicellulosic hydrolysate; Sugarcane molasses; ABE fermentation; *C. saccharoperbutylacetonicum*; Biorefinery; Biobutanol.

3.1 Introduction

The need for renewable chemical processes and bio-based products justifies large efforts for further development, innovation, and implementation of the bio-based economy. In this context, biofuels such as ethanol and *n*-butanol (hereafter referred to as butanol) appear as alternatives for petroleum-based fuels to reduce the negative environmental impacts caused by greenhouse gases (GHG) (Maiti et al., 2016).

Brazil has shown enormous potential for utilizing renewable feedstocks in biorefineries and is recognized as the second-largest producer of bioethanol in the world after the United States. Per year roughly 60 billion liters of bioethanol are produced in the US from corn sugar while Brazil produces roughly 27 billion liters per year from sugarcane (Dias et al., 2012; RFA, 2018). The Brazilian bioethanol production is based on a biorefinery model that converts the sugarcane juice and sugarcane molasses (SCM) into ethanol by *Saccharomyces cerevisiae* yeast (Mariano et al., 2013), a process known as first-generation (1G) technology. As a result, the outstanding 1G ethanol production in Brazil generates around 186 million tons of sugarcane bagasse (SCB) (Soccol et al., 2010), as well as sugarcane straw (whether taken from the field). Nonetheless, SCB is mostly burned with very low energy efficiency to generate steam and electric energy to be used in sugar and alcohol plants (Rabelo et al., 2015).

Alternatively, this lignocellulosic biomass can be hydrolyzed and fermented to produce other valuable chemicals and biofuels such as organic acids and butanol using a process known as second-generation (2G) (Mariano et al., 2013; Puro et al., 2016). For this, due to recalcitrance of the lignocellulosic biomass, a physical and/or chemical pretreatment step is required to obtain fermentable monomeric sugars by breaking the three-dimensional complex matrix of lignocellulosic biomass typically composed of 35-50% of cellulose (a glucose polymer), 20-35% of hemicellulose (a natural copolymer of pentose (C5) and hexose (C6) sugars, and 10-25% of lignin (a complex polyphenolic structure), expressed on a percent dry matter basis (Chen et al., 2018; Guilherme et al., 2015; Yu et al., 2018).

The strategy of using all the available sugars from sugarcane (juice, and sugars derived from cellulose and hemicellulose) into several products represents

an optimal biorefinery concept and can be successfully implemented in Brazil, contributing to the establishment of the circular economy concept (**Fig. 1**). The Sugarcane biorefinery platform is shown in **Chapter 1** of this thesis. Particularly, the use of sugarcane bagasse as a feedstock for a 2G platform in Brazil has a significant economic advantage, as it is already available in 1G mills, avoiding high transportation costs (Ortiz and de Oliveira Jr., 2016).

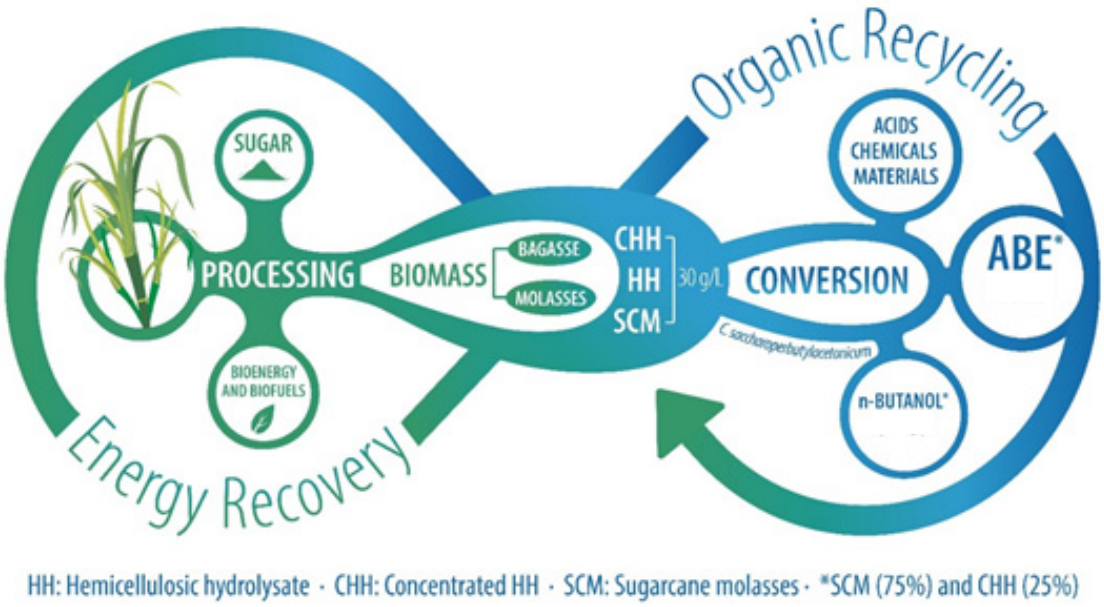

Figure 1. Circular economy concept of an integrated 1G–2G sugarcane platform with sugar, power, ethanol, butanol, acetone, and organic acids production.

In the concept of a 1G-2G sugarcane biorefinery, it is recognized that the fraction of cellulosic hydrolysate, a glucose-rich stream, will preferably be addressed to the production of ethanol combined with sugarcane molasses. However, even though hemicellulose contains a significant fraction of sugars present in sugarcane bagasse (approximately 20-25%), they cannot be naturally metabolized by the wild-type *Saccharomyces cerevisiae* yeast that is traditionally used in 1G ethanol mills (Mariano et al., 2013). One possibility to take advantage of the pentoses fraction is to use microorganisms that naturally metabolize these sugars. Thus, microorganisms capable of fermenting pentose rich-fraction are essential in the biorefinery context (Lee et al., 2008). *Clostridium* spp. are solventogenic bacteria that can metabolize a broad range of sugars (starch, sucrose, glucose, xylose, cellobiose, arabinose, lactose, pectin, among others) into solvents

(acetone, butanol, and ethanol), being butanol their main product (Jang et al., 2012). This feature is of particular relevance for the use of agro-industrial wastes as substrates in ABE fermentation in order to reduce the butanol production cost (Ezeji et al., 2007; Green, 2011).

Butanol is considered a superior biofuel which has gathered renewed attention due to its advantages when compared to ethanol, such as 30% higher energy density, lower viscosity, lower solubility in water, and less corrosive power. In addition, butanol can be mixed with gasoline in high proportions without modification or adaptation of car engines (Ni et al., 2012; Qureshi and Ezeji, 2008). Therefore, butanol has the potential to permanently impact the renewable energy sector. It is also worth noting the importance of butanol in the manufacturing of solvents, plastics, paints, polymers, thinners, among others (Jang et al., 2012; Mascal, 2012). Despite the commercial interest of bio-based butanol production, its processing is more complex than ethanol manufacturing, and its economic viability faces challenges regarding high product inhibition and low yield (Nanda et al., 2014).

Hemicellulosic hydrolysates (HH) are obtained after a pretreatment step, usually hydrothermal with an acid catalyst, and contain pentose and hexose sugars but also inhibitory compounds (ICs) formed by degradation of sugars and lignin. Those compounds can be divided into three main groups: weak acids, furans, and phenolic compounds, which have been shown to hamper the fermentation performance of many microorganisms, including *Clostridium* spp. (Jönsson and Martín, 2016; Modenbach and Nokes, 2012).

As such, we hypothesized that using a mixture of sugarcane molasses (SCM) and HH media may be an efficient strategy to circumvent the inhibitors challenge (by diluting the inhibitors), avoiding the prohibitive costs of detoxification steps which may not be cost-effective (Yang et al., 2015), as well as increasing the initial sugar concentration of the media. SCM is a residual solution after the final process of sugar crystallization that has a high nutritional value as about 50–55% consists of a mixture of sucrose and its monomers glucose and fructose, besides vitamins, suspended colloids, heavy metals, and nitrogenous compounds, among others (Liu et al., 2008). In Brazil, around 80% of its factories have integrated sugar and ethanol mills, and therefore, most of these plants utilize SCM as a carbon source for ethanol

fermentation (Ponce et al., 2016). SCM is also a typical low-cost substrate that has been used as carbon source for a wide range of biotechnological products besides ethanol (Buschke et al., 2013). Bearing all this in mind, the use of SCM for butanol production integrated to a sugarcane mill in the biorefinery concept to produce sugar, 1G-2G ethanol, electricity, and butanol is a great alternative since having the flexibility of using mixed substrates for different products is crucial for both economic and environmental aspects.

Previous works have described comparable strategies of co-fermentation to facilitate the pentose utilization to produce ethanol by *Dekkera bruxellensis* using mixed feedstocks, such as SCB and sweet sorghum bagasse (SSB) (Reis et al., 2016), corn and corn stover by both *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Chen et al., 2018), and SCM supplemented with xylose by an engineered *Saccharomyces cerevisiae* strain (Losordo et al., 2016). However, little research has addressed strategies for co-fermentation of substrates to produce butanol. A work using starch and hemicellulose from barley grain and straw, respectively, to produce ABE by *Clostridium acetobutylicum* was reported by Yang et al. (2015). Despite the tremendous potential of using SCM and HH as carbon source for ABE fermentation in an integrated 1G-2G ethanol biorefinery, most studies have only focused on the individual use of these feedstocks. Consequently, to the best of our knowledge, the co-fermentation of SCM mixed with HH as substrate for any *Clostridium* spp. has not been assessed until now.

Therefore, this work assesses whether SCM could be used not only to dilute the inhibitors generated in the pretreatment, but also to increase ABE production by increasing sugar concentrations in HH. While this strategy can reduce costs by eliminating a liquor detoxification process, the fermentation of more concentrated sugar solutions generates a more concentrated product stream, thus reducing the energy consumption in distillation and the vinasse volume. In order to test these hypotheses, and to understand the individual and synergistic effects of each component on the ABE performance, co-fermentation of mixing ratios of SCM and a non-detoxified HH as substrates to butanol production by *C. saccharoperbutylacetonicum* (a robust butanol-hyper producer strain) was assessed.

3.2 Material and methods

3.2.1 Raw materials

Sugarcane molasses and bagasse (50% w/w of moisture content) were kindly provided by a Brazilian sugarcane mill (Usina da Pedra, Serrana, São Paulo, Brazil).

3.2.2 Hemicelullosic hydrolysate production

The hemicellulosic hydrolysate (HH) was produced in the Pilot Plant for Process Development (PPDP) at the Brazilian Bioethanol Science and Technology Laboratory (CTBE) (CNPEM, Campinas, Brazil). A detailed description of the HH production has been published previously (Zetty-Arenas et al., 2019), **Chapter 2** of this thesis. In brief, the sugarcane bagasse was dried at room temperature and processed as received, i.e., the bagasse was not washed to remove ashes and residual sugars. It was submitted to a hydrothermal pretreatment at 160 °C, 60 min, and solid-to-liquid ratio of 1:10. The reaction was conducted in a 350-L Hastelloy C-276 reactor (POPE Scientific Inc., Saukville, USA). Upon completion of the pretreatment time, the reactor was slowly depressurized and cooled. The pretreated liquor was collected and filtered (Nutsche filter, POPE Scientific, USA) and subsequently transferred to the acid-post-hydrolysis step. The hydrolysis xylooligosaccharides was carried out in a 2-L stainless steel reactor (PARR Instrument Company, Moline, USA) using an H_2SO_4 aqueous solution (0.4 wt%). This reactor was operated at 130 °C and 200 rpm for 30 min.

3.2.3 Concentration process of the hemicelullosic hydrolysate

In order to have a substrate with a high sugar concentration and to minimize the amount of volatile compounds and organic acids, HH was submitted to a rota-

evaporation process under vacuum, according to the protocol described by Carvalho et al., (2006). The rota-evaporation process was carried out at 70 °C and 80 mbar. This process concentrated the HH to 5-fold and was identified as concentrated hemicelullosic hydrolysate (CHH). Hereafter, the pH of the CHH was adjusted to 7.0 using a 25% NH_4OH aqueous solution. Subsequently, the suspension was centrifuged (9000 rpm) at 10 °C for 20 min. The resulting CHH was then filtered (0.22-µm polyethersulfone top filter; Nalgene, Rochester, NY, USA) for sterilization. The filtered CHH was stored in sterile glass bottles at -4 °C until use as carbon source for the culture media preparation.

3.2.4 Culture media

The strain activation (pre-culture) was carried out on Reinforced Clostridial Medium (RCM). The mixture of SCM with HH or CHH was studied by modifying their ratios varying from 0% to 25, 50, 75, and 100%. The initial total reducing sugars (TRS) concentration in all mixtures was ~30 g/L. The substrate composition informs the percentage of total reducing sugars (TRS) coming from each carbon source: SCM, HH or CHH (for a higher ratio of hemicellulosic hydrolysate in the media, it was mandatory to concentrate HH in order to reach a TRS concentration of ~30 g/L). Media were supplemented with a modified sterile P2 solution containing (g/L): yeast extract, 5.0; KH_2PO_4, 0.75; K_2HPO_4, 0.75; NaCl, 1; $MgSO_4.7H_2O$, 0.4; $MnSO_4.H_2O$, 0.4; $FeSO_4.7H_2O$, 0.01; CH_3COONH, 4.3; *p*-aminobenzoic acid, 0.1; and biotin, 0.001. It is noteworthy that no laboratory-grade sugar was added to the media.

3.2.5 Microorganism

The wild-type *Clostridium saccharoperbutylacetonicum* DSM 14923 (N1-4) strain was purchased from the German Collection of Microorganisms and Cell Cultures (DSM). The strain was activated and propagated according to the recommendations of the supplier. Stock cultures were routinely maintained in 2-mL aliquots of 20% aqueous glycerol solution at -80 °C. Inoculum was prepared in

anoxic pre-sterilized Reinforced Clostridial Medium (RCM, Fluka, Sigma-Aldrich, Spain). Cells were cultivated anaerobically until the exponential growth phase (optical density, OD, at 600 nm = 1.0 – 1.5) measured in spectrophotometer UV-Vis (Thermo Scientific, Evolution 60S) in an anaerobic chamber (Whitley DG250 Workstation, Don Whitley Scientific Ltd., West Yorkshire, United Kingdom), as explained in our previous work (Zetty-Arenas et al., 2019). Inoculum size was 20 vol% in all fermentations. Morphological changes of the microorganisms were analyzed using microscopic inspection throughout the fermentation studies to monitor possible contaminations.

3.2.6 Fermentation conditions

All fermentation experiments were conducted in DASGIP Model Box 300-mL bioreactors with 240 mL working volume for 72 h at 30 °C in biological duplicates. The initial pH of the media was adjusted to 7.0 at the beginning of the fermentation with NH_4OH. All procedures were performed using sterile materials and performed under aseptic conditions in a laminar flow chamber, whereas procedures involving the microorganism were performed under O_2-free N_2-enriched condition in an anaerobic chamber (model DG250 Don Whitley). The strain was activated through cultivation on RCM 20% (*v/v*) inside an anaerobic chamber in a static fermentation and maintained at 30 °C until reaching the exponential growth phase (OD_{600nm} between 1.0 and 1.5) measured in spectrophotometer UV-Vis (Thermo Scientific, Evolution 60S) and subsequently was inoculated into the bioreactors. Samples were collected at 0, 3, 6, 20, 24, 30, 48, and 72 h for measurement of the cell concentration by optical density at 600 nm (OD_{600nm}) and metabolite production.

3.2.7 Analytical methods

Samples were collected and centrifuged at 8000 rpm for 10 min at 4 °C. Clean supernatants were transferred into 2 mL microtubes and stored at -4 °C until subsequent analyses for the concentration of solvents (acetone, butanol, and

ethanol), residual sugars (glucose, xylose, cellobiose, and arabinose), and acids (acetic, butyric and formic). These analyses were performed using a high-performance liquid chromatography (HPLC) (Shimadzu) equipped with an Aminex HPX-87H® 7 x 300 8 mm x 9 µm / Cation M+ Refill / IG CATION-H column and UV detector. The mobile phase consisted of acetonitrile in water (1:8) with 1% acetic acid (100 ml ACN: 800 ml Milli-Q water + 9 ml acetic acid). The operating conditions were as follows: flow: 0.8mL / min; injection Volume: 10 µL; column: Acclaim 120-C18 150x4.8mm – Thermo; column temperature: 25 °C; wavelength: 274 nm and running time: 10 min for patterns, 60 min for samples which can be extended depending on the matrix.

Fructose and sucrose were quantified using HPLC and the refractive index detector (IR) according to the following conditions: Mobile phase: Milli-Q water; flow: 0.5 mL/min; injection volume: 10µL; column: Bio-Rad Aminex HPX-87P 300x7.8mm/particle size: 9 µm; column temperature: 55 °C; detector temperature: 50 °C and running time: 30 min.

Furfural and HMF were quantified using HPLC, UV detector, and the Acclaim 120 - C18 150x4.8mm Thermo column. Operating conditions were: mobile phase: acetonitrile in water (1:8) with 1% acetic acid (100 mL ACN: 800 mL Milli-Q water + 9 mL acetic acid); flow rate: 0.8 mL/min; injection volume: 10 µL; column temperature: 25 °C, wavelength: 274 nm, run time: 10 min for standards and 20 min for samples.

3.3 Results and discussion

3.3.1 Characterization of the hemicellulosic hydrolysates and molasses from sugarcane

The composition of concentrated (CHH) and non-concentrated (HH) hemicellulosic hydrolysates, and sugarcane molasses (SCM) are shown in **Table 1**.

Table 1. Components of sugarcane molasses (SCM) and non-concentrated (HH) and concentrated (CHH) sugarcane bagasse hemicellulosic hydrolysates used as carbon sources.

	Sugarcane molasses (SCM)	Hemicelullosic hydrolysate (HH)	Concentrated hemicelullosic hydrolysate (CHH)
Xylose (g/L)	-	13.12	40.61
Glucose (g/L)	24.03	0.82	2.60
Cellobiose (g/L)	-	0.63	0.74
Arabinose (g/L)	-	2.32	6.87
Sucrose (g/L)	249.10	-	-
Fructose (g/L)	22.90	-	-
Acetic acid (g/L)	-	2.17	3.70
Formic acid (g/L)	-	0.18	0.35
HMF (g/L)	-	0.12	0.28
Furfural (g/L)	-	0.27	0.32

As expected, the composition of HH and CHH was dominated by pentoses (xylose and arabinose), whereas SCB mainly contained sucrose. Thus, mixtures of SCM with HH or CHH led to media with a wide range of sugars at different ratios accounting for ~30 g/L of total reducing sugars (TRS) **(Table 2)**. It is worth noting that CHH100% was also used for ABE fermentation. However, the strain was entirely unable to grow on this media, and therefore, it was no detected any ABE production.

A previous study using a mixture of sugars coming from synthetic hemicellulosic hydrolysate performed by Yao et al. (2017) showed the preference of *C. saccharoperbutylacetonicum* as glucose>cellobiose>xylose>arabinose, in which all the pentose and hexose sugars were depleted concomitantly during the fermentation at different sugar consumption rate. In spite of this, the consumption preference and ABE performance by any Clostridia spp. using industrial substrates

containing sucrose, glucose, fructose, xylose, and arabinose coming from SCM and HH based media has never been described before.

Table 2. Concentration of sugars present in media containing molasses (SCM) and/or concentrated (CHH) or non-concentrated (HH) hemicellulosic hydrolysate from sugarcane, before and after ABE fermentation. The overall results of the ABE performance for each media and fermentation time are also presented.

	Fermentation media				
Substrate[1]	SCM100%	SCM75% +HH25%	SCM75% +CHH25%	SCM50% +CHH50%	SCM25% +CHH75%
Fermentation time (h)	20	30	30	72	72
Sugars before fermentation (t=0 h)					
Glucose (g/L)	3.23	2.64	2.54	1.78	3.33
Fructose (g/L)	2.27	1.65	1.56	1.01	0.56
Sucrose (g/L)	22.53	16.23	17.38	13.75	7.83
Xylose (g/L)	NA	5.50	6.47	11.68	15.12
Arabinose (g/L)	NA	1.39	1.60	2.84	4.05
TRS (g/L)	28.03	27.41	29.55	31.06	30.88
Sugars after fermentation					
Glucose (g/L)	0.00	0.03	0.00	0.00	0.00
Fructose (g/L)	0.00	0.02	0.05	0.10	0.04
Sucrose (g/L)	0.14	0.00	0.00	0.00	2.69
Xylose (g/L)	NA	2.35	1.35	7.29	12.40
Arabinose (g/L)	NA	0.71	0.76	1.85	3.47
TRS (g/L)	0.14	3.11	2.16	9.24	18.59
OD_{600nm} [2]	9.30	6.09	7.80	2.15	1.42
Acetic acid (g/L)	2.65	3.21	2.60	4.70	4.57
Butyric acid (g/L)	1.92	1.72	1.67	1.57	4.47
Acetone (g/L)	0.77	1.06	1.68	0.00	0.00
Butanol (g/L)	6.33	6.82	7.79	4.07	0.54
Ethanol (g/L)	0.20	0.33	0.32	0.32	0.23
Total ABE (g/L)	7.29	8.22	9.79	4.39	0.78
Butanol yield (g/g) [3]	0.23	0.28	0.28	0.19	0.04
ABE yield (g/g) [3]	0.26	0.34	0.36	0.20	0.06
Butanol productivity (g/L h)	0.32	0.23	0.29	0.11	0.01
ABE productivity (g/L h)	0.36	0.26	0.36	0.11	0.01

[1] The substrate composition informs the percentage of total reducing sugars (TRS) coming from each carbon source: SCM, HH, or CHH (for high percentages of hemicellulosic hydrolysate in the media, it was mandatory to concentrate HH in order to reach a TRS concentration of ~30 g/L). [2] Maximum cell growth in all the media was reached at 24 h of fermentation. [3] Yield was calculated considering the butanol produced (g)/ TRS consumed (g).

3.3.2 Co-fermentation of sugars derived from hemicellulosic hydrolysates and molasses

Despite that many solventogenic *Clostridium* strains are able to utilize glucose, fructose, xylose, arabinose, lactose, sucrose, pectin, and inulin, among other sugars, some of them may be unable to utilize all the available substrates (Patakova et al., 2011). Thus, the co-fermentation of sugars derived from hemicellulosic hydrolysates and molasses by *C. saccharoperbutylacetonicum* regarding the cell growth, total reducing sugars (TRS) such as glucose, arabinose, xylose, cellobiose, fructose and sucrose, and ABE fermentation performance for each culture media remained unknown. **Table 3** summarizes the available literature on the use of molasses or hemicellulosic hydrolysates as carbon source for ABE fermentation by different *Clostridium* spp.

Fermentation of sugarcane molasses (SCM100%) as carbon source by *C. saccharoperbutylacetonicum* showed maximum cell growth compared to all media studied, thereby reaching an OD_{600nm} of 9.30, which was achieved in 24 h (**Fig. 2**).

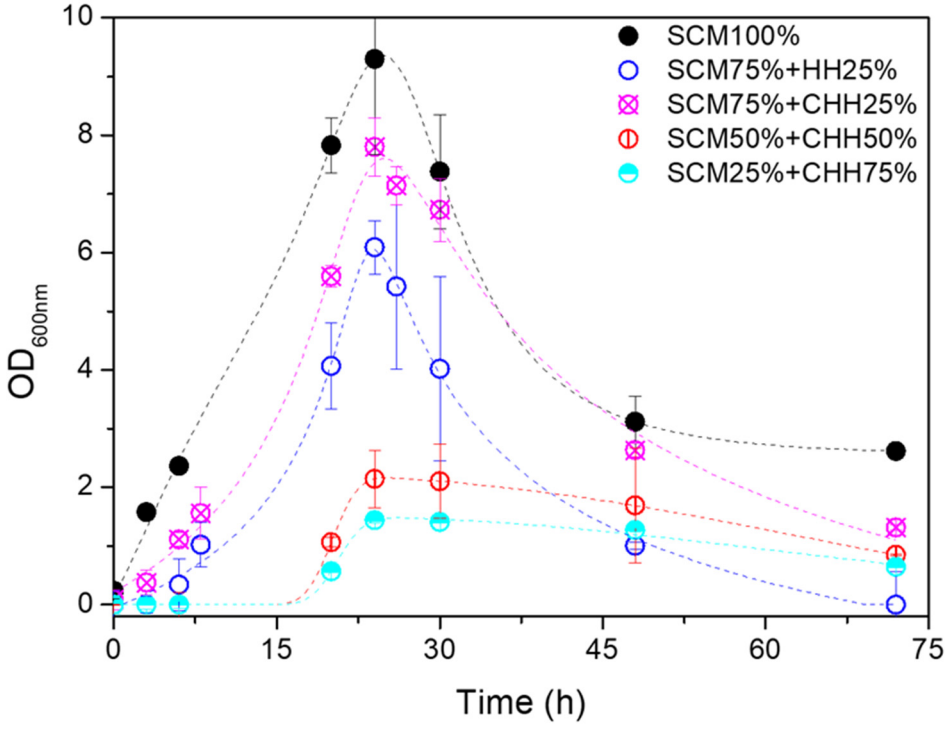

Figure 2. Cell growth profiles of *C. saccharoperbutylacetonicum* DSMZ 14923 measured as optical density at 600 nm for each culture media during 72 h of batch ABE fermentation.

Table 3. Literature review of ABE fermentation with molasses and hemicellulosic hydrolysates-based biomass.

Microorganism	Carbon source	Substrate (Initial sugar)	Condition	Inhibitor removal	Butanol (g/L)	Butanol yield (g/g)	ABE (g/L)	ABE yield (g/g)	Reference
C. saccharobutylicum DSM 13864	Molasses	Sugarcane molasses pretreated with H_2SO_4 (60 g/L)	Batch / initial pH: 6.0 / 37 °C / 250 mL flask, working volume: 150 mL / Fermentation time: 72 h	None	13.40	-	19.80	-	(Ni et al., 2012)
C. saccharobutylicum DSM 13864	Molasses	Sugarcane molasses pretreated with H_2SO_4 (60 g/L)	Batch / initial pH: 6.0/ 37 °C / 5-L bioreactor (2.5 L fermentation media) / Fermentation time: 72 h	None	11.86	-	17.88	0.33	(Ni et al., 2012)
C. beijerinckii L175	Molasses	Sugarcane molasses pretreated with H_2SO_4 (60 g/L)	Batch / 37 °C / no pH control / no agitation / Fermentation time: 84 h / 7-L reactor / Working volume: 4 L	None	11.2	-	14.9	-	(Li et al., 2013)
C. beijerinckii L175 (mutant MUT3)	Molasses	Sugarcane molasses pretreated with H_2SO_4 (60 g/L)	Batch / 37 °C / no pH control / no agitation / Fermentation time: 84 h / 7-L reactor / Working volume: 4 L	None	15.1	-	22.1	-	(Li et al., 2013)
C. acetobutylicum PTCC 23 (wild strain)	Molasses	Blackstrap molasses (60 g/L)	Batch / 32 °C / no pH control (initial pH: 6.2) / Fermentation time: 96 h	None	15.02	0.345	-	-	(Syed et al., 2008)
C. beijerinckii DSM 6422	Molasses	Beet molasses (60 g/L)	Batch / 35 °C / no pH control (initial pH: 6.2) / 2-L bioreactor / Fermentation time: 120 h	None	9.3	0.30	12.3	0.40	Bellido et al., 2018)
C. beijerinckii DSM 6422	Molasses	Beet molasses (70 g/L)	Batch / 35 °C / no pH control (initial pH: 6.2) / 2-L bioreactor / Fermentation time: 120 h	None	9.1	0.29	12.1	0.36	(Bellido et al., 2018)
C. beijerinckii DSM 6422	Molasses	Cane sugar refining molasses (60 g/L)	Batch / 35 °C / no pH control (initial pH: 6.2) / 2-L bioreactor / Fermentation time: 120 h	None	5.7	0.24	7.0	0.31	(Bellido et al., 2018)
C. beijerinckii DSM 6422	Molasses	Cane sugar refining molasses (70 g/L)	Batch / 35 °C / no pH control (initial pH: 6.2) / 2-L bioreactor / Fermentation time: 120 h	None	7.9	0.26	9.9	0.30	(Bellido et al., 2018)

Microorganism	Carbon source	Substrate (Initial sugar)	Condition	Inhibitor removal	Butanol (g/L)	Butanol yield (g/g)	ABE (g/L)	ABE yield (g/g)	Reference
C. beijerinckii BA101	Molasses	Spray-dried soy molasses (Initial sugar: 34.7 g/L)	Batch / 35 °C / Fermentation time: 96 h	None	-	-	10.7	0.31	(Qureshi et al., 2001)
C. saccharoperbutylacetonicum N1-4	Hydrolysate	Eucalyptus hydrolysate (Initial glucose: 45 g/L)	Batch / 30 °C / initial pH: 6.5	None	8.16	-	13.1	0.413	(Zheng et al., 2015)
C. saccharoperbutylacetonicum N1-4	Hydrolysate	Cassava chip hydrolysate (65.1 g/L)	Batch / 30 °C / initial pH: 6.2 / Fermentation time: 36 h / 1-L stirred reactor (0.45 L of working volume)	None	16.4	0.25	23.1	0.37	(Thang et al., 2010)
C. saccharoperbutylacetonicum N1-4	Hydrolysate	Excess sludge from sewage treatment (30-90% v/v) + glucose (40 g/L)	Batch / 30 °C / initial pH: 6.5 / 48-h of cultivation / 300-mL flask (Working volume of 200 mL)	None	9.3	-	-	-	(Kobayashi et al., 2005)
C. saccharoperbutylacetonicum N1-4	Hydrolysate	Palm kernel cake hydrolysate pretreated with H_2SO_4	Batch / 28 °C / initial pH: 6.28 / Working volume: 50 mL	Diluting by distilled water (70%)	3.59	-	5.89	-	(Shukor et al., 2014)
C. saccharoperbutylacetonicum N1-4 *C. thermocellum* ATCC 27405	Hydrolysate	Alkali-pretreated rice straw hydrolysate (40 g/L)	Batch / Fermentation time: 168 h	None	5.5	-	-	-	(Kiyoshi et al., 2015)
C. acetobutylicum ATCC 824	Hydrolysate	Concentrated detoxified sugar maple hydrolysate (Initial sugar: 60 g/L)	Batch / 35 °C / initial pH: 6.8 / Working volume: 0.7 L	Overliming	7.0	-	11.0	-	(Sun and Liu, 2012)
Clostridium saccharoperbutylacetonicum DSM 14923 (evolved strain CsCG1)	Hydrolysate	Sugarcane straw hydrolysate media supplemented with cellulose pulp/enzyme complex (around 50 g/L of total sugars)	100 mL Schott glass bottles / Working volume: 50 mL / without pH control / without agitation / 32 °C / Fermentation time: 48 h	None	Around 12.5	-	-	-	(Grassi et al., 2018)

Microorganism	Carbon source	Substrate (Initial sugar)	Condition	Inhibitor removal	Butanol (g/L)	Butanol yield (g/g)	ABE (g/L)	ABE yield (g/g)	Reference
Clostridium acetobutylicum DSM 1731	Hydrolysate	Salix (hemicellulosic hydrolysate) supplemented with starchy slurry (32.0 g/L)	Batch / 37 °C (starting temperature) / initial pH: 6.5 / Fermentation time: 120 h	None	6.7	0.22	10.6	0.35	(Yang et al., 2017)
Clostridium acetobutylicum DSM 1731	Hydrolysate	Salix (Cellulosic Hydrolysate) supplemented with starchy slurry (40.4 g/L)	Batch / 37 °C (starting temperature) / initial pH: 6.5 / Fermentation time: 120 h	Washing with water	8.1	0.21	12.4	0.33	(Yang et al., 2017)
C. beijerinckii SE-2	Hydrolysate	Corncob hydrolysate (39.7 g/L)	Batch / 37 °C / initial pH 6.8 / 100-mL bottle (Fermentation volume: 50 mL) / Fermentation time: 72 h	None	11.65	0.29	19.22	0.48	(Zhang and Jia, 2018)
C. beijerinckii SE-2	Hydrolysate	Corncob hydrolysate (39.7 g/L)	Batch / 37 °C / 100-L reactor (Fermentation volume: 50 L) / Fermentation time: 72 h	None	11.92	0.30	20.29	0.52	(Zhang and Jia, 2018)
C. acetobutylicum NCIM 2877	Hydrolysate	*Coleus forskohlii* roots hydrolysate (Concentrated to 50 g/L)	Batch / 37 °C / 125 mL bottles (working volume: 50 mL)	Overliming and Amberlite XAD-4 resine	3.3 g/L	-	5.14	0.21	(Harde et al., 2016)
C. beijerinckii DSM 6422	Hydrolysate	Enzymatic BSG (Brewer's spent grain) hydrolysates (15% BSG-Washed, around 33 g/L)	Batch / 35 °C / initial pH: 5.5 / 20 mL serum bottles / Fermentation time: 96 h	Washing	6.0	0.22	7.4	0.28	(Plaza et al., 2017)
C. beijerinckii DSM 6422	Hydrolysate	Wheat straw hydrolysates (Whole slurry originated from the steam explosion pretreatment) (around 34 g/L)	Batch / 35 °C / 2-L stirred bioreactor / no pH control / Fermentation time: 120 h	None	7.21	0.25	11.44	0.40	(Bellido, et al., 2014)

Microorganism	Carbon source	Substrate (Initial sugar)	Condition	Inhibitor removal	Butanol (g/L)	Butanol yield (g/g)	ABE (g/L)	ABE yield (g/g)	Reference
C. saccharobutylicum DSM 13864	Hydrolysate	Enzymatic hydrolysate of corncobs (Initial sugar: 52.22 g/L)	Batch / 37 °C / 125-mL erlenmeyer flask (50 mL)	Washing with tap water	12.27	-	19.44	0.35	(Gao and Rehmann, 2014)
C. saccharoperbutylacetonicum DSM 14923	Hydrolysate	Sugarcane straw hydrolysate (Initial sugar: 30 g/L)	Batch / 30 °C / Fermentation time: 100 h	None	5.18	0.15	7.64	-	(Magalhães et al., 2018)
C. saccharobutylicum DSM 13864	Hydrolysate	Sugarcane straw hydrolysate (Initial sugar: 30 g/L)	Batch / 35 °C / Fermentation time: 100 h	None	5.16	0.14	7.10	-	(Magalhães et al., 2018)
C. beijerinckii P260	Hydrolysate	Wheat straw hydrolysate (WSH) (Initial glucose: 61.2 g/L)	Batch / 50 mL bottles (35 mL of media) / 35 °C / initial pH: 6.5	None	-	-	24.98	0.42	(Qureshi et al., 2012)
C. beijerinckii BA101	Hydrolysate	Corn Fiber Hydrolysate (Initial sugar: 55.2 g/L)	Batch / 175-mL bottles (120 mL of media) / 35 °C / no pH control / Fermentation time: 88 h	Overliming	7.2	-	15.6	-	(Ezeji et al., 2007)
C. acetobutylicum DSM 1731	Mixture of hydrolysate and starch	Mixture of barley straw and grain (Initial sugar: 41 g/L)	Batch / 125-mL bottle (50 mL media) / 37 °C / initial pH: 6.5	None	7.4	0.23	11.3	0.34	(Yang et al., 2015)
C. acetobutylicum DSM 1731	Mixture of hydrolysate and starch	Mixture of grain slurry and straw hydrolysate (Initial sugar: 42.5 g/L)	Batch / 125-mL bottle (50 mL media) / 37 °C / initial pH: 6.5	None	7.8	0.17	13.5	0.29	(Yang et al., 2015)

(-): Not reported

The switch to the solventogenic phase of SCM100% started after around 10 h of fermentation (**Fig. 3b**) when the lowest pH (5.44) was achieved (**Fig. 4**), after which increased the production of acetic and butyric acid could be observed. All the TRS (glucose, fructose, and sucrose) were concomitantly depleted without any clear preference for either of them in less than 20 h of fermentation (**Fig. 3a**). The maximum production of 7.29 g/L ABE (of which 6.33 g/L were butanol) was achieved in approximately 20 h. Since molasses is a rich nutritional source, including a significant amount of mineral salts and vitamins, its use as a substrate in ABE fermentation can provide all the required compounds for fast growth and high butanol production.

As can be observed in **Fig. 3b**, **Table 2**, and **Table 3**, the butanol yield (0.23 g/g) and ABE productivity (0.36 g/L/h) achieved in SCM100% was higher than that obtained by Li et al. (2013) who showed a butanol yield around 0.20 g/g and ABE productivity of 0.18 g/L/h, using pretreated SCM by *C. beijerinckii* L175. On the other hand, the results found in this study are lower than those obtained by Ni et al. (2012) and Syed et al. (2008), which also used SCM as carbon source with a higher initial sugar concentration of 60 g/L. Ni et al. (2012) reported 19.8 g/L ABE with the corresponding yield of 0.33 g/g, in which 13.4 g/L was butanol, using *C. saccharobutylicum.* Likewise, Syed et al. (2008) used *C. acetobutylicum*, obtaining 15.02 g/L butanol with a yield of 0.34 g/g, as can be seen in **Table 3**.

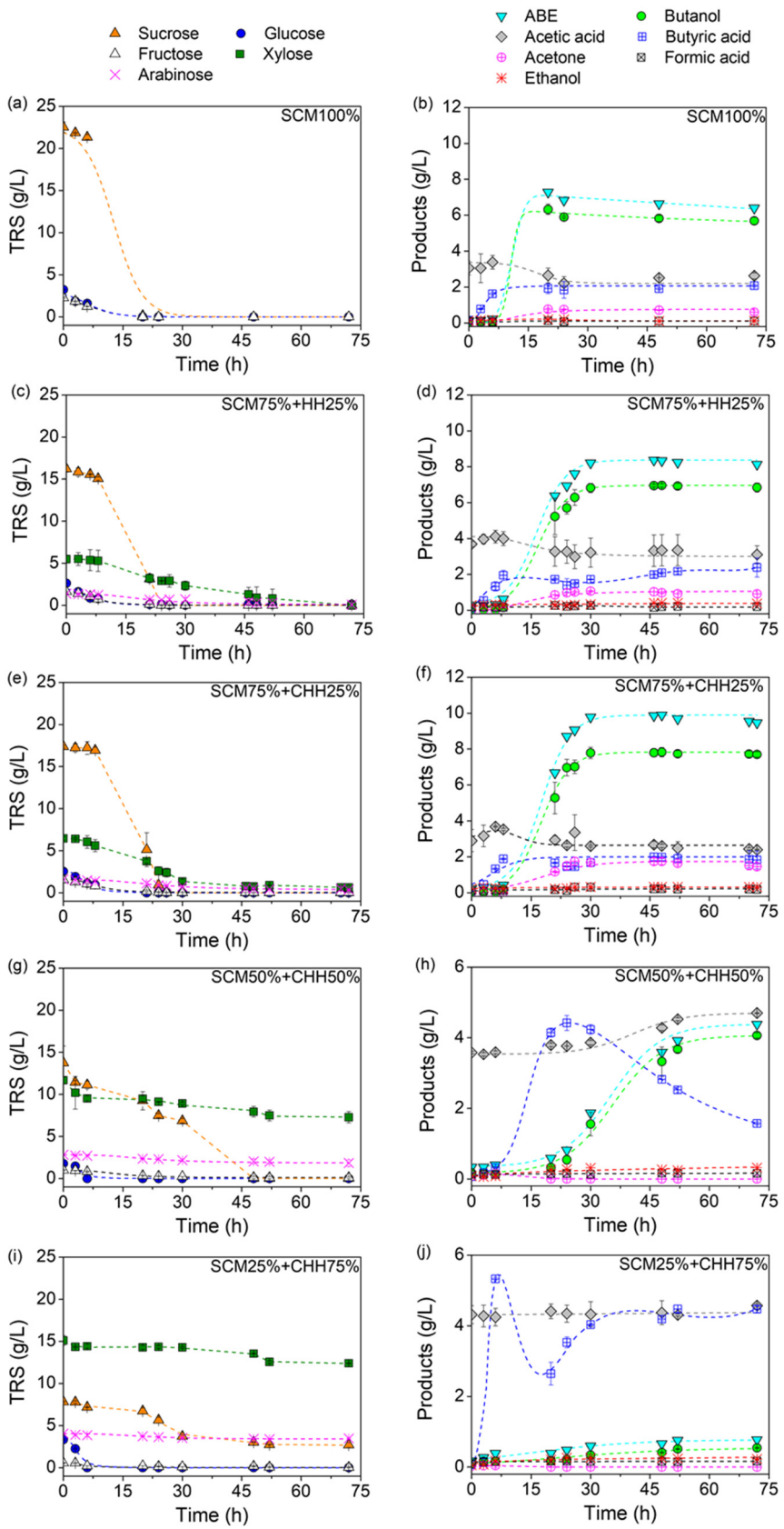

Figure 3. Total reducing sugars (TRS) consumption profiles (left side) and organic acids and ABE products (right side) of *C. saccharoperbutylacetonicum* DSMZ 14923 for each culture medium during 72 h of batch ABE fermentation.

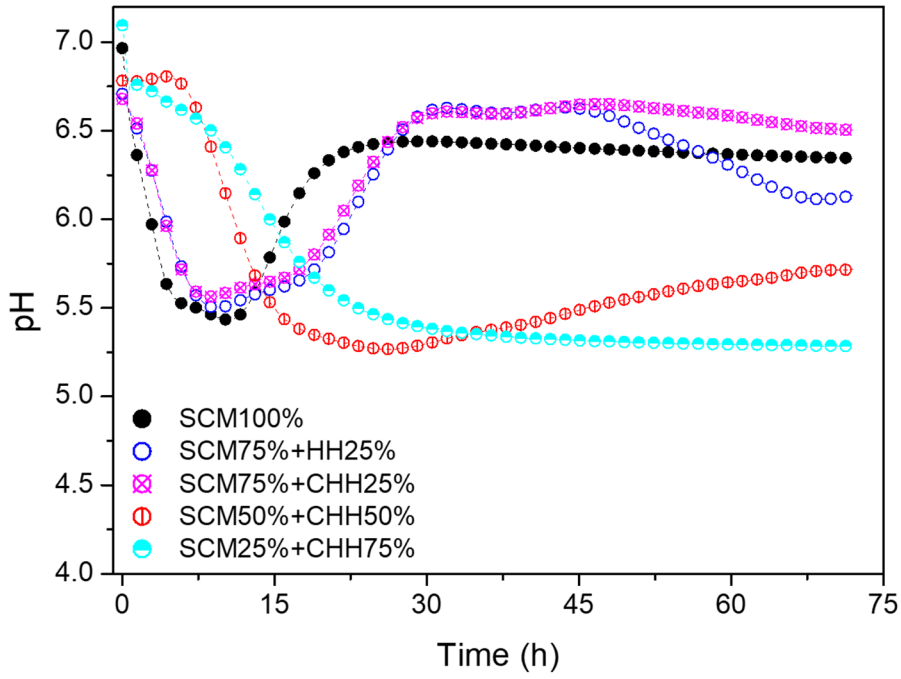

Figure 4. pH profiles of *C. saccharoperbutylacetonicum* DSMZ 14923 for each culture media during 72 h of batch ABE fermentation.

Different amounts of hemicellulose hydrolysate were added to SCM (**Table 2**). The results indicated that higher ratios of CHH added to SCM, i.e., SCM25%+CHH75% and SCM50%+CHH50% promoted a poorer ABE fermentation. **Fig. 2** shows that fermentations on both SCM50%+CHH50% and SCM25%+CHH75% exhibited a lag phase of around 10 - 20 h, and very poor cell growth, whereby maximum OD_{600nm} values of 2.15 and 1.42 were achieved after 24 h of fermentation, respectively. In fact, residual TRS were as high as 9.24 g/L for SCM50%+CHH50% and 18.59 g/L for SCM25%+CHH75% from ~30 g/L of initial TRS after 72 h of fermentation, consisting mainly of pentoses (xylose and arabinose). Glucose and fructose were fully depleted from both media, indicating that 70% and only 40% of TRS were consumed in SCM50%+CHH50% and SCM25%+CHH75%, respectively. In SCM50%+CHH50%, sucrose was fully depleted after around 48 h of fermentation, whereas only 66% of the initial sucrose was consumed in SCM25%+CHH75% remaining 2.70 g/L of this sugar after 72 h of fermentation.

The sugar utilization indicated that glucose (even present in a small amount) initiated catabolite repression of pentoses, when *C. saccharoperbutylacetonicum* was cultivated on a mixture of all the TRS present in SCM50%+CHH50% and SCM25%+CHH75%, elucidating that the preference of the strain on these media is

glucose>fructose>sucrose>xylose>arabinose, as shown in **Table 2, Fig. 3e,** and **Fig. 3g**.

As a result, we observed a decreased production of butanol (4.07 g/L) and ABE (4.39 g/L) in SCM50%+CHH50%, and hardly any production of butanol (0.54 g/L) and ABE (0.78 g/L) in SCM25%+CHH75% suggesting that when CHH was presented in high ratios, the strain was almost unable to grow, probably due to the negative impact of inhibitors on cellular metabolism. In SCM50%+CHH50%, the results also showed an increased acetic acid accumulation during the fermentation, indicating that its production rate was higher than the consumption rate. Besides the biosynthesis of acetic acid, it is important to note that it also results from the hydrolysis of acetyl groups present in the substrate, representing an important component of the hydrolysates of lignocellulosic materials (Jönsson and Martín, 2016). The butyric acid concentration steeply increased until 24 h of fermentation in SCM50%+CHH50%, after which a decrease was observed, as a result of its reassimilation for butanol production. The switch of acidogenesis to solventogenesis is confirmed by the pH increase after 24 h of fermentation. On the other hand, the fermentation of SCM25%+CHH75% showed the incapability of the strain to consume the acetic and butyric acids produced, which was reflected in its inability of switching to the solventogenic phase, resulting in an accumulation of acids, a phenomenon known as "acid crash", as depicted in **Fig. 3f**, **Fig. 3h,** and **Fig. 4**.

This phenomenon can be attributed to the ICs present in sugarcane bagasse hemicellulosic hydrolysate as a consequence of hydrolysis of hemicellulose and sugar degradation during the pretreatment, which depending on their concentration, have been shown to hamper the fermentation performance of many microorganisms including *Clostridium* spp. (Gutiérrez-Rivera et al., 2015; Jönsson and Martín, 2016; Modenbach and Nokes, 2012). ICs have been known for affecting the cell growth by damaging the DNA and/or inhibiting the enzymes or cofactors in the central metabolism (Ibraheem and Ndimba, 2013), and disturbing the redox and energy metabolism of some microorganisms (Ask et al., 2013). Therefore, the impacts observed on both viability and growth rates of the cells, as well as the lengthy lag phase are probably because of the inhibition of intracellular enzymes and from the damage produced to genetic material and cell membranes (Fillat et al., 2017).

It has been reported that the toxicity of the organic acids of lignocellulosic biomass is positively correlated with their hydrophobicity, indicating that toxicity comprises a hydrophobic target which includes the cell membrane, among others. Furthermore, it is known that phenolic compounds which come from lignin degradation during the hydrolysis, strongly contribute to the cell inhibition when lignocellulosic hydrolysates are used in high ratios in fermentation processes (Palmqvist and Hahn-Hägerdal, 2000). The main phenolic compounds of hydrolysates are 4-hydroxybenzoic acid, vanillin, and catechol (Palmqvist and Hahn-Hägerdal, 2000). Ezeji et al. (2007) identified ferulic acid, *p*-coumaric, and syringaldehyde as potent inhibitors for ABE fermentation, in concentrations as low as 0.3 g/L. Ferulic and *p*-coumaric acids have shown to be toxic to the cells by damaging the hydrophobic sites on the cell membrane, whereas syringaldehyde acts as a selective inhibitor of ABE production (with minimal effect on cell growth). Based on their preliminary results, they hypothesized that in the presence of syringaldehyde the glycolytic pathway, required for ABE production in *C. beijerinkii,* was affected, and they observed a twofold decrease in the NADH dependent butanol dehydrogenase (BDHII) activity, and an increase of 7-11 times in the sporulation genes in stage V. On the other hand, Cho et al. (2012) found that formic acid within a concentration range of 0.4–1.0 g/L resulted in massive cell death and consequently absence of ABE production in *C. acetobutylicum*. Wang et al. (2011) also reported that formic acid in a concentration range of 0.02 - 0.06 g/L, rather than acetic acid and butyric acid, triggers the "acid crash" of ABE fermentation by *C. acetobutylicum*. The presence of the inhibitory and toxic compounds mentioned above clearly shows that the complete and efficient use of C5 hydrolysates in second-generation processes is a huge challenge.

Nevertheless, our results showed a significant positive effect on the ABE fermentability by the addition of low concentration of non-concentrated and concentrated sugarcane bagasse hemicellulosic hydrolysate to SCM, i.e., SCM75%+HH25% and SCM75%+CHH25%, respectively. Thus, using the ratio 3:1 (SCM75%+HH25% and SCM75%+CHH25%), cells showed a pronounced cell growth, achieving maximum OD_{600nm} values of 6.09 and 7.80, respectively, in 24 h, comparable to those found for SCM100% although with lower cell growth (**Fig. 2**). It is possible to see that 88.7% and 92.7% of the TRS from both SCM75%+HH25% and

SCM75%+CHH25%, respectively, were consumed in around 30 h of fermentation confirming that the consumption preference of the strain on these media is *glucose>fructose>sucrose>xylose>arabinose* (**Table 2**, **Fig. 3c** and **Fig. 3e**).

As a result, when SCM75%+HH25% was used, 8.22 g/L of ABE were produced, in which 6.82 g/L corresponded to butanol, with butanol and ABE yields on TRS of 0.28 and 0.34 g/g, respectively (**Table 2**, **Fig. 3d**). Interestingly, when concentrated HH was used (SCM75%+CHH25%), a slight improvement of ABE production was found. Results showed concentrations of 7.79 g/L of butanol and 9.79 g/L of ABE obtained in SCM75%+CHH25%, with butanol and ABE yields on TRS of 0.28 g/g and 0.36 g/g, respectively, showing the highest ABE yield among the media studied (**Table 2**, **Fig. 3f**). In both cases, the addition of hemicellulosic hydrolysates HH and CHH to molasses (in a 1:3 ratio) resulted in a better fermentative performance. ABE production and its corresponding yields using the additive hemicellulosic hydrolysate were higher than those obtained in SCM100%, which indicates the advantage of mixing these substrates, probably due to the ICs dilution. Moreover, our results show that better microbial growth (SCM100%) not necessarily leads to the highest solvent production, as demonstrated by the fermentation performance of *C. saccharopebutylacetonicum* in SCM75%+HH25% and SCM75%+CHH25%.

These results are corroborated by Ezeji et al. (2007), who reported that furfural and HMF have a stimulatory effect on the ABE production by *C. beijerinckii* BA101. They found that the addition of 0.5 to 1.0 g/L of HMF to the media resulted in an increase of approximately 19% in the ABE production in comparison to the control. It has been shown that some microorganisms such as yeast and enteric bacteria are able to metabolize HMF (Wang et al., 1994) and furfural (Boopathy et al., 1993). Similarly, Qureshi et al. (2012) investigated the effect of ICs present in wheat straw hydrolysate containing 0.04–0.34 g/L of furfural and 0.12–0.88 g/L of HMF, as well as the effect of the external addition of these both compounds on the ABE fermentation performance of *C. beijerinckii* P260. Despite that both furfural and HMF had an inhibitory effect on the cell growth, they found that the ABE concentration and productivity were significantly improved by concentrations between 0.1 to 1.0 g/L of furfural and HMF. They stated that there is no a clear mechanism by which furfural and HMF enhance ABE production and productivity.

A possible reason could be that there is an increase in the enzymatic activities involved in ABE fermentation, or that furfural and HMF are precursors to this fermentation. Hence, they highlighted the advantage of using lignocellulosic biomass in ABE fermentation as long as the HMF and furfural concentrations are kept at sufficiently low levels **(Table 3)**. We hypothesize that *i*) the low levels of ICs in these media (SCM75%+HH25% and SCM75%+CHH25%) hampers the rate and efficiency of cell growth, resulting in an increased carbon flux towards ABE production and/or *ii*) that a stress response of the cells led to higher maintenance energy requirements and therefore increased ABE production.

Nevertheless, it is noteworthy that in some cases a synergistic effect of ICs results in increased toxicity (affecting both cell growth and productivity) compared to the individual effects of HMF and/or furfural, suggesting that the observed stimulatory action depends on the concentration of all the compounds in the media. However, a negative response could be seen when higher ratios of CHH were used (SCM50%+CHH50% and SCM25%+CHH75%) in this study, which agrees with that presented by Zaldivar et al. (1999), who found that the synergistic effect of furfural with some aldehydes was more toxic to the growth of *E. coli* KO11 and *E. coli* KO11 LY01. Still, this observation is in contrast with the results obtained in this study using SCM75%+CHH25% and SCM75%+HH25%, evidencing that the positive effect of HMF and furfural has a threshold from which it starts to have an adverse effect as well as is affected by the presence of other ICs, such as acids and especially phenolic compounds. Probably, the dilution of the ICs in these media could relieve their inhibitory effects on ABE fermentation.

3.4 Conclusions

Fermentation of sugarcane molasses (SCM100%) by *C. saccharoperbutylacetonicum* resulted in maximum cell growth compared to fermentation of mixtures of sugarcane molasses and hemicellulosic hydrolysates. Nevertheless, the addition of non-detoxified hemicellulosic hydrolysates (HH and CHH) to sugarcane molasses (SCM) in a ratio 1:3 resulted in an enhanced conversion of hexoses and pentoses into ABE compared to the SCM100% media. The sugar

preference of the strain on these mixed media was *glucose>fructose>sucrose>xylose>arabinose*. The best fermentation performance in terms of ABE concentration (9.79 g/L) and yield (0.36 g/g) was obtained when the HH was concentrated (SCM75%+CHH25%). However, increased ratios of hemicellulosic hydrolysate to molasses resulted in decreased production of 4.39 g/L ABE in SCM50%+CHH50%, and an almost nonexistent production of 0.78 g/L ABE in SCM25%+CHH75%, evidencing that in these conditions, the strain was almost unable to grow, metabolize sugars and, consequently, produce ABE due to the toxicity of the inhibiting compounds (ICs) present. Our results clearly showed that the effect of ICs on the growth and ABE production strongly depends on their concentration, whereby low levels of HMF and furfural probably had a positive effect on the bioconversion of concentrated C5 hydrolysates to butanol in terms of both, higher yields and productivities. Hence, *C. saccharoperbutylacetonicum* DSMZ 14923 showed to be a robust organism co-fermenting a mixture of hemicellulosic hydrolysates (without any detoxification step) and sugarcane molasses at ratios 1:3, respectively, which is a crucial factor in the economic viability of second-generation biorefineries based on sugarcane.

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Chapter

4

Vacuum evaporation for in-situ product recovery during ABE fermentation by *Clostridium saccharoperbutylacetonicum* using sugarcane by-products increased ABE productivity

This chapter has been submitted to a scientific journal

Abstract

The Acetone-Butanol-Ethanol fermentation is product limited due to butanol toxicity to solventogenic Clostridia spp. In this work, extractive batch ABE fermentation by *C. saccharoperbutylacetonicum* DSMZ 14923 was investigated in which intermittent vacuum cycles were applied for *in-situ* butanol removal. A condensation system and vacuum pump were coupled in series with the bioreactor. Firstly, an ABE standard solution was used to characterize the vacuum recovery process at absolute pressures 56 mmHg and 25 mmHg at 30 °C. Subsequently, the lowest pressure was chosen to assess the impact of the presence of acetic acid, 5- hydroxymethylfurfural (HMF), and furfural on evaporation from the standard ABE solution during vacuum recovery. Finally, ABE fermentations using sugarcane molasses (SCM) mixed with sugarcane bagasse hemicellulosic hydrolysate (CHH) containing a total of ~36 g/L of sugars coupled or not (control) with *in-situ* product recovery by cycles of 2-h at an absolute pressure of 25 mmHg and 4-h of atmospheric pressure were performed. Clearly, *in-situ* product recovery by vacuum evaporation during ABE fermentation by *C. saccharoperbutylacetonicum* resulted in a decrease of the fermentation time, almost complete depletion of total sugars, improved cell growth, and increased ABE productivity and ABE yield.

Keywords: Butanol, *In-situ* product recovery, vacuum ABE fermentation, *Clostridium saccharoperbutylacetonicum,* sugarcane molasses, sugarcane hemicellulosic hydrolysate.

4.1 Introduction

Butanol is a valuable chemical also considered as a superior biofuel with the potential to substitute gasoline by reason of its high energy density, low miscibility with water, and low vapor pressure and flammability. Moreover, butanol can be added to gasoline or diesel oil in high ratios without the need to modify the current vehicle engines (Qureshi and Ezeji, 2008; Rathour et al., 2018). Despite the interest in bio-butanol production via acetone-butanol-ethanol (ABE) fermentation, one important bottleneck of this process is the product inhibition that butanol causes to *Clostridium* spp. cells (Patakova et al., 2018; Zetty-Arenas et al., 2019). Butanol toxicity results in low productivity and yield, as well as process energy inefficiency, as a consequence of the product recovery from a diluted stream (lower than 13 g/L of butanol in batch fermentation) (Nanda et al., 2014), and degeneration of the butanol-producing strains (Kumar et al., 2012). However, the strain used in this work, i.e., *C. saccharoperbutylacetonicum* DSMZ 14923 has shown a higher butanol tolerance, which lies around 15-17 g/L, compared to other solventogenic *Clostridium* spp., as reported in our recent work (Zetty-Arenas et al., 2019).

On the other hand, in a second-generation (2G) process from the low-cost pre-treated lignocellulosic materials such as sugarcane bagasse, ABE fermentation is impacted by the presence of inhibitory compounds generally found in those streams. The presence of inhibitory compounds, such as organic acids (acetic, levulinic, and formic acids), furans (5-hydroxymethylfurfural (HMF) and furfural), and phenolic compounds leads to reduced cell growth as well as inhibition of ABE production (Jönsson et al., 2013; Tramontina et al., 2017; Zetty-Arenas et al., 2019).

The development of microorganisms with increased butanol tolerance and simultaneous butanol removal from the broth during ABE fermentation appear to be effective strategies to overcome butanol inhibition (Wang et al., 2014). Vacuum fermentation is a promising technique for *in-situ* product recovery, whereby the product is evaporated at the fermentation temperature by the application of vacuum, and subsequently recovered by condensation. Cysewski and Wilke (1977) proposed this technique for the first time for ethanol fermentation. However, the application of vacuum recovery for ABE fermentation had been disregarded due to

the fact that the boiling point of water (100 °C) is lower than that of butanol (118 °C), as stated by Roffler et al., (1987).

Nevertheless, Mariano et al. (2011) demonstrated for the first time that this technique was viable to ABE fermentation, once butanol and water form a heteroazeotropic mixture that, at concentrations below 70 g/L, boils at a lower temperature (or higher pressure in isothermal processes) than the boiling point of either butanol or water. In effect, the vapor phase is always more butanol-rich than the liquid mixture. Later, Mariano et al. (2012b) studied *in-situ* removal of butanol by applying continuous and intermittent vacuum using synthetic media containing glucose as carbon source. They found that both vacuum modes led to improved performance of ABE fermentations with total glucose depletion, enhanced productivity, and cell growth, whereby a highly concentrated butanol stream was recovered. These results were further improved when the vacuum was operated for 1.5-h at 4.0-h intervals of fermentation at atmospheric pressure, which is favorable to decrease the energy demands of the vacuum technology and to increase the ABE concentration in the condensate, as less water is vaporized under this operating condition. In addition, using this technique, no additional tanks are required to promote product separation, and there is no need to add separation agents. Driven by these advantages, this work aimed to assess the performance of ABE co-fermentation integrated with intermittent product recovery by the hyper-butanol producer *C. saccharoperbutylacetonicum* DSMZ 14923 using sugarcane molasses (SCM) and concentrated hemicellulosic hydrolysate from sugarcane bagasse (CHH), as carbon sources. The advantages of using the co-fermentation of these substrates are addressed in **Chapter 3**.

4.2 Material and methods

4.2.1 Assessment of the vacuum recovery process with a standard solution

The vacuum recovery process was conducted at 30 °C using 1-L of ABE standard solution containing: (g/L) ethanol, 1.1; acetone, 4.8; and butanol, 10.2 (concentrations usually reached in batch ABE fermentations) to evaluate the application of absolute pressures of 56 and 25 mmHg for ABE recovery from a dilute

solution. For this, a 3-L bioreactor (New Brunswick Bioflo®/Celligen®115) was connected in series to a condensation system at -10 °C, and to an oil-free vacuum pump (Edwards XDS 5 pump, AC motor, 250 W). A water bath was used to keep the temperature of the ABE solution at 30 °C. Vacuum was applied to the reactor containing the ABE standard solution for a period of 1-h, whereafter the vacuum was released to withdrawn duplicate samples of 1-mL from the reactor for quantification of the solvent concentrations. This procedure was repeated three more times, such that in total 4-h of vacuum was applied to the reactor. In a second evaporation experiment, 1-L of ABE standard solution was supplied with the intermediate acetic acid, and the inhibitors HMF and furfural. This solution contained (g/L): acetic acid, 4.9; ethanol, 0.9; butanol, 8.4; acetone, 2.3; HMF, 0.2; furfural, 0.4. In this experiment, vacuum was applied for a period of 0.5 h, whereafter the vacuum was released to withdrawn duplicate samples of 1-mL from the reactor for the quantification of the solvent concentrations. This procedure was repeated three more times, such that in total 2-h of vacuum was applied to the reactor.

At the end of all experiments, mass and volume of the solution remaining in the reactor, as well as the condensates at -10 °C were measured, and the fed compounds were quantified. Mass balances were made over the experiments. A schematic diagram of the reactor set-up for the recovery experiments and ABE fermentations with *in-situ* product recovery by vacuum is shown in **Fig. 1**.

4.2.2 Microorganism, culture maintenance, and inoculum preparation

C. saccharoperbutylacetonicum DSM 14923 [collection of cultures from the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ)] was used to carry out the ABE fermentation study. The maintenance of the culture and the inoculum preparation were performed, as explained previously (Zetty-Arenas et al., 2019). In brief, working stock cultures were stored in aqueous 20% glycerol at −80 °C. The preparation of the inoculum was performed in anaerobic pre-sterilized Reinforced Clostridial Medium (RCM, Fluka, Sigma-Aldrich, Spain). Cells were grown in a glass flask in an anaerobic chamber (Whitley DG250 Workstation, Don Whitley Scientific Ltd., West Yorkshire, United Kingdom) until reaching the

exponential growth phase, i.e., an optical density, OD_{600nm} =1.0–1.5, and inoculated into the bioreactor.

4.2.3 Conventional batch ABE fermentation

Batch fermentation experiments were carried out in a 3-L bioreactor (New Brunswick, model BioFlo 115) with a working volume of 1-L, at an initial pH 7.0 (adjusted with sterile 25% NH_4OH aqueous solution) and 30 °C, with 200 rpm agitation. The bioreactor was inoculated with a 20 vol% of inoculum (200 mL of preculture). The initial concentration of total sugars was ~36 g/L [75 wt% and 25 wt% came from sugarcane molasses (SCM) and concentrated hemicellulosic hydrolysate from sugarcane bagasse (CHH), respectively]. The components of SCM and CHH substrates used as carbon sources as well as the advantages of the co-fermentation of CHH with SCM as a result of the dilution of the inhibitors present in the former substrate, and the presence of some components in SCM that promote the growth of *C. saccharoperbutylacetonicum* can be found in **Chapter 3.** Anaerobic conditions were maintained through nitrogen sparging (1 L/min, i.e., 1 vvm). Nitrogen sparging was started 2-h prior to inoculation, to ensure fully anaerobic conditions at the start of each fermentation. All experiments were conducted in duplicate.

4.2.4 *In-situ* product recovery by cyclic vacuum during ABE fermentation

Initially, each batch fermentation was run for a period of 20 h, after which the vacuum evaporation was started. During the application of the vacuum the medium in the bioreactor boiled at 30 °C generating ABE and water vapors. The vacuum was cyclically applied to the fermentor, as follows: a period of 2-h vacuum (25 mmHg) followed by a period of 4-h without vacuum (atmospheric pressure). After applying the vacuum, the pressure inside the bioreactor was restored to atmospheric pressure by stopping the vacuum and restarting the N_2 flushing. During this period, samples were withdrawn from the bioreactor. ABE and water vapors were condensed at -10 °C, as shown in **Fig. 1**. To keep a constant liquid volume, i.e., 1-L (to compensate for the water losses due to evaporation), oxygen-free sterile water

was added to the bioreactor at different intervals. During the vacuum application, foam was formed in the bioreactor, which was collapsed by applying 2-seconds pulses of N_2 gas for 10 min, as well as by adding antifoam when necessary. Samples were withdrawn at various intervals for further analysis.

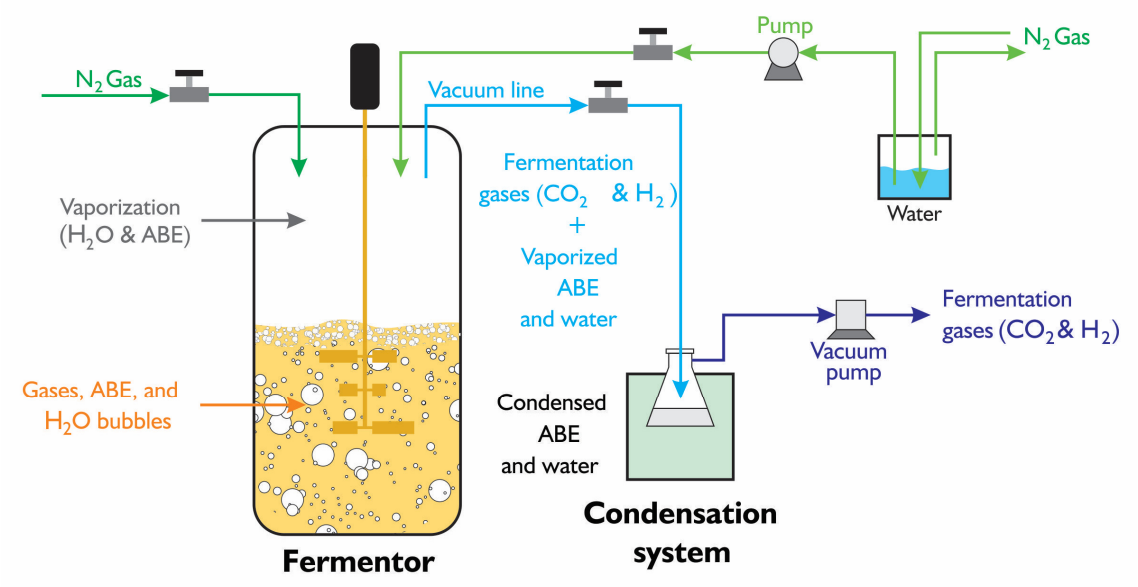

Figure 1. Schematic diagram of batch ABE fermentation and recovery by intermittent vacuum application (products of ABE fermentation boil off at 30 °C and 25 mmHg, and are recovered by a condensation system at -10 °C.

4.2.5 Analytical methods

The samples of the fermentation broth were collected and centrifuged for 10 min at 9000 g and 4 °C. Clean supernatants were transferred into 2 mL microtubes and stored at -4 °C until subsequent analyses for quantification of solvents (acetone, butanol, and ethanol), acids (butyric and acetic) and sugars (glucose, xylose, cellobiose, fructose, sucrose, and arabinose). Likewise, the samples taken from the reactor and condensate in the vacuum recovery experiments using the standard solutions were used for the analysis of acetone, butanol, ethanol, acetic acid, and furans (HMF, and furfural). These analyses were performed using a high-performance liquid chromatography (HPLC) (Shimadzu) equipped with an Aminex HPX-87H® 7 x 300 8 mm x 9 µm / Cation M+ Refill / IG CATION-H column and UV detector. The mobile phase consisted of acetonitrile in water (1:8) with 1% acetic

acid (100 ml ACN: 800 ml Milli-Q water + 9 ml acetic acid). The operating conditions were as follows: flow: 0.8 mL/min; injection volume: 10 µL; column: Acclaim 120-C18 150x4.8mm – Thermo; column temperature: 25 °C; wavelength: 274 nm and running time: 10 min for patterns, 60 min for samples.

Fructose and sucrose were quantified using HPLC and the refractive index detector (IR) according to the following conditions: Mobile phase: Milli-Q water; flow: 0.5 mL/min; injection volume: 10µL; column: Bio-Rad Aminex HPX-87P 300x7.8mm/particle size: 9 µm; column temperature: 55 °C; detector temperature: 50 °C and running time: 30 min.

Furfural and HMF were quantified using HPLC, UV detector, and the Acclaim 120 - C18 150x4.8mm Thermo column. Operating conditions were: mobile phase: acetonitrile in water (1:8) with 1% acetic acid (100 mL ACN: 800 mL Milli-Q water + 9 mL acetic acid); flow rate: 0.8 mL/min; injection volume: 10 µL; column temperature: 25 °C, wavelength: 274 nm, run time: 10 min for standards and 20 min for samples.

4.3 Results and discussion

4.3.1 Characterization of the vacuum recovery process

To characterize the ABE recovery during the intermittent vacuum application, initial experiments were conducted to evaluate the technology using an ABE standard solution at absolute pressures of 56±2 mmHg and 25±2 mmHg at different vacuum recovery times. Subsequently, the effects of the presence of acetic acid, HMF, and furfural in the ABE recovery efficiency were evaluated. The butanol recovery was determined through the ratio of butanol mass on the condensate and butanol mass in the feed.

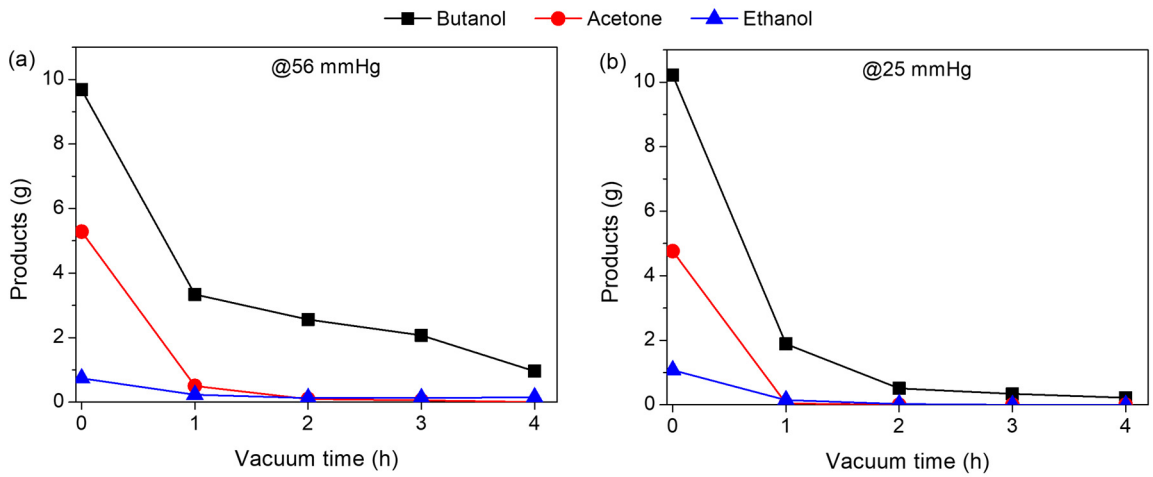

Figure 2. Decrease in ABE amounts in the reactor following different vacuum recovery times at absolute pressures of (A) 56±2 mmHg and (B) 25±2 mmHg at 30 °C.

Fig. 2 depicts the decrease of the ABE concentrations of the standard solution in the reactor after the application of vacuum recovery for 1, 2, 3, and 4 h. As expected, the amounts of all solvents in the reactor decreased over time, as a result of the vacuum evaporation.

After 1 h of vacuum applied at absolute pressures of 56 mmHg (**Fig. 2A**) and 25 mmHg (**Fig. 2B**), both ethanol and acetone, were almost completely extracted, and butanol was for a large part removed. After the first hour of vacuum, the butanol removal rate slowed down for both pressures, whereby at the lowest pressure the butanol removal was negligible after 2-h, as the amount remained stable at around 0.5 g.

For butanol, 4-h of vacuum at an absolute pressure of 56 mmHg were required to remove 90% of the butanol initially present, while 95% and 98% of the butanol was removed after 2-h and 4-h of vacuum in absolute pressure of 25 mmHg, respectively, showing that butanol removal was more efficient at the lowest pressure. After 4-h of vacuum, the recovered condensate contained 81% and 85% of the butanol, 64% and 66% of the acetone, and 70% and 75% of the ethanol of the initial solution fed in the reactor at 56 mmHg and 25 mmHg, respectively. Because at a constant temperature, the vapor pressure of the butanol-water mixture is lower at 25 mmHg than at 56 mmHg, the butanol-water mixture evaporates faster in the former condition because it is in the region of superheated vapor, giving more suitable conditions for butanol extraction.

Based on the results of the batch vacuum extraction experiments, 25 mmHg of vacuum applied during 2-h was chosen as the operating condition for determining the effect of three common components present in the sugarcane bagasse hemicellulosic hydrolysate, namely 5-hydroxymethylfurfural (HMF), furfural, and acetic acid (which is also formed during the acidogenic phase) on the butanol recovery. During these two-hours of evaporation, the vacuum was released every half hour to take a sample for quantification of the ABE solvents, acetic acid, and inhibitors. The ABE solvents and acetic acid were partly removed during the vacuum extraction while the removal of the inhibitory compounds namely furfural and 5-hydroxymethylfurfural was insignificant (**Fig. 3**). The butanol amount decreased from the initial amount of 8.4 g to 1.4 g after 2-h of vacuum, which corresponded to a decrease of 83%, which was less than in the absence of the three additional components (almost 95% butanol removed after 2-h).

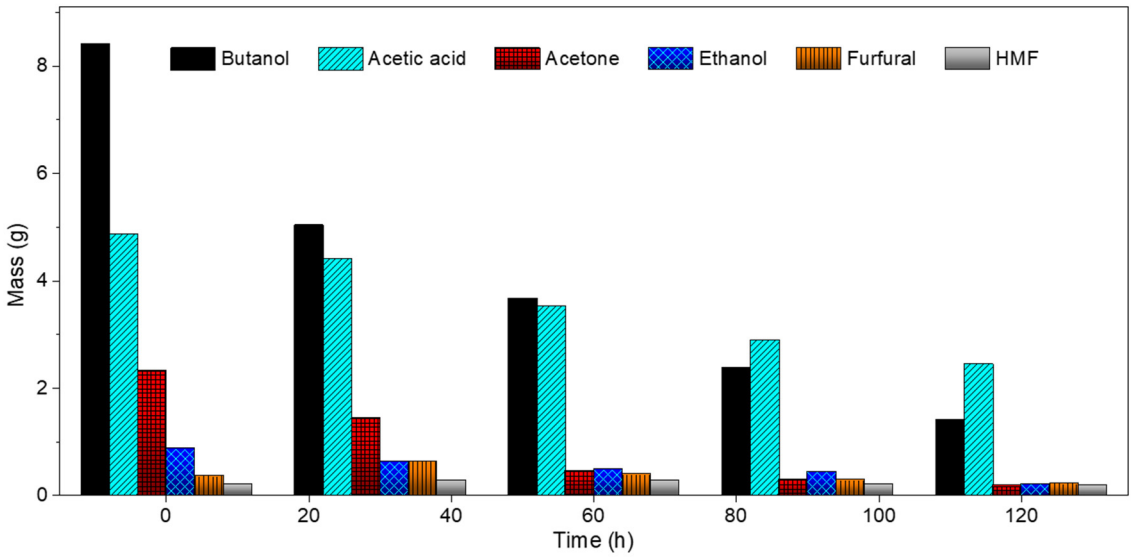

Figure. 3. Change in the amounts of the compounds of the ABE standard solution during vacuum recovery at 25 mmHg.

The recovered condensate stream was evaluated in order to assess the efficiency of the condensation system. After 2 h of vacuum, the condensate contained, as percentages of the solvents present in the initial solution: 83% of the butanol, 56% of the acetone, and 72% of the ethanol. Remarkably, the amounts of butanol and ethanol in the condensate were almost not impacted by the presence of

HMF, furfural, and acetic acid. While 85% of butanol and 75% of the ethanol in the standard ABE solution (without additives) were condensed in 4 h, 83% and 72% of the butanol and ethanol were condensed in 2 h from the ABE solution with the presence of these additives (HMF, furfural, and acetic acid), respectively. Nevertheless, although acetone was effectively evaporated from the solution, it was less efficiently trapped in the condensation system. Only 56% and 66% of acetone were condensed from the ABE standard solution with and without the above-mentioned additives, respectively.

These overall results are in accordance with observations by Mariano et al. (2012b) who reported that butanol evaporation during the recovery by means of vacuum was impacted by the presence of acetate and butyrate in the ABE standard solution, however, they did not affect the recovery of butanol in the condensate.

4.3.2 Conventional batch ABE fermentation

To compare the performance of ABE fermentation by *C. saccharoperbutylacetonicum* with *in-situ* product recovery by intermittent vacuum in a 3-L bioreactor (1-L working volume), it was firstly assessed the conventional production of ABE in batch mode (control) using a mixture of a concentrated sugarcane bagasse hemicellulosic hydrolysate (CHH) and sugarcane molasses (SCM) in the best ratio found in **Chapter 3,** i.e., 75% and 25% of the total sugars coming from SCM and CHH, respectively.

Fig. 4A depicts the growth of *C. saccharoperbutylacetonicum* and total sugars throughout 60 h of batch fermentation. The maximum optical density (OD_{600nm} = 6.46) was reached after 30 h of fermentation, while the remaining sugar concentration was 4.1 g/L, and did not decrease further, which might indicate that growth had ceased as a result of butanol toxicity and/or the presence of inhibitory compounds in the CHH substrate. The fermentation products were only quantified at the end of the cultivation, i.e., after 60 h. The primary solvent formed was butanol, followed by acetone and a small amount of ethanol (**Fig. 4B**). The ABE productivity and yield on the consumed sugar were 0.16 g/L/h and 0.31 g/g, respectively (**Table 1**).

Low butanol concentration in the broth and low productivity are usually obtained in ABE fermentation because butanol is toxic for wild-type Clostridia spp., and lethal in a concentration range of 10–12 g/L (Mariano et al., 2016). Although we recently reported that the strain used in this work, i.e., *C. saccharoperbutylacetonicum* exhibits a higher tolerance to butanol, which becomes lethal around 15 g/L (Zetty-Arenas et al., 2019), ABE fermentation is disturbed due to butanol toxicity, and consequently, the depletion of sugar is not reached.

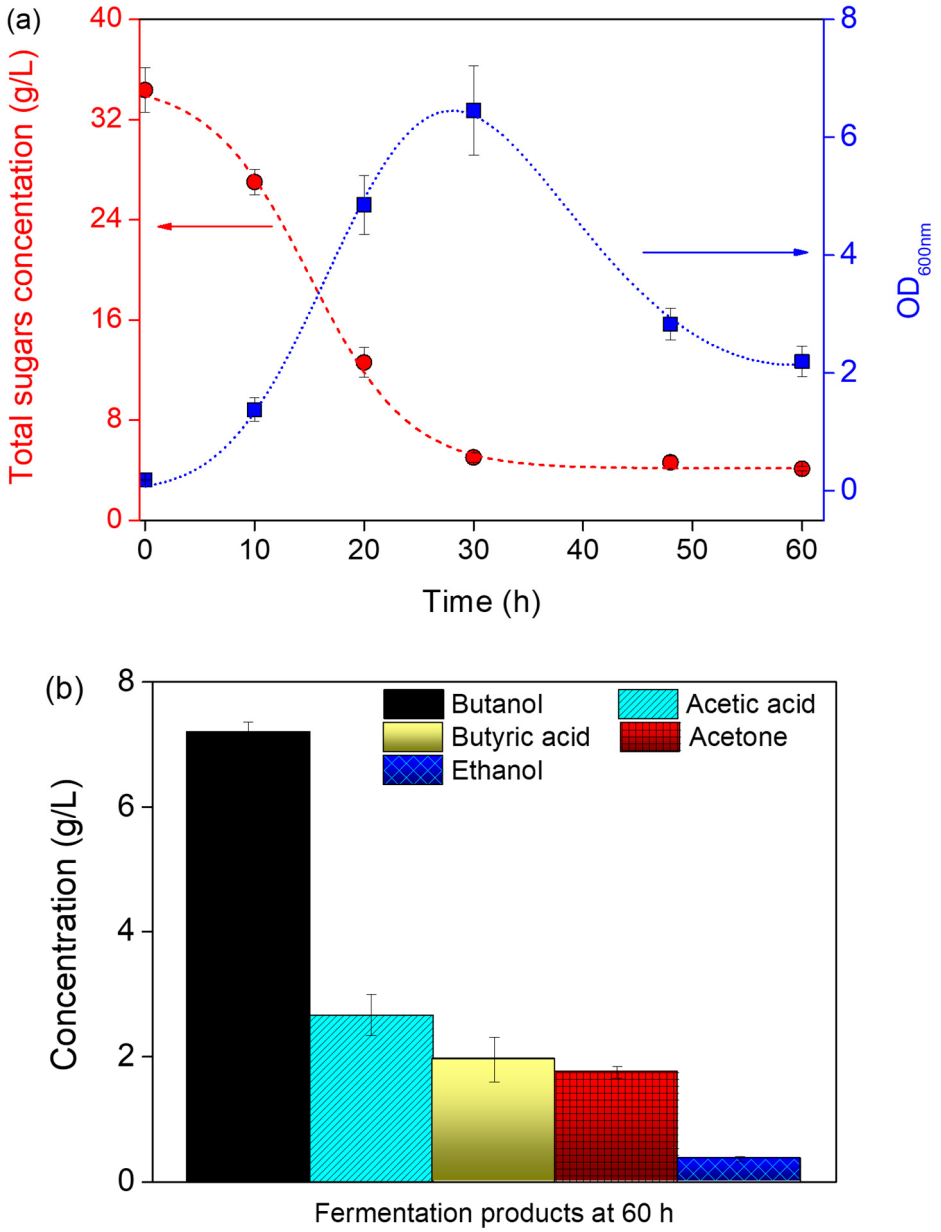

Figure. 4. ABE production in control fermentation. (A) Total sugars concentration and cell growth of *C. saccharoperbutylacetonicum* in batch fermentation using SCM added with CHH. (B) Products of batch ABE fermentation at 60 h.

Table 1. Performance of ABE fermentation and product recovery by cyclic vacuum.

Parameters	Control experiment	Cyclic vacuum fermentation
Total ABE (g)	9.3	12.4
Total acetone (g)	1.8	1.7
Total butanol (g)	7.2	10.4
Total ethanol (g)	0.37	0.37
Maximum cell density (OD_{600nm})	6.5	8.2
Initial total sugars (g/L)	34.4	39.9
Residual total sugars (g/L)	4.1	1.1
Total sugars utilized (%)	88	97
Butanol productivity (g/L/h)	0.12	0.26
ABE productivity (g/L/h)	0.16	0.31
ABE yield (g/g)	0.31	0.32
Acetone yield (g/g)	0.06	0.04
Butanol yield (g/g)	0.24	0.27
Ethanol yield (g/g)	0.01	0.01
Fermentation time (h)	60	40
Vacuum time (h)	-	8
Condensate volume (L)	-	0.112
Acetone concentration in condensate (g/L)	-	13.8
Butanol concentration in condensate (g/L)	-	78.9
Ethanol concentration in condensate (g/L)	-	3.3
Acetate concentration in condensate (g/L)	-	0.40
Butyrate concentration in condensate (g/L)	-	0.10

4.3.3 ABE fermentation with vacuum product recovery

Subsequently, the performance of ABE fermentations integrated with *in-situ* product recovery by means of intermittent vacuum evaporation was assessed. Prior to applying the vacuum evaporation cycles of 2-h vacuum (absolute pressure 25 mmHg) followed by 4-h atmospheric pressure, the ABE fermentation was run without any disturbance for 20 h, during which *C. saccharoperbutylacetonicum* got well established in the bioreactor, and the butanol concentration reached values above 5 g/L. These experiments were designed in light of the data obtained from the standard ABE solution experiments, and that reported by Mariano et al., 2011; Mariano et al., 2012a. The patterns of optical density, concentrations of total sugars, acetic and butyric acids, and ABE products over time are shown in **Figs. 5A and 5B**.

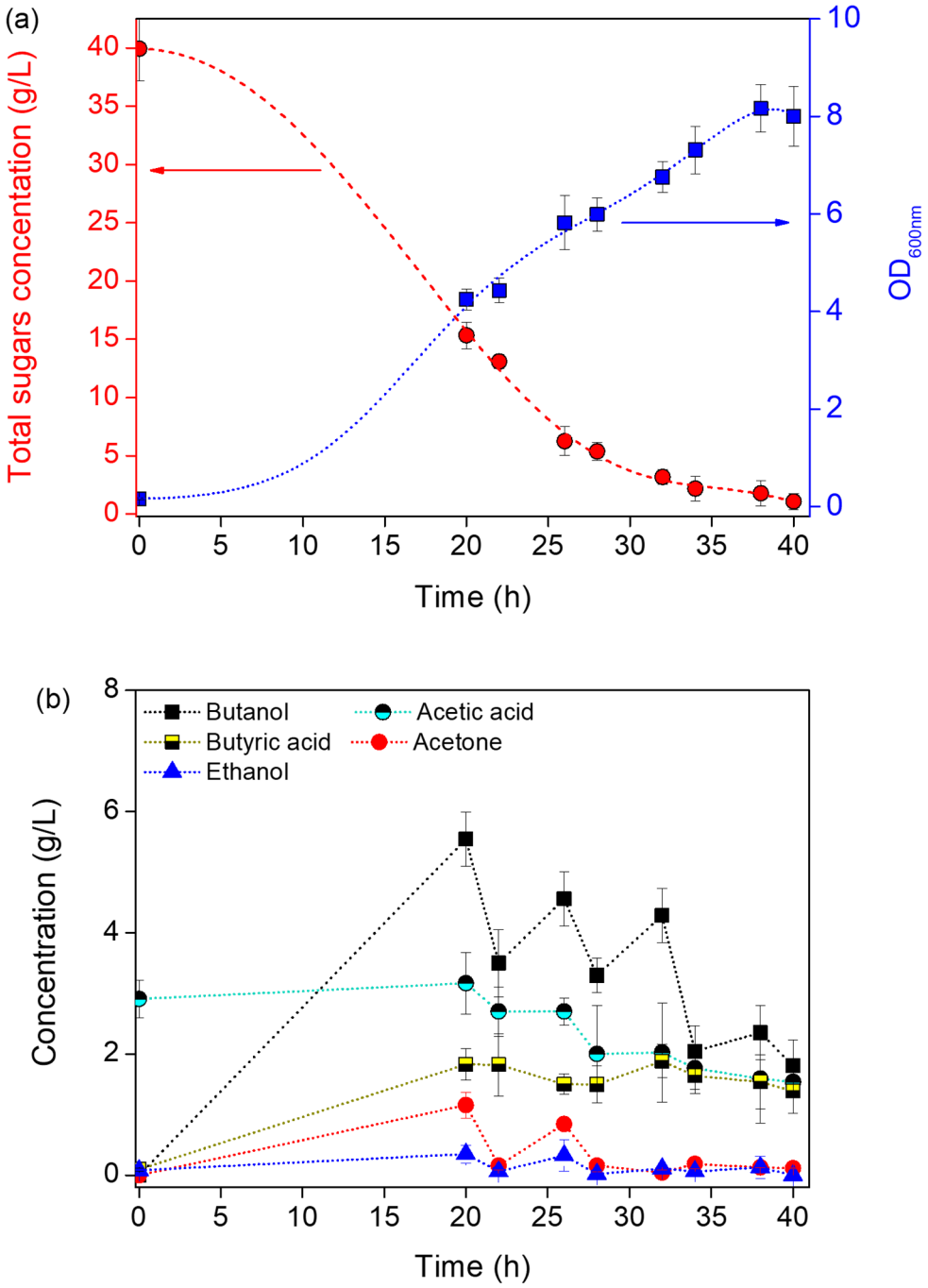

Figure 5. ABE fermentation and *in-situ* product recovery by 2-h of cyclic vacuum separated by 4-h intervals of fermentation (at atmospheric pressure) by *C. saccharoperbutylacetonicum* in batch fermentation using SCM added with CHH. Fermentation was allowed to progress for 20 h before beginning vacuum recovery. Vacuum was on during the 20 – 22 h, 26 – 28 h, 32 – 34 h, 38 – 40 h of fermentation. (A) OD_{600nm} and total sugars; (B) ABE and acids concentration in the bioreactor over time.

The *in-situ* product recovery by vacuum evaporation during the ABE fermentations on the non-synthetic medium, i.e., sugarcane molasses mixed with sugarcane bagasse hemicellulosic hydrolysate was effective. After 20 h of fermentation, the cyclic application of vacuum evaporation leads to an oscillatory behavior of the butanol, acetone, ethanol, and acetic acid concentrations, whereby the concentrations decreased during the vacuum and increased thereafter. In contrast, butyric acid did not present this behavior likely as a result of the fact that it has the highest boiling point of all quantified components.

The butanol concentration in the broth decreased to as low as to 1.8 g/L and increased up to 4.6 g/L after and before vacuum application, respectively **(Fig. 5B)**. Therefore, vacuum cycles were suitable to keep the butanol concentration below the toxicity threshold for Clostridia spp. which favored *C. saccharoperbutylacetonicum* to reach a maximum optical density of 8.2, which was higher than that obtained in the control experiment (**Figs. 4A and 5A, and Table 1**). This is in accordance with the findings of Mariano et al. (2012a), who observed even a 1.9 times increase in the cell density of *C. beijerinckii* 8052 measured as OD_{540nm} when the fermentation was subjected to intermittent vacuum compared to the control experiment. These results clearly show that, as observed for *C. beijerinckii* 8052, the exposure of *C. saccharoperbutylacetonicum* to repetitive vacuum cycles did not negatively affect the cells.

After 40 h of fermentation, the total sugars were practically completely depleted (97% of the initial amount), while this was only 88% in the control experiment (**Figs. 4A and 5A, and Table 1**). This gain in the sugar consumption found in this study is considered high because we used a mixture of sugarcane molasses and sugarcane bagasse hemicellulosic hydrolysate as substrate. In **Chapter 3,** we have shown that the sugar preference of *C. saccharoperbutylacetonicum* on this mixed sugarcane substrate was glucose>fructose>sucrose>xylose>arabinose. Indeed, the last sugars consumed were xylose and arabinose (data no shown), indicating the same profile in both sugar consumption and preference, as detailed in **Chapter 3**.

It is worthy to note that even if all sugars in the fermentation without vacuum (control) were consumed, the butanol concentration would not reach toxic levels. The strain used in this study has a higher tolerance to butanol compared to other

solventogenic *Clostridium* spp., as shown in our previous work (Zetty-Arenas et al., 2019). Therefore, the main factor for the bacteria not to deplete sugars could have been the presence of inhibitors. On the other hand, vacuum evaporation results in significantly lower butanol levels, and thus reduced butanol stress, which might have allowed the bacteria to perform better, even in the presence of inhibitors.

Acetic acid was slightly recovered, and its concentration in the condensate was only 0.40 g/L while butyric acid was practically unrecovered, showing only 0.1 g/L in the condensate. These results are desirable since the acids produced by the cells are available to be metabolized into ABE (**Fig. 5A and Table 1**). The total ABE produced in the 1-L fermentation with vacuum extraction was 12.4 g, resulting in a twofold increase in productivity of 0.31 g ABE/L/h, but a similar yield of 0.32 g ABE/g compared to the control experiment (**Table 1**). It should be noted, however, that the yield of acetone on total sugars declined from 0.06 g/g in the control experiment to 0.04 g/g in the vacuum fermentation. This has most likely occurred because acetone is the most volatile compound and could not be efficiently trapped in the condensation system. Previous works indicated that acetone might have been accumulated in the vacuum pump (Assumpção et al., 2018; Mariano et al., 2011; Mariano et al., 2012b, 2012a). **Table 1** summarizes the main performance parameters of control and vacuum fermentations.

To the best of our knowledge, this is the first study using *Clostridium saccharoperbutylacetonicum* in a co-fermentation of sugarcane molasses mixed with sugarcane bagasse hemicellulosic hydrolysate integrated with *in-situ* product recovery by vacuum. A comparison of the ABE performance of the vacuum evaporation technology for *in-situ* product recovery is summarized in **Table 2**, showing that our results are in agreement with that found by other researchers using ABE fermentation coupled with vacuum recovery.

Table 2. Literature review of ABE fermentation studies using vacuum evaporation technology for *in-situ* product recovery.

Vacuum evaporation

Bacteria	Substrate	Method description	Butanol production	ABE production	ABE productivity	$Y_{P/S}$	Reference
Clostridium saccharoperbutylacetonicum DSMZ 14923	Sugarcane molasses mixed with sugarcane bagasse hemicellulosic hydrolysate (40 g/L)	Batch reaction with an intermittent vacuum (2 h with 4 h interval); Fermentation time: 40 h. 3 L bioreactor; Working volume: 1 L	10.37 g	12.39 g	0.31 g/L/h	0.32 g/g (ABE yield)	This work
Clostridium beijerinckii P260	Glucose (60 g/L)	Batch reaction with intermittent vacuum (1.5 h with 4 h interval); Fermentation time: 46 h. 14 L bioreactor; Working volume: 7 L	85.1 g	108.6 g	0.28 g/L/h	0.26 g/g (ABE yield)	(Mariano, Qureshi, & Filho, 2011)
Clostridium beijerinckii P260	Glucose (60 g/L)	Batch reaction with intermittent vacuum (1.5 h with 6 h interval); Fermentation time: 63 h. 14 L bioreactor; Working volume: 7 L	103.0 g	122.8 g	0.34 g/L/h	0.29 g/g (ABE yield)	(Mariano, Qureshi, & Filho, 2011)
Clostridium beijerinckii NCIMB 8052 (ATCC 51743)	Eucalyptus wood hydrolysate (ratio 1.5 between cellulosic and hemicellulosic hydrolysate) (total initial sugars: 60 g/L)	5-L bioreactor; Working volume: 2 L; 35 °C; Vacuum sessions (10 kPa) started after 20 h of fermentation (2-h-vacuum with 4 h of intervals); Fermentation time: 50 h	53.3 g/L (condensate)	69.3 g/L (condensate)	0.25 g/L/h (Butanol productivity)	0.26 g/g (Butanol yield)	(Assumpção et al., 2018)
Clostridium beijerinckii 8052	Glucose (65.4 g/L)	Continuous vacuum application (vacuum range 711–737 mmHg) starting at 20 h of fermentation; Fermentation time: 44 h; 35 °C. 14 L bioreactor; Working volume: 7 L	106 g	132.4 g	0.43 g/L/h	0.29 g/g (ABE yield)	(Mariano et al., 2012b)
Clostridium beijerinckii NCIMB 8052 (ATCC 51743)	Glucose (66.1 g/L)	Cyclic vacuum (2-h of vacuum and 4 h of intervals, vacuum range 711-737 mmHg) starting at 18 h of fermentation; Fermentation time: 55 h; Vacuum time: 12 h. 14 L bioreactor; Working volume: 7 L	120.1 g	141.2 g	0.37 g/L/h	0.34 g/g (ABE yield)	(Mariano et al., 2012a)

4.4 Conclusions

This work proved that the intermittent vacuum evaporation technique can be successfully applied for *in-situ* butanol recovery through ABE fermentations using *C. saccharoperbutylacetonicum*. The butanol concentration in the recovered condensate was more than ten times greater than that in the fermentation broth, which significantly facilitates the downstream processing. Furthermore, the application of vacuum recovery during the ABE fermentation decreased the fermentation time, resulted in higher cell density, almost complete consumption of total sugars of the sugarcane molasses/bagasse hemicellulosic hydrolysate-based media, and increased ABE productivity and production. The strain was not negatively affected by the vacuum application. Therefore, intermittent vacuum evaporation is a promising technology to improve the performance of solventogenic Clostridia-based butanol fermentation processes.

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Chapter

5

Clostridium saccharoperbutylacetonicum revealed biofilm formation: characterization of the extracellular polymeric substances (EPS) and quantitative proteomics of sessile and planktonic cells

This chapter will be submitted to a scientific journal

Abstract

Cells in biofilms have shown to have a higher tolerance to stress factors in harsh environments and improvements in butanol production when they are used for continuous acetone-butanol-ethanol (ABE) fermentation. This work reveals the first evidence for the biofilm composition of the extracellular polymeric substances (EPS) and proteomic analysis of sessile cells and aggregates compared to planktonic cells in any solventogenic *Clostridium* spp. Firstly, the ability of the hyper-butanol producing *Clostridium saccharoperbutylacetonicum* to form biofilms during chemostat cultivation with a gradually increasing dilution rate from 0.11 h^{-1} to 0.41 h^{-1} under acidogenesis was investigated. Secondly, spectroscopy, chromatography, morphological, and colorimetric analysis, along with quantitative proteomics of sessile and planktonic cells, were combined to obtain an in-depth molecular characterization of these biofilms. The highest biofilm growth was observed at D = 0.28 $h^{-1,}$ and the main products were butyric acid and acetic acid with a maximum production rate of 1.07 g/L/h and 0.39 g/L/h, respectively. Autofluorescence was only observed in the EPS of the mature biofilm which was likely to be related to the presence of tryptophan that was identified by FT-IR spectroscopy. HPAEC-PAD sugar analysis showed the same saccharides in all the three samples (planktonic cells, aggregates, and biofilm), with the exception of xylose and mannose that were only found in the biofilm sample. Biofilm, aggregates, and planktonic cells resulted in around 4 wt%, 7 wt%, and 11 wt% sugar mixture equivalents, and 99 wt%, 84 wt%, and 53 wt% BSA equivalents, respectively. Based on these results, it can be stated that the biofilm formed by *C. saccharoperbutylacetonicum* is dominated by protein/polypeptides. Quantitative proteomics identified 164 proteins enriched in the biofilm samples compared to the planktonic cells, of which 124 were identified based on homology (47%) or sequence similarity (53%), and 40 were not further assigned to any function. Here, it was surprisingly revealed the presence of the Beta-lactamase domain protein, which is related to antibiotic resistance.

Keywords: chemostat ABE fermentation, solventogenic Clostridia, extracellular polymeric substances, biofilm characterization, proteomics, FT-IR.

5.1 Introduction

Growing concerns about the negative environmental impacts of the application of fossil-fuel derived products, global energy crisis, and economic security have made bio-based fuels, and chemicals gain increasing attention as alternatives to move towards a more sustainable economy (Zhao et al., 2018).
n-Butanol (hereafter referred to as butanol), produced via the acetone-butanol-ethanol (ABE) fermentation, is considered as a valuable chemical building block and a superior biofuel due to its physical and chemical properties close to those of gasoline (Dürre, 2007; Wang et al., 2017). The demand for butanol is expected to increase to 122 million tons per year by 2020 (Ibrahim et al., 2017).

Despite the remarkable advantages of bio-based butanol, its production by fermentation requires new cost-affordable strategies to become economically feasible on an industrial scale. The main reasons for this are feedstock cost and availability, low cell density, low butanol titer, due to its toxic effect on the producing organism *Clostridium* spp. (cells do not accumulate more than 13 g/L in a batch reactor), and consequently, high downtimes and low productivities (Mao et al., 2010).

Biofilm reactors have been reported as one of the most useful strategies for continuous large-scale ABE fermentation, as they have several advantages compared to planktonic cells such as: (i) higher cell density; (ii) higher productivity; (iii) cell stability; (iv) flexibility in reactor design for longer-term continuous operation at high dilution rates without cell washout; and (v) easier and lower-cost downstream processing (Patakova et al., 2018; Qureshi et al., 2005; Raganati et al., 2016).

Cells in biofilms have shown to have a higher tolerance to stress factors in harsh environments by providing a barrier that slows down the diffusion of toxic substances and protects the cells against phages and bacteriocides (Costa et al., 2016). Moreover, the uptake of harmful compounds in biofilms is diminished because the cells in the interior generally have lower growth rates. Thus, biofilms for biotechnological industrial applications are receiving increasing attention in recent years (Limoli et al., 2015; Patakova et al., 2018; Qureshi et al., 2005). Recently, a combined approach, including proteomic and Fourier transform infrared (FT-IR)

spectroscopy analysis, has assisted the investigation of the distinctiveness of biofilm formation for some microorganisms, such as *Bordetella pertussis* and *Escherichia coli* (Koerdt et al., 2011).

Biofilms are microbial communities with the natural ability to attach to a surface or stick to each other as suspended biomass. These microbial cells are embedded within a slimy self-produced complex three-dimensional matrix of extracellular polymeric substances (EPS) which consist of proteins (enzymes and structural proteins), polysaccharides, lipids, and nucleic acids (Limoli et al., 2015; Seviour et al., 2019; Zhuang et al., 2016). Besides the mechanical stability, it has been reported that biofilms have digestive capabilities due to enzyme retention (Guellil et al., 2001), resource capture through sorption (Flemming, 1995), antibiotic tolerance and/or resistance (Olsen, 2015), amongst other properties. The transition from free-living cells to a sedentary biofilm requires the coordinated regulation of several genes (Costa et al., 2016). To have an in-depth characterization of the biofilms is crucial to identify EPS molecules and address them to the specific functions. This demands a combined multidisciplinary approach by using different tools to elucidate this phenomenon, as described in **Fig. 1**.

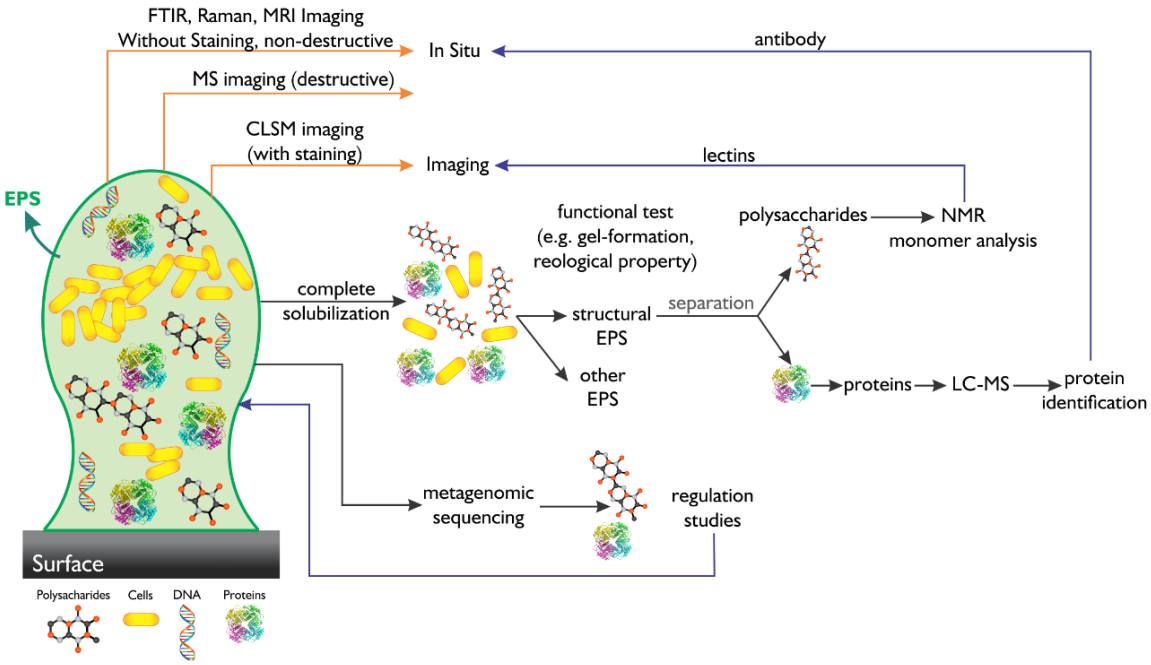

Figure 1. Proposed multidisciplinary roadmap for resolving the identities and functions of extracellular polymeric substances in biofilms, involving complementary chemical, biophysical, and 'omic' analysis of biofilms and isolated constituents (Adapted from Seviour et al., 2019).

Although it is known that the understanding of biofilm formation and composition is essential for the design and optimization of biofilm-based processes and that EPS play a crucial role in the internal protection of cells against environmental stressors (Seviour et al., 2019), few researchers have investigated biofilms of *Clostridium* spp. in comparison with biofilms formed by other bacteria such as *Staphylococcus* spp. or *Bacillus* spp., raising the question of whether *Clostridium* spp. could actually develop a proper biofilm or only be recruited into a pre-existing biofilm (Pantaléon et al., 2015). Most structural studies involving *Clostridium* spp. have been done only with pathogens such as *Clostridium botulinum, Clostridium difficile*, and *Clostridium perfringens* or by non-pathogens, present in the gut microbiota such as *Clostridium clostridioforme* and *Clostridium malenominatum* (Pantaléon et al., 2014). Concerning biofilm formation in solventogenic *Clostridium* spp., researchers have mostly addressed only the application of immobilized cells for enhanced ABE production and butanol tolerance (Qureshi et al., 2005). In fact, the only study focusing on EPS production by solventogenic *Clostridium* spp. so far was carried out by Zhuang et al. (2016). Increased butanol production by biofilm grown of *C. acetobutylicum* CGMCC 5234 was reported, in addition to improved tolerance to butanol and acetic acid in biofilms, when compared to planktonic cells through cellular morphology and fermentative performance. Later, Liu et al. (2016) found that in biofilms, there is a kind of stress in which sporulation is limited using *C. acetobutylicum* CGMCC 5234, showing a delay in the up-regulation of the sporulation genes in sessile cells when compared to planktonic cells. Still, the knowledge on the mechanisms of biofilm formation and its regulation in solventogenic *Clostridium* spp. is extremely limited, and the biofilm composition in terms of EPS has never been followed up by in-depth molecular characterization (Patakova et al., 2018).

Particularly, *C. saccharoperbutylacetonicum* has been reported as a robust butanol hyper producer strain which uses hexoses and pentoses as carbon sources (Del Cerro et al., 2013) and has been identified as one of the most promising strains for second-generation butanol production from low-cost lignocellulosic feedstocks (Zetty-Arenas et al., 2019). Despite the robustness and importance of *C. saccharoperbutylacetonicum*, to the best of our knowledge, the formation of biofilms by this organism has not been reported.

Therefore, the first aim of this study was to investigate the ability of wild-type *C. saccharoperbutylacetonicum* to form biofilms. Secondly, it was carried out a comparative study of planktonic and biofilm cells using proteomic analysis, and EPS characterization by combining FT-IR spectroscopy and High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD) for monosaccharides determination, as well as total carbohydrates and protein quantification. With these analyses, it is aimed to gain a first insight into the composition and potential properties of *C. saccharoperbutylacetonicum* biofilms.

5.2 Material and methods

5.2.1 Microorganism and culture conditions

Clostridium saccharoperbutylacetonicum DSM 14923 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was activated and propagated according to the procedure recommended by the supplier at 30 °C under anaerobic conditions. Stock cultures were preserved in Reinforced Clostridia Medium (RCM, Fluka, Sigma-Aldrich, Spain) containing 20% vol. of glycerol and stored at - 80 °C.

5.2.2 Media preparation

Fermentation experiments were conducted using a modified P2 medium containing in (g/L): yeast extract, 5.0; KH_2PO_4, 0.75; K_2HPO_4, 0.75; NaCl, 1; $MgSO_4.7H_2O$, 0.4; $MnSO_4.H_2O$, 0.4; $FeSO_4.7H_2O$, 0.01; CH_3COONH_4, 4.3; EDTA 0.05; *p*-aminobenzoic acid, 0.1; and biotin, 0.001. Glucose was the carbon source for the biofilm reactor: 60 g/L for the initial batch, and the first 290 h of the chemostat cultivation; and 20 g/L thereafter. The culture medium was divided into two parts for sterilization, as follows: yeast extract, KH_2PO_4, K_2HPO_4, and NaCl were autoclaved at 121 °C for 20 min, while the remaining components were filter sterilized through 0.2 µm Sartopore® 2 membrane capsule (Sartorius Stedim Biotech, Germany), and subsequently mixed with the autoclaved part of the medium under sterile conditions.

5.2.3 Support material

Mutag BioChips 25™ (Umwelttechnologie AG; Germany) were used as a high-performance biofilm carriers. These carriers are made of polyethylene containing 3,000 m^2 of active surface area per m^3 of material with pores in which the biofilm can grow as medium components can diffuse into the biofilms down to about 0.5 mm depth on both sides of the chips. Previous experiments conducted with the carriers showed that they do not have any inhibitory or stimulatory effects on the ABE fermentation by *Clostridium saccharoperbutylacetonicum*.

5.2.4 Reactor set up and operating conditions

The chemostat cultivations were conducted in two biological duplicates at 30 °C and 200 rpm in the setup shown schematically in **Fig. 2**. Fifteen *Mutag BioChip 25™* carriers were dried at 70 °C and subsequently cooled down at room temperature in desiccators and individually weighted. Then, the carriers were loaded in a 2-L bioreactor (Applikon, Delft, The Netherlands) with 1-L of working volume, equipped with temperature, pH, and dissolved oxygen probes, and subsequently autoclaved at 121°C for 20 min. No chemical treatment was used to promote biofilm formation on the carrier. Water from an external circulating water bath was fed into the jacket of the reactor to control the temperature at a value of 30±0.01 °C.

To maintain anaerobic conditions throughout the fermentation, the reactor was continuously sparged with sterile nitrogen gas, obtained by filtration through a sterile 0.22 µm plate filter, at a rate of 0.4 L/min which was controlled by a mass flow controller (Brooks 58505 calibration at 0 °C and 1 bar). Nitrogen sparging was started 1 h before initiating the batch fermentation to establish fully anaerobic conditions. The sterile medium was fed at the top of the reactor by means of a peristaltic pump. The exhaust gas of the bioreactor was dried by passing through a condenser at 4 °C and a Nafion dryer (Permapure, Toms River, USA) before entering an NGA 2000 off-gas analyzer (Rosemount Analytical, Anaheim, USA) to quantify the carbon dioxide produced and to verify the complete absence of oxygen.

On-line data acquisition was carried out with MFCS/win 3.0 software. Chemostat cultivations were conducted in two steps: an initial batch phase followed by the chemostat cultivation.

5.2.4.1 Batch phase

The initial pH of the batch medium was adjusted to 7.0 using sterile 2 M NaOH. Subsequently, the reactor was inoculated with *C. saccharoperbutylacetonicum* DSM 14923, grown exponentially under anaerobic conditions in Reinforced Clostridial Medium until an OD_{600nm} between 1.0 and 1.2. Twenty percent (v/v) of the working volume of the reactor was inoculated with the actively growing culture (t = 0 h). During the batch cultivation, which lasted for about 20 hours, the pH was not controlled.

5.2.4.2 Chemostat cultivation

After the batch phase was finished, which was observed from a sudden decrease of the CO_2 production, the reactor was switched to chemostat mode. At that time, planktonic cell samples were withdrawn and centrifuged at 5000×g for 5 min at 4 °C. Pellets were processed for further analysis, as explained below. During chemostat cultivation, the pH was controlled at 6.50±0.05 using sterile 2 M NaOH. The initial dilution rate was 0.11 h^{-1}. Subsequently, the dilution rate was increased to 0.4 h^{-1} in 5 steps (0.16, 0.20, 0.28, 0.35, and 0.41 h^{-1}). After each increase of the dilution rate, the chemostat was run until the CO_2 production remained constant for at least 5 residence times. Subsequently, 2 mL-samples were collected throughout the fermentation and immediately frozen at -80 °C for further analysis.

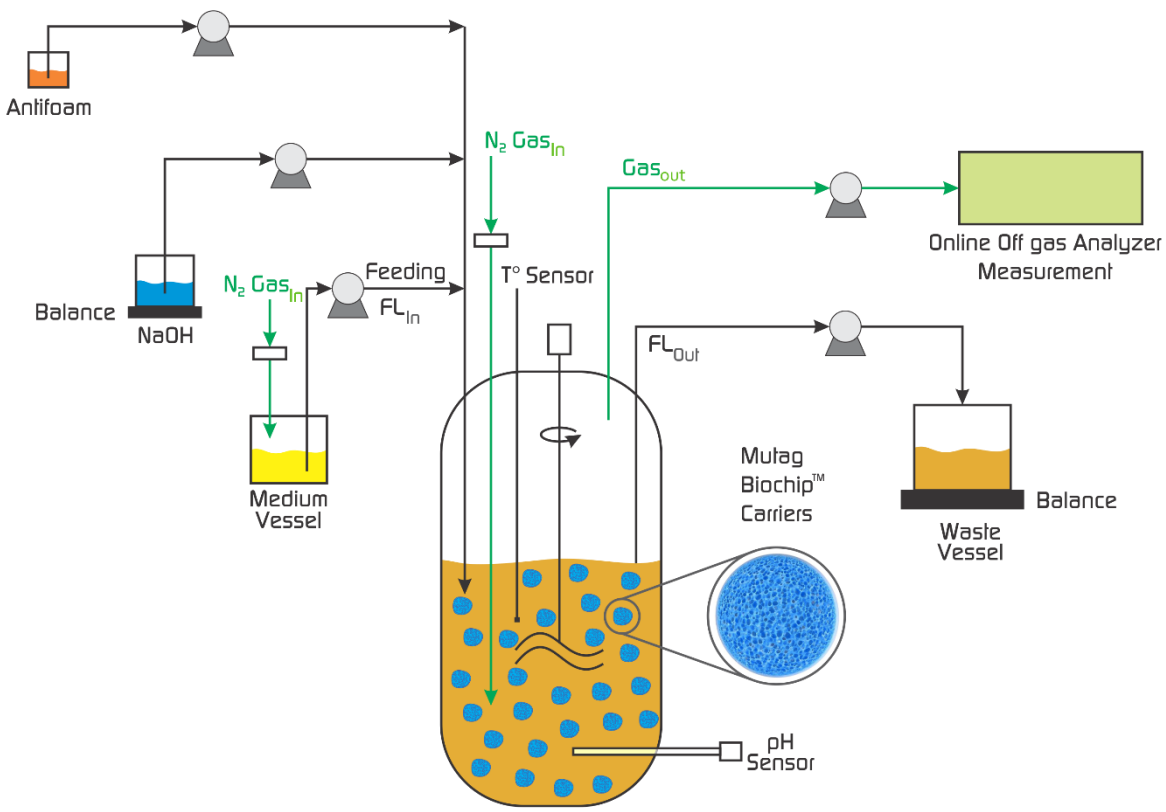

Figure 2. Outline of the chemostat setup equipped with biofilm carriers.

5.2.5 Biofilm harvesting and quantification

The biofilm mass in the reactor was measured at the end of the experiment by sacrificing the fermentation as follows: for the biofilm harvest, all the broth (containing aggregates) was removed from the reactor and subsequently, the biofilm was scraped from the reactor glass wall and weighted for further dry cell weight (DCW) analysis. Then, both biofilm and broth were centrifuged at 5000×g and 10000×g for 5 min at 4 °C, respectively. Subsequently, the biofilm and aggregates obtained as well as the planktonic cell pellets (see section 5.2.4.2) were treated according to the requirements of further studies, as follows: for proteomic analysis, the pellets were washed once with phosphate-buffered saline (PBS), whereas for the remaining pellets were washed once with ultra-pure water. Subsequently, all washed pellets were stored at -80 °C until further analysis.

For the quantification of the total biofilm dry cell weight (DCW), the *Mutag BioChip 25™* carriers were pre-dried at 70 °C and subsequently cooled down at room temperature in desiccators, and weighted before loading them into the

reactor. Likewise, the biofilm on the carriers and wall was harvested and dried at 70 °C and subsequently cooled down at room temperature in desiccators in order to reach a constant weight. The dry weight of the biofilm in the reactor was calculated as the difference between the dry weight of the carriers containing the biofilm and the empty carriers, as well as the dry weight of the biofilm scraped from the reactor wall. The concentration of biofilm in the reactor was calculated as the ratio between the dry weight of the biofilm and the liquid volume in the reactor.

5.2.6 Calculation of net consumption and production rates

Net consumption and production rates of compounds only present in the liquid phase of the chemostat were calculated from their liquid phase mass balances:

$$\frac{d(V \cdot C_i)}{dt} = F_{L,in} \cdot C_{i,in} - F_{L,out} \cdot C_{i,out} + R_i \quad (1)$$

Wherein V is the working volume of the chemostat, $F_{L,in}$ and $F_{L,out}$ are the medium in- and broth outflow rates (L/h), C_i, $C_{i,in}$, and $C_{i,out}$ are the concentrations of compound "i" in the chemostat and the inflows and outflows, respectively (g/L), and R_i is the net conversion rate (production or consumption) of compound "i" (g/h).

For a constant volume steady state chemostat this can be rewritten to give the volumetric conversion rate (g/L/h):

$$r_i = \left(F_{L,out} \cdot C_{i,out} - F_{L,in} \cdot C_{i,in}\right) / V \quad (2)$$

Yields of produced compounds on consumed glucose ($g_i/g_{glucose}$) were calculated as:

$$Y_{i/G} = \frac{r_i}{-r_G} \quad (3)$$

5.2.7 Biofilm characterization

5.2.7.1 Fourier transformation infrared spectroscopy (FT-IR)

The FT-IR spectrum of the lyophilized free cells, aggregate cells, and biofilm cells of *C. saccharoperbutylacetonicum* was recorded on a FT-IR spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with a wavenumber range from 600 cm^{-1} to 4000 cm^{-1}.

5.2.7.2 High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for monosaccharide analysis

Monosaccharide analysis was performed using HPAEC-PAD, as described previously by Felz et al. (2019). In brief, biomass samples corresponding to the pellets of biofilm, aggregates, and planktonic cells (explained in section 5.2.4.2) were lyophilized. Samples were suspended with a concentration of 10 mg/mL in 1 M HCl and hydrolyzed at 105 °C for 8 h in caped reaction tubes. The hydrolyzed samples were centrifuged at 10000×g for 5 min. and the obtained supernatants were neutralized with 1 M sodium hydroxide. The neutralized supernatants were diluted 1:5 with ultrapure water and filtered through a 0.45 µm PVDF filter. Analysis of the samples was conducted using a Dionex ICS 5000+ HPAEC-PAD with an AminoTrap pre-column (Dionex) and a PA20 column (Dionex).

5.2.7.3 Phenol sulfuric acid method for sugar quantification

The saccharides in planktonic cells, aggregates, and biofilm were measured as total sugar equivalents using the phenol sulfuric acid assay (Dubois et al., 1956) in triplicate. The standard used was a sugar mixture containing equal amounts of the neutral sugars: fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose, resembling the sugar composition of previously analyzed EPS, as reported by Felz et al. (2019) which is a more suitable standard than only glucose in sugar EPS analysis. Sugar quantification was performed in triplicate by measuring the absorbance in cuvettes at 482 nm.

5.2.7.4 Bicinchoninic acid (BCA) assay for protein quantification

Proteins in lyophilized planktonic cells, aggregates, and biofilms were measured as Bovine Serum Albumin (BSA) equivalents using the bicinchoninic acid (BCA) method (Interchim Uptima BC assay quantitation kit) as reported by Smith et al. (1985). Standard lines were prepared with BSA in a concentration range of 20 mg/L-1000 mg/L, and the absorbance was measured at 562 nm.

5.2.7.5 Label-free quantification (LFQ) by shot-gun proteomics

Protein extraction. A modified protocol from Hansen et al. was used to prepare whole protein extracts (Hansen et al., 2014). Briefly, approx. 20 mg biomass (wet weight) were lysed using B-PER reagent and bead-beating followed by centrifugation at 14000xg under cooling to collect the protein supernatant. The proteins were precipitated using TCA and incubation in the cold followed by washing twice using ice-cold acetone.

Trypsin digestion. The protein pellet was re-dissolved in 200 mM ammonium bicarbonate containing 6M Urea, reduced in a 10 mM DTT solution and further alkylated using a 20 mM IAA. The solution was diluted to below 1 M Urea and digested using Trypsin at ratio protease to protein of 1:50. Before analysis, peptides were desalted using an Oasis HLB solid-phase extraction sorbent (Waters) according to the manufacturer protocols.

Large-scale shot-gun proteomics. An aliquot corresponding to approx. 300 ng protein digest was analyzed in duplicates using an one dimensional shot-gun proteomics approach (Köcher et al., 2012). Briefly, 1 µL of sample were analyzed using a nano-liquid-chromatography system consisting of an ESAY nano LC 1200, equipped with an Acclaim PepMap RSLC RP C18 separation column (50 µm x 150 mm, 2µm), and a QE plus Orbitrap mass spectrometer (Thermo). The flow rate was maintained at 300 nL/min over a linear gradient from 5% to 30% solvent B over 90 min, and finally to 75% B over 25 min. Solvent A was H_2O containing 0.1% formic acid, and solvent B consisted of 80% acetonitrile in H_2O and 0.1% formic acid.

The Orbitrap was operated in data depended acquisition mode acquiring peptide signals form 350 -1400 m/z, where the top 10 signals were isolated at a window of 2.0 m/z and fragmented using a NCE of 30.

Database search and label-free quantification and visualization. Data were analyzed against the proteome database from Clostridium saccharoperbutylacetonicum N1-4(HMT) (Uniprot, Tax ID: 931276, July 2018) using PEAKS Studio 8.5 (Bioinformatics Solutions Inc) allowing for 20 ppm parent ion and 0.02 m/z fragment ion mass error, 2 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q deamidation as variable modifications. Peptide spectrum matches were filtered against 1% false discovery rate (FDR) and protein identifications with ≥ 2 unique peptides were accepted. Changes in protein abundances between planktonic cells, aggregates and biofilm were further evaluated using the label-free quantification (LFQ) option provided by the PEAKS Q software tool (Bioinformatics Solutions Inc). A pairwise comparison of the above-mentioned conditions was performed on identified peptide spectra filtered against 0% FDR, a mass error equal or less 10 ppm and a maximum RT shift between runs of 2.5 min. Peptides with variable modifications were excluded. The significance method was set to ANOVA with a significance level threshold of ≥15, 1.5-fold change and 2 unique peptides per protein. Data were further visualized in log2 (ratio) volcano plots and hierarchical clustered protein profile heatmaps (Hansen et al., 2017).

5.2.8 Analytical methods for cell growth and extracellular metabolites

During the chemostat cultivation, triplicate samples were taken after reaching a steady-state at each dilution rate. Samples were collected and centrifuged at 5000×g for 5 min and 4 °C. The clean supernatant was transferred into 2 mL microtubes and stored at -20 °C until further analysis. Before analysis all samples were filtered using 0.22-µm Millipore Millex-HV PVDF membrane filters.

The concentrations of the solvents (acetone, *n*-butanol, and ethanol) and glucose were determined by high-performance liquid chromatography (HPLC) using an Aminex ®HPX-87H column (BioRad) coupled to a refractive index (RI) detector (Waters 2414) at 30 °C, and for organic acids (acetic and butyric) coupled to a UV-Vis (Waters 2489) detector at 210 nm. As mobile phase 1.5 mmol/L phosphoric acid in Milli-Q water was used with a flow rate of 0.6 mL/min. The injection volume was 10 µL; run time: 10 min for standards and 50 min for samples.

5.3 Results

5.3.1 *C. saccharoperbutylacetonicum* showed biofilm formation in acidogenic chemostat culture

To promote biofilm formation, *C. saccharoperbutylacetonicum* was grown in chemostat culture whereby the dilution rate was increased stepwise. To increase the surface to volume ratio the reactor was loaded with *Mutag BioChip 25™* carriers. The pH was controlled at 6.5 to keep the fermentation in the acidogenic phase and to facilitate biofilm formation over planktonic cell growth. After a batch phase of 20 h. the reactor was switched to chemostat mode. The initial dilution rate was set at 0.11 h^{-1} and was stepwise increased until 0.41 h^{-1} (**Fig. 3**), approaching the wash-out condition since the $\mu_{max.}$ of this strain was found to be 0.398 h^{-1} using glucose as substrate (Alalayah et al., 2010).

After about 10 days of chemostat cultivation, a visible biofilm layer was formed onto the porous matrix of the *Mutag BioChip 25™* carriers and on the reactor wall. It was thereby showed the ability of *C. saccharoperbutylacetonicum* to form a biofilm for the first time. The fastest biofilm growth was observed between 400 h to 630 h at D = 0.28 h^{-1}. The total concentration of biofilm dry cell weight grown on the carriers and on the wall of the reactor measured at the end of the experiment (after 800 h of cultivation) was 10.71 g DCW/L broth of which 7.47 g corresponded to biofilm grown on the carriers.

5.3.2 Cell growth, glucose consumption, and metabolite production

The time patterns of cell growth, glucose, and metabolite concentrations measured during the chemostat cultivation are shown in **Fig. 3**. It is important to note that the glucose concentration in the chemostat feed was 60 g/L during the first 290 h of the chemostat cultivation ($D = 0.20\ h^{-1}$); and was lowered to 20 g/L thereafter, because of the high concentration of residual glucose. Shortly after the glucose concentration in the feed was decreased to 20 g/L the residual glucose concentration decreased to a low value (0.3 g/L). When the dilution rate was further increased to $D = 0.28\ h^{-1}$ the residual glucose concentration increased again and coincided with a decreased butyric acid concentration, which might have been caused by mass transfer limitation of glucose into the biofilm.

During the entire chemostat cultivation the ABE productivity was low (0.21 g/L/h or less, **Fig. 4**) and the main products were butyric acid and acetic acid with a maximum total production rate of 1.07 g/L/h and 0.39 g/L/h, respectively. These results confirm that the culture was maintained in the acidogenic phase.

The metabolite productivities as a function of the dilution rate during the fermentation are shown in **Fig. 4**. There was a gradual increase in the production rates of all the metabolites, except for ethanol, with increasing dilution rate until $D=0.20\ h^{-1}$ from which the productivities started to decrease. The ethanol production rate was highest at the two highest dilution rates, $D = 0.31$ and $0.41\ h^{-1}$.

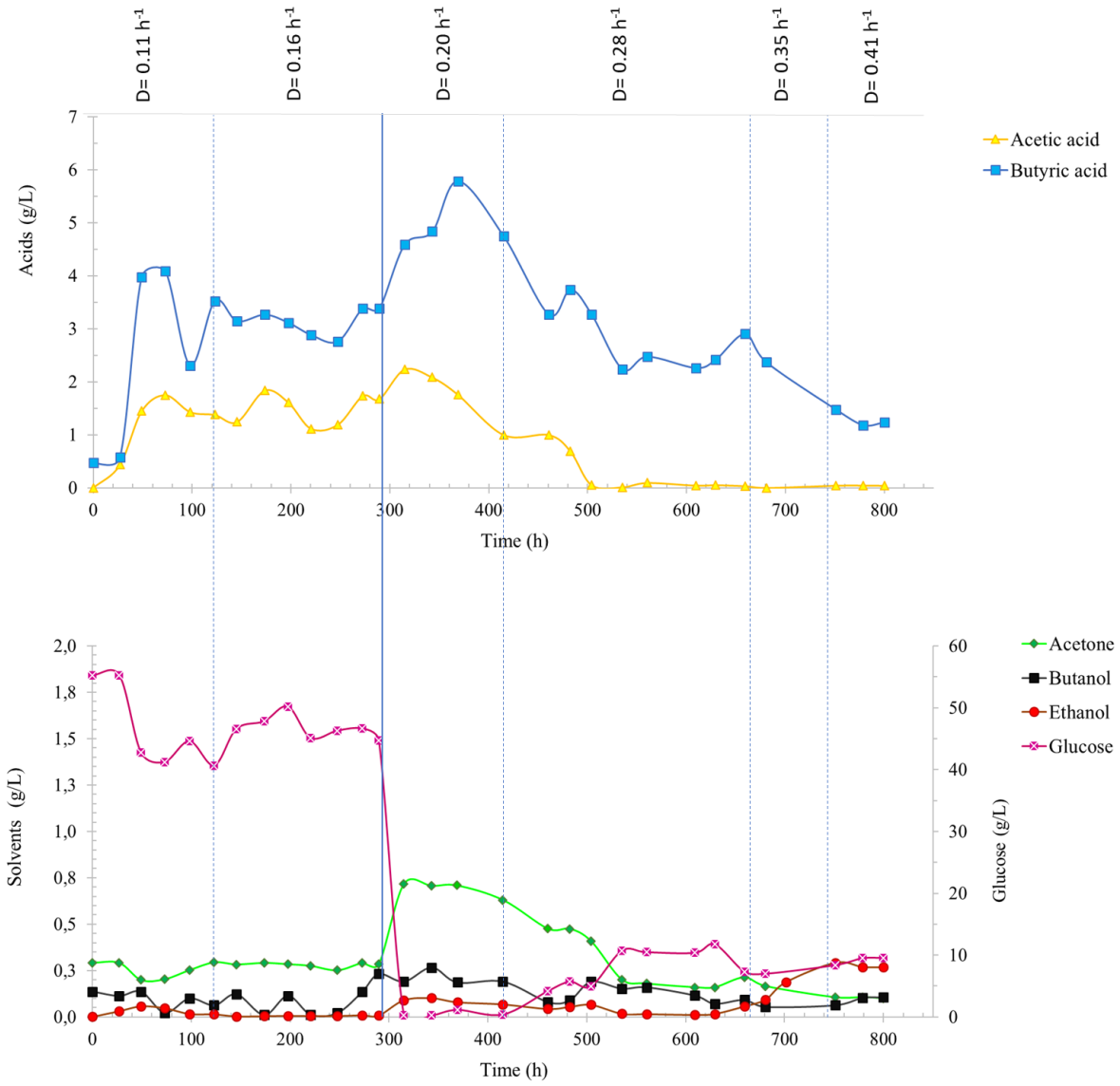

Figure 3. Main data measured during the start-up of the chemostat biofilm reactor under acidogenesis conditions (pH control at 6.5) at different dilution rates (vertical dashed lines). The vertical solid line marks the instant at which the glucose concentration in the feeding was decreased from 60 to 20 g/L.

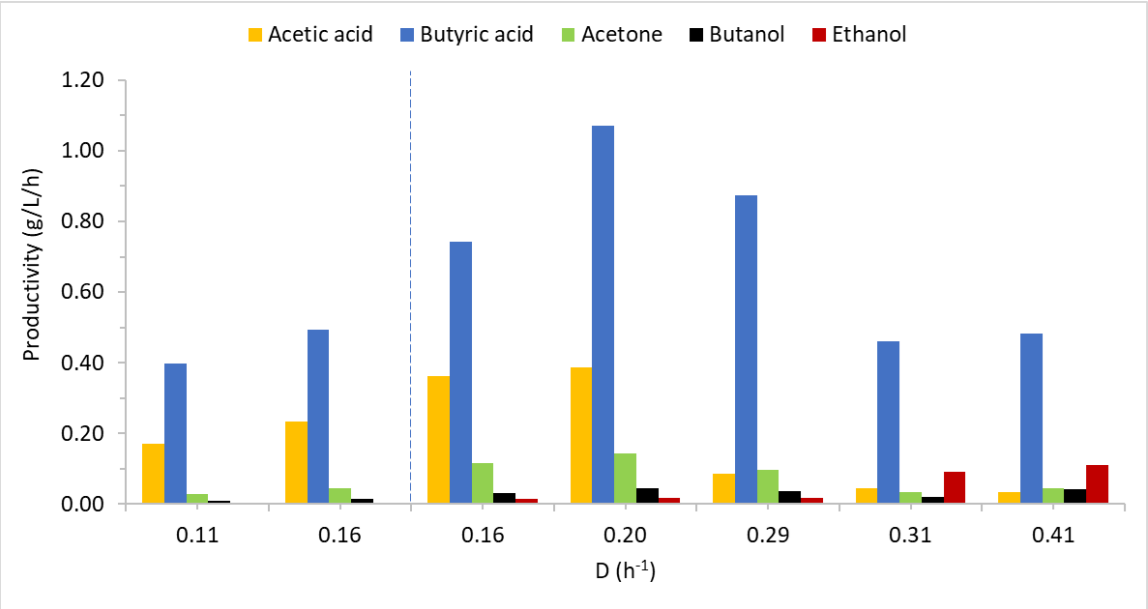

Figure 4. Production rates of metabolites under steady-state conditions of *C. saccharoperbutylacetonicum* using a continuous biofilm reactor under acidogenic conditions. The vertical dashed line marks the instant at which the glucose concentration in the feeding was decreased from 60 to 20 g/L.

5.3.3 Cell morphology

Large morphologic differences between planktonic, aggregates, and sessile cells were observed (**Fig. 5**). At the beginning of the fermentation, during the batch and the first 24 h of continuous cultivation at the initial dilution rate (0.11 h^{-1}), we exclusively observed planktonic cells (**Fig. 5a**). Subsequently, as the fermentation progressed, cells started to attach to the surface and formed aggregates, which remained suspended in the fermentation broth and became coarser and bigger in time compared to the suspended cells (**Fig. 5b**). Finally, the attached cells formed a mature biofilm (**Fig. 5c-d**). It was possible to observe EPS accumulation in the aggregates and also in the sessile cells (**Fig. 5c-d**). Interestingly, some components present in the EPS of the mature biofilm showed autofluorescence which was not detected for the other growth forms (planktonic cells or aggregates).

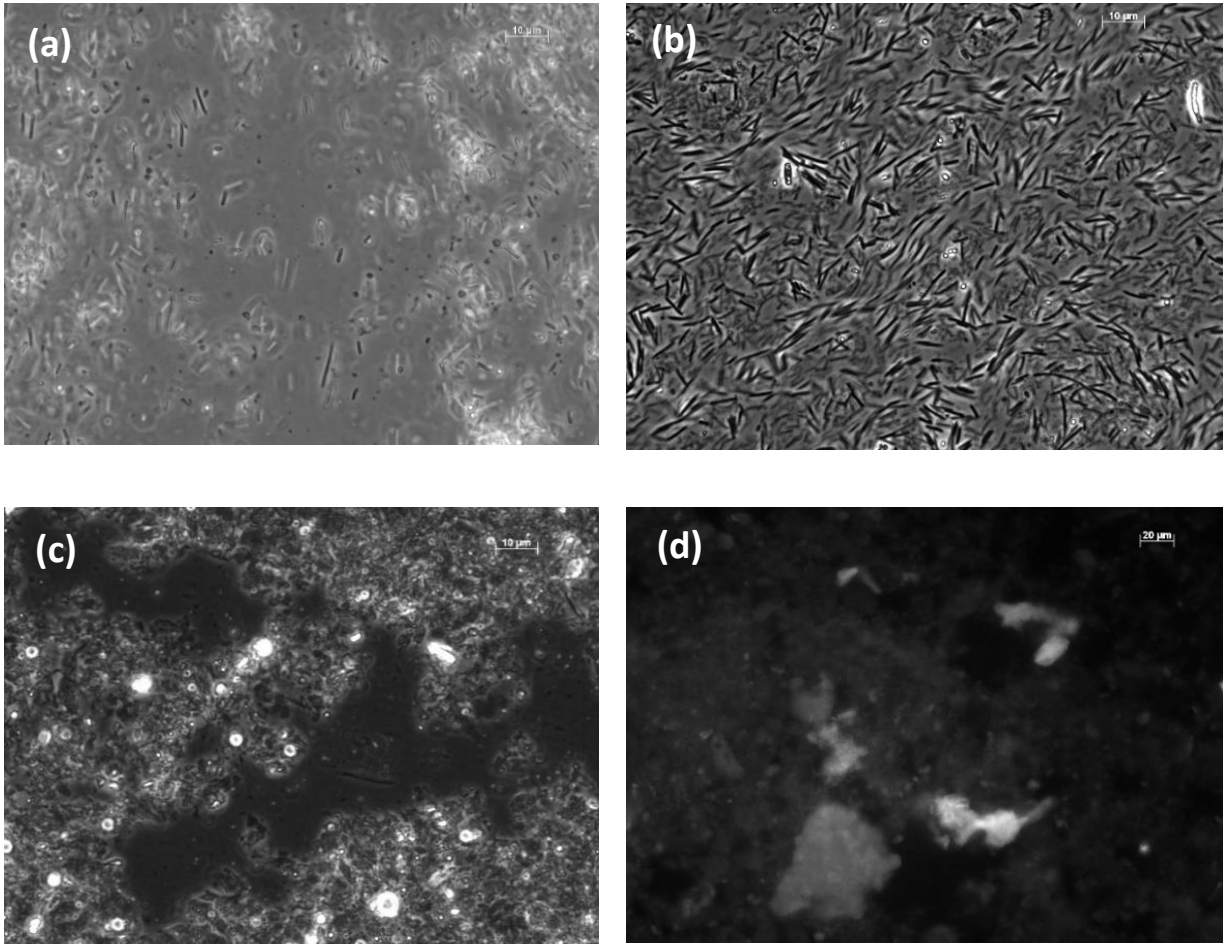

Figure 5. Optical microscope images of the biofilm growth process of *C. saccharoperbutylacetonicum* from planktonic to sessile stage cultivated in continuous conditions. Light microscopy image without autofluorescence of (a) planktonic cells; (b) aggregates cells; (c-d) autofluorescence of the biofilm matrix (excitation wavelength 436 nm and emission wavelength 477 nm).

5.3.4 Biofilm characterization

5.3.4.1 Fourier transformation infrared spectroscopy (FT-IR) of biofilm, aggregates, and planktonic cells

FT-IR is a rapid, nondestructive technique that has been effectively applied to characterize the molecular composition of many biological systems, including biofilms (Koerdt et al., 2011). The potential chemical functional groups within the FT-IR spectrum between 600 cm^{-1} to 4000 cm^{-1} were evaluated for *C. saccharoperbutylacetonicum* cell samples grown either in the biofilm, aggregates, or planktonic lifestyle. FT-IR results provided spectroscopic evidence to support the hypothesis that *C. saccharoperbutylacetonicum* has specific characteristics that distinguish sessile cells from aggregates and planktonic cells.

Fig. 6 depicts a comparative analysis of spectral data of planktonic cells, aggregates, and biofilm of *C. saccharoperbutylacetonicum*. In general, the sharp peak at 3250 cm^{-1} indicates that, the three samples contain large amounts of proteins. Specifically, the biofilm sample is different from the other two samples in: 1) A shoulder peak at 3080 cm^{-1} which appears only in the spectra of the biofilm sample, and is assigned to aromatic –CH groups, indicating the presence of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine. 2) A relatively high peak intensity at around 1547 cm^{-1} in the spectra of biofilm sample which is indicative of the abundance of proteins with side chains. 3) The amid I band in the spectra of the biofilm has peaks at both 1650 cm^{-1} and 1629 cm^{-1}, with the peak intensity at 1629 cm^{-1} slightly higher than that of 1650 cm^{-1}, which indicates that the proteins in biofilm have both α-helices and cross-β sheet secondary structures, while the cross-β sheet structure is more dominant than the α-helices. 4) The peak at 1400 cm^{-1} is much stronger in the spectra of the biofilm than those in the spectrum of aggregates and planktonic cell samples.

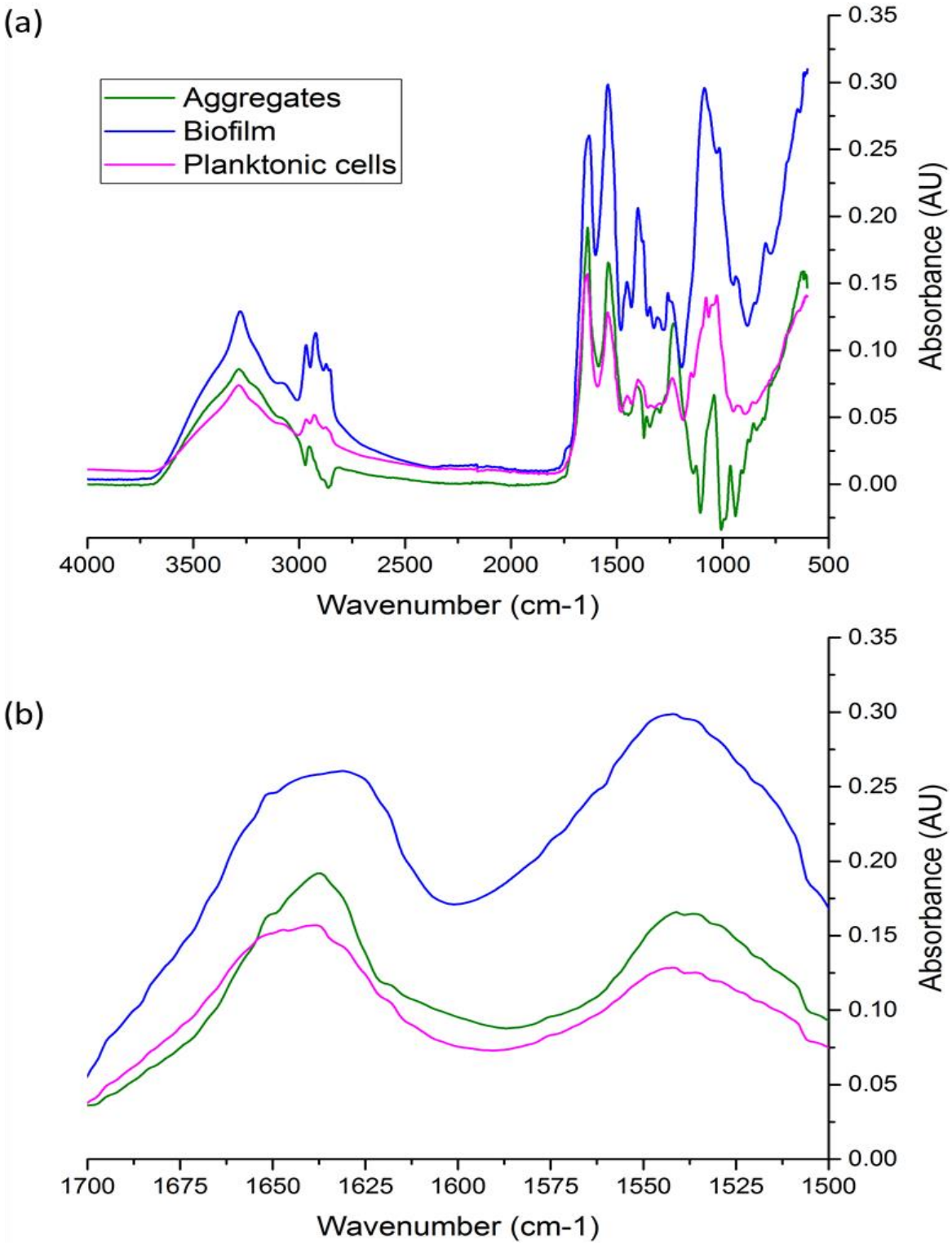

Figure 6. FT-IR spectrum of planktonic cells, aggregates, and biofilm of *C. saccharoperbutylacetonicum*. a) Full-spectrum from 4000 cm^{-1} to 600 cm^{-1}; b) Part of the same FT-IR spectrum as (a) but zoomed in on the region between 1700 cm^{-1} and 1500 cm^{-1}.

5.3.4.2 HPAEC-PAD monosaccharide analysis

Several sugars were found in planktonic cells, aggregates, and biofilm of *C. saccharoperbutylacetonicum* detected by qualitative analysis with HPAEC-PAD analysis. **Fig. 7a** depicts the overlay of the hydrolyzed biofilm, aggregates, and planktonic cells for sugar alcohols, neutral and amino sugars. These results show that biofilm, aggregates, and planktonic cell samples contain one sugar alcohol (glycerol), two amino sugars (glucosamine and galactosamine), and four neutral sugars (rhamnose, galactose, glucose, and ribose). It is interesting to see that xylose and mannose were only found in the biofilm sample, indicating that those sugars are most likely present in the EPS. Mannose could be part of glycoproteins which probably appeared once biofilm formation was initiated. In addition, elution with sodium acetate/sodium hydroxide displayed the absence of galacturonic and glucuronic acids (**Fig. 7b**).

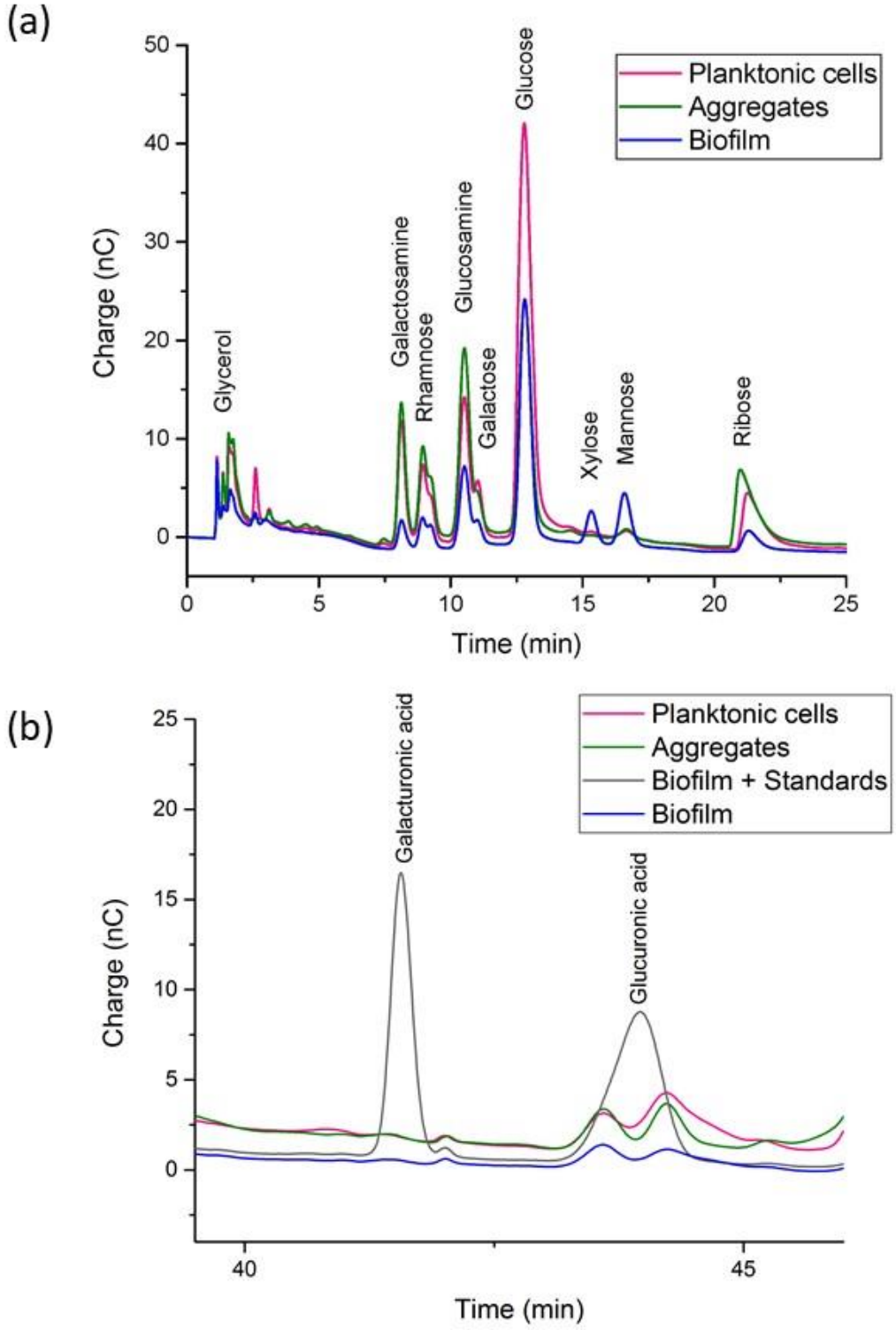

Figure 7. Qualitative analysis of monosaccharides in acid hydrolyzed biofilm, aggregates, and planktonic cells of *C. saccharoperbutylacetonicum* using HPAEC-PAD. Elution with NaOH showed the presence of sugar alcohols, neutral and amino sugars (a), and almost no uronic acids (b).

5.3.4.3 Total sugar and protein quantification

In order to get an impression on the overall amount of sugars in the samples, the phenol sulfuric acid method for total sugar quantification was carried out using a sugar mix as standard. The sugar mixture as standard compound showed a good linearity in the here used concentration range of 5 mg/L - 100 mg/L with a R^2 of 0.9970.

Total protein was quantified with the bicinchoninic acid (BCA) assay using BSA as standard. The BSA standard showed a good linearity in the here used concentration range of 20 mg/L - 1000 mg/L with a R^2 of 0.9959.

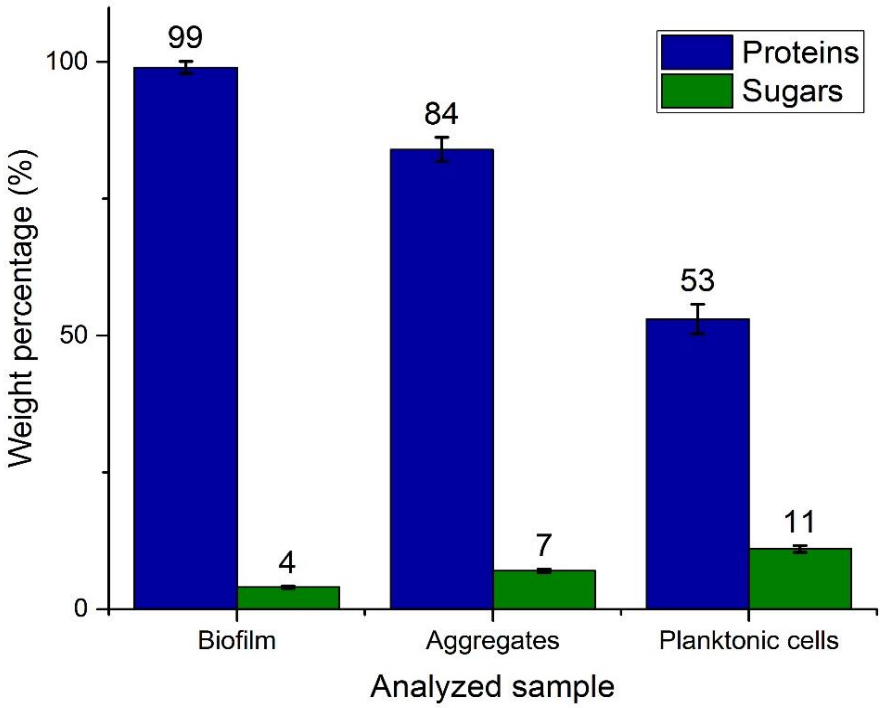

Figure 8. Sugar and protein composition of planktonic, aggregates, and biofilm cells of *C. saccharoperbutylacetonicum* measured as sugar mix equivalent at 482 nm and BSA equivalent at 562 nm, respectively, presented in weight percentage of the organic fraction of the samples.

From the results (**Fig. 8**) it can be seen that the sugar mix equivalent concentrations decreased from a planktonic lifestyle to aggregates into microcolonies, and mature biofilm confirming the results obtained in the HPAEC-PAD analysis. In contrast to this, the total protein quantification showed an opposite trend, with the highest weight percentage of proteins detected in the biofilm. These results show that *C. saccharoperbutylacetonicum* EPS mainly consists of proteins/polypeptides and contains only a very small amount of sugars.

5.3.5 Quantitative proteomics

In total 164 proteins were enriched in the biofilm samples compared to the planktonic cells, of which 124 were identified based on homology (47%) or sequence similarity (53%), and 40 were not further assigned to any function. Of the identified proteins 41 have a metabolic function, 19 are involved in genetic information processing, 29 are membrane transport proteins or components thereof, 24 are related to environmental information processing and motility, 4 are phage related, 3 are membrane/structural proteins, and 1 is related to antibiotic resistance (**Table 1**).

Table 1. Proteins that were more abundant in *C. saccharoperbutylacetonicum* biofilm compared to planktonic cells (**AN:** accession number, **ORF**: open reading frame, **PE**: protein evidence, 3 = protein inferred from homology, 4 = protein predicted).

Function	Protein	AN	ORF	PE
Carbohydrate metabolism	4-alpha-glucanotransferase	M1M820	malQ1	3
	6-phospho-beta-glucosidase AbgA	M1LNI1	abgA1	3
	Acetate kinase	M1MAU4	ackA1	3
	Alpha-amylase	M1MLV9	Cspa_c51730	3
	Butyrate--acetoacetate CoA-transferase subunit A	Q7X4B6	ctfA	4
	Citrate lyase subunit beta	M1MW20	citE1	3
	Iron-containing alcohol dehydrogenase	M1MP80	Cspa_c27830	4
	NAD-dependent aldehyde dehydrogenase	M1MTG7	Cspa_c56880	4
	Probable acetoacetate decarboxylase	Q7X4B4	adc	3
	Protein LacX chromosomal	M1M7H3	lacX	4
	Transketolase subunit B	M1MN25	tktB	4
Energy metabolism	Electron transfer flavoprotein subunit alpha	M1MCN2	etfA2	4
	Electron transfer flavoprotein subunit beta	M1MRE6	etfB2	4
	Glycolate oxidase subunit GlcD	M1M888	glcD1	4
Nitrogen metabolism	Nitrogen fixation protein NifU	M1LQ45	nifU1	4
Sulfur metabolism	Sulfide dehydrogenase subunit alpha	M1MJQ9	sudA1	4
	Thioredoxin	M1MP56	trxA3	3
	Thioredoxin	M1MLZ3	trxA4	3
Lipid metabolism	Cyclopropane-fatty-acyl-phospholipid synthase	M1MTD6	Cspa_c56530	4

Function	Protein	AN	ORF	PE
Nucleotide metabolism	2-polyprenylphenol hydroxylase-like oxidoreductase	M1MYR2	Cspa_c27840	4
	2-polyprenylphenol hydroxylase-like oxidoreductase	M1N603	Cspa_c50710	4
	Cytidylate kinase	M1MGK7	cmk	3
	N5-carboxyaminoimidazole ribonucleotide mutase	M1MEX1	purE	3
	Trifunctional nucleotide phosphoesterase protein YfkN	M1LZ12	yfkN	3
	Xanthine phosphoribosyltransferase	M1MKJ6	xpt	3
Glycine, serine and threonine metabolism	Serine-pyruvate aminotransferase/archaeal aspartate aminotransferase	M1LP68	Cspa_c08580	3
	Phosphoserine aminotransferase	M1M7H9	serC	3
Lysine biosynthesis	Diaminopimelate decarboxylase	M1M0X0	lysA	3
	Phosphoribosyl-ATP pyrophosphatase	M1MKH9	hisE	3
Arginine biosynthesis	Acetylornithine aminotransferase	M1MDV8	argD	3
Histidine metabolism	PHP family phosphohydrolase histidinol phosphatase	M1MPY5	Cspa_c44900	4
Glycan biosynthesis and metabolism	D-alanyl-D-alanine carboxypeptidase	M1MA52	Cspa_c10430	3
	Glycosyltransferase involved in cell wall biogenesis	M1MMN0	Cspa_c54090	4
	S-layer domain-containing protein	M1MCZ9	Cspa_c20080	4
	3D domain-containing protein	M1ME56	Cspa_c24290	3
	Penicillin-binding protein A	M1MN16	pbpA2	4

Function	Protein	AN	ORF	PE
Riboflavin metabolism	6 7-dimethyl-8-ribityllumazine synthase	M1MUB3	ribH	3
	Riboflavin biosynthesis protein RibBA	M1MJY6	ribBA	3
	Riboflavin biosynthesis protein RibD	M1MB11	ribD1	3
Vitamin B6 metabolism	Pyridoxal kinase	M1MJ26	Cspa_c25530	3
Transcription	RNA polymerase sigma factor SigA	M1MIS	rpoD2	3
	NAD-dependent protein deacetylase	M1M157	cobB2	3
Translation	30S ribosomal protein S6	M1N856	rpsF	3
	50S ribosomal protein L11	M1LMF9	rplK	3
	50S ribosomal protein L17	M1MGE0	rplQ	3
	50S ribosomal protein L4	M1MQV3	rplD	3
	50S ribosomal protein L6	M1MGC6	rplF	3
	Putative translation initiation inhibitor yjgF family	M1MW26	Cspa_c19340	4
Folding, sorting and degradation	Leucyl aminopeptidase	M1LYU2	Cspa_c47120	4
	M18 family aminopeptidase	M1N739	apeB	3
	Methionine aminopeptidase	M1N0N2	map2	3
	Peptidyl-prolyl cis-trans isomerase	M1MFX4	rotA	3
	Peptidylprolyl isomerase	M1LMB1	prsA1	4
RNA degradation	Peptidyl-tRNA hydrolase	M1MBX3	pth	3
	Polyribonucleotide nucleotidyltransferase	M1MFG5	pnp	3
	Ribonuclease 3	M1MAV0	rnc	3
	Ribonuclease	M1ML99	Cspa_c49360	3
	Ribonuclease Y	M1MUA3	rny	3
Replication and repair	Anaerobic ribonucleoside-triphosphate reductase-activating protein	M1MBU7	nrdG	3

Function	Protein	AN	ORF	PE
Membrane transport	Cystine-binding periplasmic protein FliY	M1MJ90	fliY1	3
	Energy-coupling factor transporter ATP-binding protein EcfA	M1MC70	cbiO2	3
	Energy-coupling factor transporter transmembrane protein EcfT	M1M7V1	cbiQ1	3
	Histidine-binding protein HisJ	M1MSU3	hisJ	4
	Ion-translocating oxidoreductase complex subunit G	M1MDJ8	rnfG	3
	Leucine- isoleucine- valine- threonine- and alanine-binding protein BraC	M1MNL2	braC	4
	Oligopeptide-binding protein OppA	M1MN88	oppA	4
	Peb1A: major cell-binding factor	M1MBT1	Cspa_c16190	4
	Protein translocase subunit SecY	M1MGD2	secY	3
	Putative D-methionine transport system permease protein MetI	M1MRR9	metI3	3
	Rhodanese-like sulfurtransferase	M1MGF2	Cspa_c32430	4
	RND family efflux transporter MFP subunit	M1LX14	Cspa_c39930	3
	Sodium:dicarboxylate symporter	M1LYM3	Cspa_c46270	3
	Sulfate-binding protein Sbp	M1MPT2	sbp	4
	Sulfate-transporting ATPase	M1MFP4	cysA	3
ABC transporters	Amino acid ABC transporter membrane protein 2 PAAT family	M1MN51	Cspa_c38780	3
	Amino acid ABC transporter substrate-binding protein PAAT family	M1N3A8	Cspa_c41890	4
	Amino acid ABC transporter substrate-binding protein PAAT family	M1MQC6	Cspa_c46350	4
	Amino acid ABC transporter substrate-binding protein PAAT family	M1MMU0	Cspa_c54590	4
	Amino acid/amide ABC transporter substrate-binding protein HAAT family	M1MRD9	Cspa_c35450	4
	Amino acid/amide ABC transporter substrate-binding protein HAAT family	M1M1I7	Cspa_c57590	4
	High-affinity branched-chain amino acid transport ATP-binding protein LivF	M1N1F0	livF1	4

Function	Protein	AN	ORF	PE
Antibiotic resistance	Beta-lactamase domain protein	M1MMG8	Cspa_c53590	4
Multidrug resistance	ABC-type multidrug transport system ATPase and permease component ABC-type multidrug transport system ATPase component ABC-type uncharacterized transport system periplasmic component Cation/multidrug efflux pump Multidrug resistance efflux pump	M1MGS5 M1MSU9 M1MAH2 M1MSS8 M1MCF9	Cspa_c33630 Cspa_c08700 Cspa_c11730 Cspa_c39940 Cspa_c02900	4 4 4 3 4
Phosphotransferase system (PTS)	PTS system D-glucose-specific IID component Man family Phosphotransferase system cellobiose-specific component IIA	M1N5C5 M1MSK4	Cspa_c48790 Cspa_c53780	4 4
Signal transduction	Hemerythrin-like metal-binding protein Hemerythrin-like metal-binding protein Signal peptidase I Stress responsive alpha-beta barrel domain protein	M1MI17 M1LUG5 M1MMU5 M1MT20	Cspa_c22030 Cspa_c29490 lepB3 Cspa_c55080	4 4 3 4
Cellular community - prokaryotes	Lipoprotein	M1N623	metQ3	3

Function	Protein	AN	ORF	PE
Cell motility	Chemotaxis protein chew	M1MUI9	cheW	4
	Flagellar assembly factor FliW	M1MUH5	fliW	3
	Flagellar hook-associated protein 3	M1N4G6	flgL	4
	Flagellar motor switch protein FliG	M1MK52	fliG	4
	Flagellar protein FlbD	M1MUC7	flbD	4
	Flagellar protein FliL	M1LYE3	fliL	3
	Flagellar secretion chaperone FliS	M1N4G3	fliS	3
	Flagellin	M1MK87	hag4	3
	Motility protein B	M1MSY3	motB2	4
	Methyl-accepting chemotaxis protein McpB	M1N6J7	mcpB3	4
	Methyl-accepting chemotaxis protein	M1MCL8	Cspa_c18840	4
	Methyl-accepting chemotaxis protein	M1N2V7	Cspa_c40000	4
	Methyl-accepting chemotaxis protein	M1MKJ8	Cspa_c47110	4
	Methyl-accepting chemotaxis protein	M1MRP6	Cspa_c50670	4
	Methyl-accepting chemotaxis protein	M1MLU7	Cspa_c51580	4
	Methyl-accepting chemotaxis protein	M1MX43	Cspa_c53960	4
	Methyl-accepting chemotaxis protein TlpA	M1MBZ2	tlpA3	4
	Methyl-accepting chemotaxis sensory transducer	M1M9M1	Cspa_c08720	4
	Methyl-accepting chemotaxis sensory transducer	M1MUQ2	Cspa_c46580	4
Phage related proteins	Phage tail sheath protein	M1LNX0	Cspa_c07630	4
	Phage-like element PBSX protein XkdK	M1MI34	xkdK	4
	Phage-like element PBSX protein XkdM	M1MSJ7	xkdM	4
	Tail sheath subunit	M1MNZ2	Cspa_135p00240	4

Function	Protein	AN	ORF	PE
Membrane proteins/structural proteins	Band7 family protein	M1MEW0	Cspa_c11370	4
	Major structural protein	M1M1T6	Cspa_135p00250	4
	Outer membrane protein	M1MKL5	Cspa_c47310	4
Miscellaneous	Radical SAM-superfamily protein	M1MY41	Cspa_c25520	4
	Rubrerythrin	M1MIZ8	Cspa_c10350	4
	Selenium metabolism protein YedF	M1M8Q4	yedF	4
Uncharacterized proteins	UPF0145 protein Cspa_c05950	M1MS21	Cspa_c05950	3
	Putative regulatory protein	M1MAR9	Cspa_c12770	3
	Uncharacterized protein	M1MTX9	Cspa_135p00030	4
	Uncharacterized protein	M1MTZ6	Cspa_135p00180	4
	Uncharacterized protein	M1N8G7	Cspa_135p00670	4
	Uncharacterized protein	M1N8I7	Cspa_135p00820	4
	Uncharacterized protein	M1M7G2	Cspa_c00740	4
	Uncharacterized protein	M1LMM0	Cspa_c02370	4
	Uncharacterized protein	M1LMT5	Cspa_c02970	4
	Uncharacterized protein	M1MI77	Cspa_c08140	4
	Uncharacterized protein	M1MA10	Cspa_c09930	4
	Uncharacterized protein	M1MJA9	Cspa_c11600	4
	Uncharacterized protein	M1MG47	Cspa_c15430	4
	Uncharacterized protein	M1MV03	Cspa_c15720	4
	Uncharacterized protein	M1MG74	Cspa_c15730	4
	Uncharacterized protein	M1MKN0	Cspa_c16010	4
	Uncharacterized protein	M1LRP3	Cspa_c18400	4
	Uncharacterized protein	M1MCN1	Cspa_c18940	4
	Uncharacterized protein	M1MHQ8	Cspa_c20770	4
	Uncharacterized protein	M1LSB6	Cspa_c20790	4
	Uncharacterized protein	M1LTE6	Cspa_c25150	4

Function	Protein	AN	ORF	PE
Uncharacterized proteins	Uncharacterized protein	M1MY88	Cspa_c26020	4
	Uncharacterized protein	M1MEM7	Cspa_c26040	4
	Uncharacterized protein	M1MNU5	Cspa_c26310	4
	Uncharacterized protein	M1MJJ9	Cspa_c27330	4
	Uncharacterized protein	M1MMF0	Cspa_c36370	4
	Uncharacterized protein	M1MRU6	Cspa_c37110	4
	Uncharacterized protein	M1N236	Cspa_c37870	4
	Uncharacterized protein	M1LWP6	Cspa_c38650	4
	Uncharacterized protein	M1MJX9	Cspa_c44560	4
	Uncharacterized protein	M1LY85	Cspa_c44620	4
	Uncharacterized protein	M1LYM0	Cspa_c46220	4
	Uncharacterized protein	M1MLW9	Cspa_c51830	4
	Uncharacterized protein	M1N6W2	Cspa_c53870	4
	Uncharacterized protein	M1M0P5	Cspa_c54000	4
	Uncharacterized protein	M1MX71	Cspa_c54210	4
	Uncharacterized protein	M1N719	Cspa_c54370	4
	Uncharacterized protein	M1MMR7	Cspa_c54390	4
	Uncharacterized protein	M1MSS6	Cspa_c54430	4
	Uncharacterized protein	M1N7S0	Cspa_c56970	4

5.4 Discussion

5.4.1 *C. saccharoperbutylacetonicum* showed biofilm formation during acidogenic chemostat cultivation

This work reveals the first evidence of biofilm composition in terms of the content of EPS and proteomic analysis of sessile cells compared to planktonic cells in any solventogenic *Clostridium* spp. The biofilm formation ability of the wild-type *C. saccharoperbutylacetonicum* was observed for the first time and was successfully obtained during chemostat cultivation under acidogenic conditions.

It is well known that during growth Clostridia pass through two different physiological phases, the acidogenic and solventogenic phase. During the former, the cells grow exponentially, and the substrate is converted into acetic and butyric acids by highly motile cells while the culture pH decreases. After the substrate is depleted the cells enter the solventogenic phase whereby the produced acids are converted into acetone-butanol-ethanol (ABE), and cell division ceases. In continuous or fed-batch cultivations, the cells can be maintained in an actively growing acidogenic state by a continuous supply of substrate and control of the cultivation pH. To keep *C. acetobutylicum* in the acidogenic phase the pH was controlled at either 5.0 (Napoli et al., 2011) or 5.5 (Raganati et al., 2016). We determined from preliminary experiments (data not shown) that a pH of 6.5 was required to keep chemostat cultivations of *C. saccharoperbutylacetonicum* in an actively growing acidogenic state.

As expected, the main products during the acidogenic chemostat cultivation were butyric and acetic acid, while ABE production was almost absent. The total acid (acetic and butyric) yield at the end of the fermentation was 0.12 g acids/g glucose which is lower than that reported by Raganati et al. (2016) and Raganati et al. (2013) who found a total yield of 0.45 $g_{acid}/ g_{glucose}$, and 0.35 $g_{acid}/g_{lactose,}$ respectively, both using *C. acetobutylicum* DSM 792 in packed bed biofilm reactors under acidogenesis conditions at D=0.8 h^{-1}. The difference between the acid yield measured in this study and those reported by the previously mentioned authors is likely due to the different substrates (lactose and cheese whey) and that the strain utilized in these previous studies typically produces more acids than *C. saccharoperbutylacetonicum*.

Regarding the changes in the biofilm morphology, the results of this work show a number of differences with the findings of Zhuang et al. (2016) who observed shorter sessile-cells compared to free-cells of *C. beijerinckii*. It is important to highlight that the transition from free-living cells to the biofilm lifestyle forces microorganisms to undergo a series of different dynamic changes, such as production of secondary metabolites and intensifies the resistance toward biological, chemical, and physical attacks (Costa et al., 2016), facilitated by the embedding of the cells in an EPS matrix. It should be noted that the regulation of EPS production is still poorly understood and is influenced by many factors such as type of microorganism and substrate, nutrient levels, bacterial growth phase, external conditions, among others (Sheng et al., 2010).

5.4.2 Biofilm characterization

This study provided the first evidence of the composition of sessile, aggregates, and planktonic cells of the wild-type *C. saccharoperbutylacetonicum.* Because biofilms are composed of microorganisms and EPS, a thorough molecular characterization of their biomass is essential to elucidate the biofilm structure, stability, and functionality. For this, we combined FT-IR spectroscopy, HPAEC-PAD monosaccharide determination, and total carbohydrate and protein quantification. Combining the obtained results, it can be stated that the biofilm formed by *C. saccharoperbutylacetonicum* is dominated by proteins/polypeptides. It is known that proteins are unique in showing autofluorescence as an intrinsic property among biopolymers which is specifically associated with the aromatic amino acids. Especially tryptophan has been reported as the major fluorophore in proteins with an excitation wavelength higher than 295 nm (Diaspro, 2011; Lin et al., 2018) (**Fig. 5c-d**). Autofluorescence was observed only in the biofilm samples which is clearly shown in **Fig. 5c-d** indicating the likely presence of tryptophan. Subsequent FT-IR analysis confirmed the possible presence of this aromatic amino acid by the shoulder peak at 3080 cm^{-1} shown in **Fig. 6.**

On the other hand, it is worthy to note that although it has been reported that total carbohydrate measurement by the phenol sulfuric acid method is sensitive to the presence of galacturonic acid and humic acid (Felz et al., 2019) it showed

negligible interference to proteins. In the initial publication (Dubois et al., 1956) was reported that the presence of uronic acids results in an increased absorbance intensity using this method. Humic acid and galacturonic acid can result in an overestimation of the measured carbohydrate content. Fortunately, as proved in the HPAEC-PAD analysis, there was no presence of uronic acids neither in the biofilms, aggregates, nor planktonic cell samples analyzed in this study. Therefore, the sugar quantification using the phenol sulfuric acid method for this samples was considered reliable and was corroborated with the HPAEC-PAD and the BCA assay results.

Considering the differences between the biofilm, aggregates, and planktonic cell samples, the content of glycerol, galactosamine, glucosamine, rhamnose, galactose, and ribose was the highest in aggregates and planktonic cells which resulted in a similar pattern, while glucose was significantly higher in planktonic cells. It is important to highlight that xylose and mannose were only found in the biofilm sample, indicating that those sugars are present in the EPS. Similar results showing xylose present in EPS have been reported for some bacteria, such as *Paenibacillus* and *Idiomarina* species (Aguilera et al., 2001; Mata et al., 2008), and for aerobic granular sludge (Felz et al., 2019). Interestingly, Jiao et al. (2010) found that xylose production in EPS is likely a function of biofilm maturation and may be related with lipopolysaccharides. They found this monosaccharide only in EPS from mature and not from a mid-developmental stage of biofilms grown on acid mine drainage which are in agreement with that previously found by Irie et al. (2006) who observed xylose production only in the stationary phase of *Bordetella bronchiseptica* biofilm.

The BCA assay resulted in very high protein values, which likely originated from the principle of this assay as it only represents the measurement of the peptide bond and four amino acids in the samples related to the standard compound, bovine serum albumin. If the composition of the protein in the samples varies from this standard, values in this range can be obtained. The fluorescence of the proteins indicates a high tryptophan content in the biofilm EPS which explains the relatively high protein measurement.

Thus, it can be stated that there is a clear trend that the protein content is higher in the biofilm compared to aggregates with planktonic cells showing the

lowest protein content. Similar observations have been made for anammox biofilms and aerobic granular sludge. Boleij et al. (2018) reported 599 mg proteins/g EPS and 49 mg carbohydrates/g EPS in anammox biofilms. Likewise, Felz et al. (2019) verified a protein dominated EPS extracted from aerobic granular sludge showing 38.1 wt% and 13.8 wt% of BSA equivalent and sugar mix equivalents of the organic mass, respectively.

Additionally, to have a comparison on the amount of sugars obtained after the hydrolysis in comparison to other EPS, an overlay of hydrolyzed ALE with the biofilm and aggregates was carried out (data not shown). Based on these results, in contrast to ALE extracted from aerobic granular sludge reported by Felz et al. (2019), neither aggregates nor biofilm contained fucose.

5.4.3 Proteomic analysis

Proteomics is the large-scale study of proteins and proteomes that enables the identification, characterization, and quantification of proteins and has been applied to study biofilms with success (Köcher et al., 2012; Koerdt et al., 2011). Since bacterial biofilms of any solventogenic Clostridia had never been analyzed until now, this work has elucidated that the transition from a planktonic lifestyle to a sedentary biofilm lifestyle requires the coordinated regulation of genes involved in the biofilm formation of *C. saccharoperbutylacetonicum*.

5.4.3.1 Chemotaxis and cell motility

Remarkably, in the biofilm samples, 19 proteins related to cell motility were more abundant than in the planktonic cells, namely 8 flagella and 11 chemotaxis proteins. This seems counterintuitive as the biofilm cells are immobilized and do not need their motility apparatus anymore. Nevertheless, previous works have shown that the flagellum plays a role in the sensing of surfaces (Belas, 2014), the acceleration of surface adhesion, and biofilm formation in several other bacteria (Karatan and Watnick, 2009). The observation that a mutation in the flagellin gene of *Clostridium difficile* significantly decreased biofilm formation of this organism (Dapa et al., 2013) indicates that this also occurs in Clostridia. From the 11

overexpressed chemotaxis proteins, 10 belong to the methyl accepting class, the most common sensors in bacteria and archaea, which have been reported to be also involved in biofilm formation in *Pseudomonas aeruginosa* (Hickman et al., 2005).

5.4.3.2 Antibiotic resistance

Amongst the 29 membrane transport proteins, 4 multidrug export systems were more abundant in the biofilm cells of *C. saccharoperbutylacetonicum*. Furthermore, a beta-lactamase domain protein was present. These findings indicate that cells of this organism grown in biofilms might have acquired increased antibiotic resistance. Bacteria grown as biofilms have been reported to be up to 1000-fold more resistant to antibiotics than planktonic cells. It has been suggested that this could be attributed to the fact that the cells are embedded in an EPS matrix which hampers the transport of toxic molecules to the cells, phenotypic changes, inactivation of the antibiotics by extracellular polymers or the lower growth rate of biofilm cells (Davey and O'toole, 2000). Recently it has been shown that due to an increased mutation frequency, *Escherichia coli* cells grown as a biofilm accumulated antibiotic-resistant mutants, even in the absence of antibiotics (France et al., 2019).

5.4.3.3 Moonlighting proteins

It has been reported for both gram-positive and -negative bacteria that several intracellular proteins have adhesive moonlighting functions on the cell surface (Amblee and Jeffery, 2015; Kainulainen and Korhonen, 2014). Identification of *C. acetobutylicum* biofilm matrix proteins revealed that the three most abundant proteins were GroEL, a molecular chaperone, the structural surface layer (S-layer) protein, and the oxidative stress protein rubrerythrin (Liu et al., 2018). In this work, it was found that the latter two proteins were also overexpressed in biofilm cells of *C. saccharoperbutylacetonicum*, together with electron transfer flavoprotein subunit beta and flagellin which were in the top 30 of most abundant proteins identified by Liu et al. (2018) in *C. acetobutylicum* biofilm matrix.

5.4.3.4 Proteins putatively related to EPS synthesis

The main components of the extracellular polymeric substances (EPS) of which the biofilm matrix consists are polysaccharides, proteins, lipids, and DNA (Limoli et al., 2015; Seviour et al., 2019). Several proteins related to the synthesis of these polymers were significantly more abundant in the biofilm cells of *C. saccharoperbutylacetonicum*, namely 5 enzymes related to glycan synthesis and metabolism, 6 enzymes of amino acid biosynthesis pathways and 6 enzymes related to nucleotide synthesis.

5.5 Conclusions

Biofilm formation was successfully induced in the hyper-butanol producing *Clostridium saccharoperbutylacetonicum* DSM 14923 for the first time, bringing entirely novel results about the ability of this strain to grow as a biofilm. Also, the first analysis of biofilm composition in terms of extracellular polymeric substances (EPS) of any solventogenic Clostridia spp. was revealed. Hereby, we applied cutting-edge analytical techniques along with quantitative proteomics of sessile and planktonic cells to obtain an in-depth characterization of these biofilms. The fastest biofilm growth was observed at a high dilution rate ($D = 0.28\ h^{-1}$) during chemostat cultivation under acidogenic conditions. Autofluorescence revealed the likely presence of tryptophan. A comparison between planktonic cells, aggregates, and biofilm showed that xylose and mannose were only present in the biofilm sample. Biofilm, aggregates, and planktonic cells contained respectively 4 wt%, 7 wt%, and 11 wt% sugar mix equivalents, and respectively 99 wt%, 84 wt%, and 53 wt% BSA equivalents. Therefore, it was concluded that the biofilm formed by *C. saccharoperbutylacetonicum* is dominated by polypeptides/proteins. A total number of 164 proteins were enriched in the biofilm samples when compared with the planktonic cells, of which 124 were identified, either based on homology (47%) or sequence similarity (53%), and 40 could not be characterized. Most remarkably, a β-lactamase homolog was identified in the biofilm sample, indicating possible antibiotic resistance of biofilm grown cells.

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Chapter

6

Further Discussion, Conclusions, and Outlook

Nowadays, the world´s energy supply is mainly based on fossil sources, i.e., crude oil, mineral coal, and natural gas. Energy from fossil sources has been rethought over the years due to the fact that they are non-renewable, beyond the high price fluctuations of the petroleum barrel in the market, as well as the environmental impacts caused by greenhouse gas (GHG) emissions (Choi et al., 2014). In fact, the early 21st century faced a dramatic increase in petroleum prices. As a result, and along with environmental policy incentives, major investments have been made in biotechnology to develop cleaner fuels from renewable feedstocks (Albers et al., 2016). These investments resulted in significant achievements, and now several equivalent petroleum-based products can be produced from renewable resources (sugars, lignocellulosic materials, waste streams, etc.) via fermentation processes (Biddy et al., 2016), and constant developments are occurring.

Thus, the concept of biorefinery emerged, implying the conversion of all sugars present in the renewable raw materials into several products analogous to fossil refineries (Hasunuma et al., 2013). Biorefineries are classified according to the raw material used as first- (1G), second- (2G), or third-generation (3G). The raw material utilized in biorefineries depends on the geographic location of the industry, for reasons of availability and logistics of transportation, as explained in **Chapter 1.**

Among all bio-based products that can be produced in biorefineries, butanol stands out as an important industrial chemical and superior biofuel due to its features close to gasoline. Since lignocellulosic feedstocks are the most abundant sugar sources in the world, 2G biorefineries using this non-food feedstock appear to be enormously promising for butanol production. Nevertheless, this kind of feedstock requires pre-treatment to remove compositional and structural barriers of lignocellulose, leading to an improvement in the efficiency of hydrolysis to obtain monomer sugars that can be subsequently fermented. However, besides fermentable sugars, inhibitory compounds are also formed during pre-treatment processes.

Sugarcane bagasse from sugarcane mills is one of the main lignocellulosic raw materials produced in Brazil and, therefore, has received considerable attention to produce 2G ethanol (also called cellulosic ethanol). The use of bagasse as raw material has a number of advantages: it is already processed in the mills and available in large quantities; it is low cost, and is ready for use on-site, avoiding increased cost due to transportation (Soccol et al., 2010). Regarding that, a significant fraction of the sugars from the bagasse, the hemicellulosic hydrolysate stream that is pentoses-rich, are not metabolized by the conventional *S. cerevisiae* yeast, its adequate use remains to be addressed. Among several possibilities of using this pentose-rich material as feedstock, the conversion to butanol appears to be suitable. Nevertheless, the application of hemicellulosic hydrolysates as feedstocks has the disadvantage that inhibitory compounds such as phenolic compounds, organic acids, and furan derivatives are present, which negatively affect growth and product synthesis. In view of the challenges of second-generation butanol production becoming economically feasible, this thesis aimed to develop different strategies for acetone-butanol-ethanol (ABE) production using low-cost sugarcane by-products (hemicellulosic hydrolysate and molasses) as carbon sources, with focus on pentoses (C5 sugars) valorization.

In **Chapter 2** of this thesis, it is presented a scientific paper published in May 2019. In this work, it is proved that the strain screening strategy was essential for the identification of the wild-type solventogenic Clostridia with more potential to produce butanol from xylose as carbon source by ABE fermentation. Through this strategy, two strains were found to present high performance in the conversion of glucose and xylose into butanol: *C. saccharoperbutylacetonicum* DSM 14923 and *C. saccharobutylicu*m DSM 13864. Since *C. saccharoperbutylacetonicum* DSM 14923, presented the highest butanol yield among the strains studied, it was selected for further studies, i.e., butanol toxicity tolerance and ABE fermentation using sugarcane molasses mixed with sugarcane bagasse hemicellulosic hydrolysate without any detoxification process.

It is important to highlight the superior butanol tolerance of this strain that was found to lie around 15-17 g/L, compared to other solventogenic *Clostridium* spp. that have a butanol toxicity threshold around 12 g/L, becoming a paramount strain for both the academy and industry. These results highlight the potential of

this strain for further studies, such as exploring its capability to develop even more tolerance to butanol and to the inhibitory compounds present in non-detoxified hemicellulosic hydrolysates, using strategies such as adaptive laboratory evolution (ALE) and metabolic engineering approaches (Liu et al., 2013; Moon et al., 2016) which could bring great advances for the bio-based butanol production.

On the other hand, *C. saccharobutylicum* DSM 13864 showed a similar performance to that of *C. saccharoperbutylacetonicum* DSM 14923. Therefore, conducting an in-depth study of the performance of *C. saccharobutylicum* DSM 13864 in hemicellulosic hydrolysates from sugarcane bagasse should be addressed. Bearing all this in mind, it is highly recommended exploring this strain since it has also shown a high ability to metabolize sugars present in sugarcane straw hydrolysates (Magalhães et al., 2018).

Finally, a great advantage of both strains was identified in this chapter for these both strains, namely *C. saccharoperbutylacetonicum* DSM 14923 and *C. saccharobutylicu*m DSM13864, was the minor production and re-consumption of acids, mainly butyrate. It indicated that butanol was metabolized by an alternative route called "*hot pathway"* in which the synthesis happens via a direct route from acetyl-coenzyme A (CoA) and butyryl-CoA (Jang et al., 2012; Zetty-Arenas et al., 2019). Thus, additional optimization of their metabolism in order to decrease the production of by-products, such as butyric acid, which eventually diverts the carbon that could be used in butanol formation, should be addressed in further research.

Because the feedstock costs represent about two-thirds of the whole bio-based butanol production process (Jiang et al., 2015), its efficient use is of paramount importance. A strategy to achieve this was developed in **Chapter 3** and is based on combining sugarcane bagasse hemicellulosic hydrolysate with sugarcane molasses as carbon source for ABE fermentation. The approach of utilizing all the available sugars from sugarcane, i.e., juice, molasses, and sugars derived from cellulose and hemicellulose, into a portfolio of bio-based products represents the wisest concept of biorefinery. This co-fermentation strategy can circumvent the concentration of the inhibitory compounds present in the sugarcane bagasse hemicellulosic hydrolysate, thus avoiding the prohibitive costs of detoxification steps, as well as increase the sugars in the media by the addition of sugarcane molasses. This process alternative can be successfully implemented in

countries like Brazil, contributing to the establishment of the circular economy concept in the already available sugarcane mills.

Additional process strategies for the feedstock bottleneck can be further studied such as the usage of hemicellulosic hydrolysates obtained in softer pre-treatment conditions without the post-hydrolysis step, in order to test the ability of this strain to consume xylo-oligomers, which would result in the generation of less inhibitory compounds, greater economy due to the reduction of unit operations, as well as a more environmentally friendly process. Likewise, the use of local lignocellulosic raw materials and exploration of alternative pre-treatment methods, as detailed by Morone and Pandey (2014), should be of great value. On the other hand, besides the furans and organic acids studied in **Chapter 3**, the assessment of the phenolic compounds present in the lignocellulosic biomass during ABE fermentation would be of great value to understand their synergistic effect on Clostridia spp. metabolism.

Since high toxicity of butanol is one crucial factor that prevents high butanol titers and thus limits the use of concentrated sugar solutions, *in-situ* product recovery techniques are highly desirable. In **Chapter 4**, it was investigated as a strategy to circumvent the butanol toxicity problem. Hereby the optimum feedstock composition identified in **Chapter 3** (i.e., 75% of SCM and 25% of CHH) was successfully coupled with cyclic vacuum evaporation for *in-situ* product recovery during ABE fermentation. The results showed an increase in ABE productivity as well as a decrease in the fermentation time, 97% depletion of total sugars, and improved cell growth of *C. saccharoperbutylacetonicum,* indicating that the cells were not negatively affected by the vacuum evaporation.

However, a more efficient condensation system able to trap all the ABE solvents, especially acetone (the most volatile compound) is required. This can be achieved by utilizing steel tubing and/or lower temperature in the condenser. It was possible to observe that the use of cyclic vacuum evaporation integrated to ABE fermentation brings design and process gains. Some advantages of this technique are the easy operation and implementation, and the absence of the need for agitation and membrane systems that increase the capital and operating costs. In addition, the produced gases during fermentation facilitate the stripping of the solvents as was also reported by Mariano et al. (2016).

Nevertheless, final decisions about the application of vacuum for ABE fermentations are not definitive, as there is not enough data to support this technique. Vacuum evaporation has the potential to significantly enhance the sugar utilization and productivity of the ABE fermentation, but without an efficient product condensation, the benefits are limited as significant product is lost. Further studies using semi-batch mode could result in an even greater increase in the process productivity.

Finally, one of the great achievements obtained in this thesis was provided in **Chapter 5**. Here, it was revealed for the first time, the ability of *C. saccharoperbutylacetonicum* DSM 14923 to grown in biofilms, as well as provided the first analysis of biofilm composition in terms of extracellular polymeric substances (EPS) and proteomic analysis of sessile cells and aggregates compared to planktonic cells in any solventogenic *Clostridium* spp. It is known that cells in biofilms have shown higher tolerance to stress factors in harsh environments and improvements in butanol production when they are used for continuous ABE fermentation, thus appearing as one of the most useful strategies for large-scale butanol production, as shown in **Chapter 1 (Table 2)** and **Chapter 5.**

In spite of the fact that the understanding of biofilm formation and composition is essential for the design and optimization of biofilm-based processes (Seviour et al., 2019) and that the extracellular polymeric substances (EPS) play a crucial role in the internal protection of cells against environmental stressors, most structural studies involving *Clostridium* spp. have been done only with pathogens or by non-pathogens present in the gut microbiota (Pantaléon et al., 2014). With respect to biofilm formation in solventogenic *Clostridium* spp., researchers have mostly addressed only the application of immobilized cells for enhanced ABE production and butanol tolerance. Actually, the only study focusing on EPS production by solventogenic *Clostridium* spp. so far was carried out by Zhuang et al. (2016), who investigated cellular morphology and fermentative performance analyses. They found increased butanol production by biofilm grown *C. acetobutylicum* CGMCC 5234, in addition to improved tolerance to both butanol and acetic acid when compared to planktonic cells. They called the attention to the recent increased interest in obtaining more understanding about the mechanisms of EPS related to the enhanced tolerance to adverse environmental stress in cells

grown in biofilms. Summarizing the above, it can be stated that the regulation of EPS production in Clostridia spp. is still poorly understood.

Besides the contributions given by this thesis for understanding both the formation and EPS composition of biofilms compared to planktonic cells, further research in this field is relevant. In-depth knowledge of the regulation and composition of EPS matrices and their specific functions will allow developing improved strategies for enhancement or suppression of EPS formation, depending on whether EPS production is advantageous or disadvantageous. For both cases, changing the mechanical properties of biofilms could enhance the process control and resource recovery in the different biofilm-based systems (Seviour et al., 2019).

For instance, it is expected that the findings presented in this study give the basis not only for bio-based butanol production using biofilm reactors containing toxic compounds in the media but also for other fields using multi-species biofilms involving Clostridia. Therefore, additional studies using a multidisciplinary approach for determining the identities and roles (configuration and interactions among constituents) of EPS molecules in biofilms, including the corresponding biochemical, biophysical and 'omic' analysis of biofilms and isolated components as proposed by Seviour et al. (2019) would be of huge value.

Future works deriving from this biofilm investigation can be approached towards an enhanced second-generation butanol production, such as:

- Identify the forty (40) proteins enriched in the biofilm compared to the planktonic cells that were not further assigned to any function.
- Investigate the possible presence of both sialic acids and glycoproteins in the biofilm samples compared to the planktonic cells.
- Thoroughly explore the Beta-lactamase domain protein which is related to antibiotic resistance that was identified in biofilm samples.
- Investigate the tolerance and performance of biofilm after the addition of inhibitory compounds such as organic acids, furans and phenolic compounds commonly present in hemicellulosic hydrolysates, as well as the use of sugarcane bagasse hemicellulosic hydrolysate as carbon source during a batch, feed-batch, and continuous ABE fermentation.

Finally, as an overall overview, as a strategy towards making the ABE fermentation process economically feasible, it is suggested continuing exploring the

usage of low-cost renewable feedstocks such as sugarcane by-products, in an intensified process, using two or more consecutive fermenters with biofilms to split the two phases, namely acidogenesis and solventogenesis. The latter could be integrated into a product recovery system and maintained at a lower dilution rate since the coupled product recovery will avoid the cells to be exposed to high butanol concentrations. In parallel to this, the development of robust *C. saccharoperbutylacetonicum*, able to tolerate higher inhibitory compounds concentration and higher butanol concentration, has demonstrated strong potential to contribute consolidating the economic viability of the whole process.

Remarkably, the potential and robustness of *C. saccharoperbutylacetonicum* were evidenced in this thesis. The impact of butanol production using biofilms and vacuum product recovery on the bioenergy sector as well as the importance of using sugarcane bagasse hemicellulosic hydrolysate (renewable, low-cost and abundant substrate), were highlighted as strategies to partly solve the problem of the economic viability of second-generation ABE fermentation. Nevertheless, challenges such as low yield and energy-intensity of the recovery process need to be addressed in future investigations to make second-generation butanol production on the industrial scale become reality.

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«If you want to go fast, go alone
If you want to go far, go together.»

African Proverb

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Ana María Zetty Arenas

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Curriculum Vitae

Curriculum Vitae

Ana Maria Zetty Arenas was born on 25th March 1986 in Palmira, Valle, Colombia. In 2003, she graduated with honors from Liceo Sagrada Familia, where she performed her high-school studies. In the same year, she enrolled in the B.Sc. program in Agroindustrial Engineering at Universidad Nacional de Colombia (UNAL) in Palmira. In 2008, she graduated with distinction, receiving an *Honor Degree*.
During her bachelor studies, she was a beneficiary of a loan-grant provided by the Universidad Nacional de Colombia, which was fully condoned in reason of the award obtained.

During her bachelor's degree, she performed a project on the effect of 1-methylcyclopropene on the physical and chemical properties of yellow pitaya. During this time, she also enrolled in a research group on Energy Efficiency and Alternative Energies (GEAL) carrying out a study of ethanol production from tropical sugar beet.

In 2010, she moved to Brazil and started her M.Sc. Degree in Chemical Engineering at Escola Politécnica da Universidade de São Paulo (USP). She graduated in 2012 with a project focused on the development of cassava starch-based biofilms as potential indicators of pH change. From 2012 to 2013, she moved back to her home country, Colombia, to work as a research assistant studying the bioethanol production from cassava, at CLAYUCA in the International Center for Tropical Agriculture (CIAT).

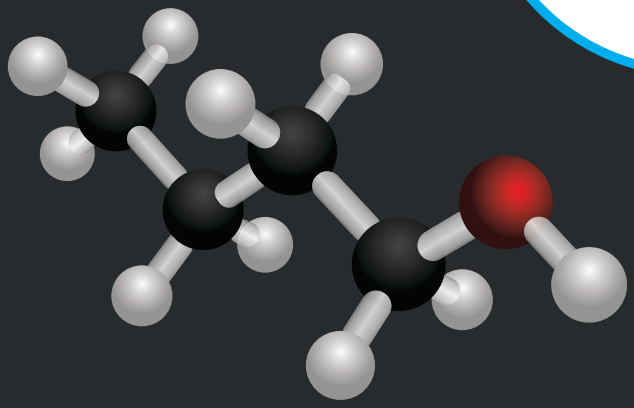
In 2014, she decided to move to Brazil again to start her Doctoral degree in the Bioenergy Ph.D. program at the Universidade de Campinas (UNICAMP), in the context of an agreement on joint doctoral supervision between UNICAMP and Delft University of Technology (TU Delft), the Netherlands. In Brazil, she conducted a large part of her doctoral thesis in the Brazilian Center for Research in Energy and Materials (CNPEM) in the CTBE laboratory, currently called Brazilian

Biorenewables National Laboratory (LNBR), and also in the School of Chemical Engineering, UNICAMP, under supervision of Dr. Sindelia Freitas and Prof. Dr. ir. Rubens Maciel Filho. In 2017, she moved to the Netherlands to join to the Environmental Biotechnology (EBT) group, Department of Biotechnology, Faculty of Applied Sciences, TU Delft, under the supervision of Dr. Walter van Gulik and Prof. Dr. ir. Mark van Loosdrecht. The results of this work are described in this thesis.

Biofuels

Pharmaceuticals

Paints



n-Butanol Applications

Rubbers

Cosmetics

Other Chemicals