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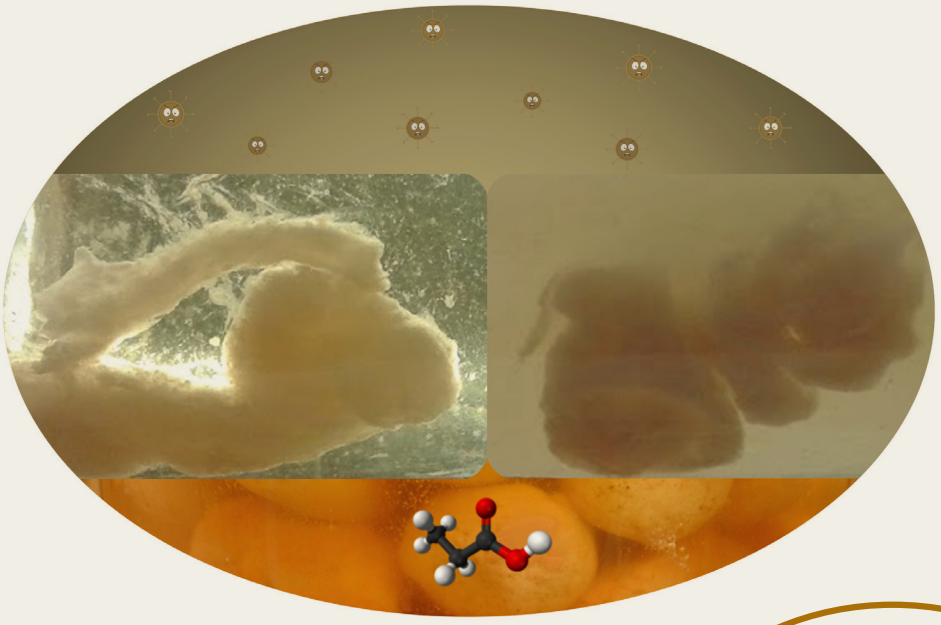

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Abstract

Propionic acid (PA) is a naturally occurring short chain fatty acid produced by microbial fermentation of the indigestible foods in the intestine, having a positive effect on the gastrointestinal health. PA inhibits the growth of pathogenic bacteria and molds; hence it is widely used as preservative for food, feed and grains. It is also used as a component in several industrial products like polymers, cosmetics and pharmaceuticals. Its industrial production still depends on petrochemistry, however microbial fermentation as a route for production from renewable resources has attracted attention but is not yet competitive with the existing route. Dairy propionibacteria, used as starters for the maturation of Swiss-type cheeses and also known for their probiotic properties, produce propionic acid from different sugars or glycerol via Wood-Werkman cycle under anaerobic conditions. The fermentation process has a long lag phase and is also subject to product inhibition, resulting in low productivity and yield.

This thesis involves studies on high cell density fermentations and development of acid tolerant strain as ways to improve the efficiency of propionic acid fermentation. Studies have been performed using Propionibacterium acidipropionici DSM 4900 (ATCC 25562), a bacterium that is generally regarded as safe (GRAS). Glycerol, a by-product of biodiesel production process, was used as the carbon source for fermentations.

Different strategies of high cell density fermentations including sequential batch cultivation, cyclic batch cultivation, cell immobilization and cell retention using membrane filter, were evaluated. Propionate yield and productivity of 93 mol % and 0.53 g/L/h were obtained in cyclic batch while maximum productivity of 1.63 g/L/h was obtained from 90 g/L glycerol during sequential batch cultivations with free cells. P. acidipropionici cells were immobilized on plastic based AnoxKaldnes® and recycled glass based Poraver® carriers, either by polyethyleneimine (PEI) treatment or by biofilm formation. Exposing the cells to stress factors like 30 mM citric acid and 1 M NaCl was beneficial for increasing the Biofilm Forming Capacity index (BFC) and for production of exopolysaccharides (EPS), both indicators of biofilm formation. Fermentations in the immobilized packed-bed bioreactors using the bacteria pre-exposed to citric acid and NaCl gave propionic acid productivities of 0.7 and 0.78 g/L/h, respectively, with Poraver® matrix, and the corresponding values for AnoxKaldnes® were 0.39 and 0.43 g/L/h, respectively. Continuous propionic acid fermentations by cell retention in a ceramic membrane filter, gave productivity of 2.35 g/L/h in a medium with 60 g/L glycerol and 20 g/L yeast extract.

A tolerant strain able to grow in a culture medium supplemented with 20 g/L of PA, was obtained by adaptive laboratory evolution. It exhibited 1.4-fold higher growth rate, 3.7-fold higher propionic acid production kinetics, 1.2-fold higher yield and almost 2-fold higher titer in batch cultivations compared to the parental cells in a basal cultivation medium. It was immobilized to PEI-treated Poraver® and used for fermentations in recycled batch mode with increasing glycerol concentration and decreasing pH, respectively, in packed-bed bioreactors. Glycerol up to a concentration of 100 g/L was completely consumed, resulting in 58 g/L propionic acid and product yield of 0.64 mol/mol. Progressive decrease in pH values gave increased propionic acid yield and productivity from 20 g/L glycerol, while maintaining the product titer nearly constant at pH 5.0.

The exposure to stress factors for biofilm formation and to high propionic acid concentration during cultivation of P. acidipropionici showed trehalose to play a protective role. Following the expression of the trehalose synthesis-related genes treY and otsA, and by Real Time-qPCR revealed treY expression (but not otsA) to be significantly increased during biofilm formation under the influence of citric acid. Expression of the biofilm-related luxS gene was also detected by fluorescence in situ hybridization (FISH). Expression of treY was increased 82-fold in the tolerant strain obtained by adaptive evolution with respect to the 16S rRNA gene used as a control.

Key words Biorefinery; platform chemical; fermentation; propionic acid; *Propionibacterium acidipropionici*; high cell density; cyclic/sequential batch fermentation; immobilization; biofilm formation; stress conditions; trehalose production; adaptive evolution; acid tolerance; cell retention fermentation; membrane-based fermentation.

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A la memoria del ser que me dio la vida y siempre apostó por mi formación: Paqui. Al niño que ilumina mis días: Míkael…

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Abstract

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Popular Summary

Propionic acid is a short chain fatty acid currently produced via chemical synthesis from fossil-based resources. Because of its inhibitory effect on molds and many bacteria, it is commonly used for preservation of foods, especially bread and other baked goods, feed and grains, and also as herbicide. Other important applications of propionic acid are as ingredients in plastics and pharmaceuticals. The industrial production of propionic acid, estimated to be over 400 000 metric tons globally, depends on petroleum resources that undergo chemical refinement and synthesis with procedures that are not environmentally friendly. Its growth by 2026 is expected to be over 500 000 metric tons.

Propionic acid is also produced by microorganisms in the intestines fermenting the indigestible foods; the short chain fatty acids formed possess positive effects on the gastrointestinal health. Several microorganisms produce propionic acid via fermentation of different sugars like glucose, xylose, and maltose. Among the important ones are dairy propionibacteria that are used as starter cultures for maturation of Swiss-type cheeses. Propionibacteria are also attracting attention for their promising probiotic features, which are partly attributed to the production of propionic acid. Using propionibacteria for the industrial production of propionic acid from renewable resources would thus provide an environmentally benign alternative to the current fossil-based production. However, for this to be realized, there is a need to overcome the bottleneck posed by the inhibitory effect of propionic acid on the bacteria, especially at high concentrations, that results in low yields and productivity, and high costs. This is possible to be achieved by developing a robust process based on the use of large amount of cells that can be recycled and also evolving the cells by adaptation to high propionic acid concentration.

This thesis studies different ways of the so-called high cell density fermentations using wild type as well as adapted bacteria for propionic acid production. *Propionibacterium acidipropionici*, a bacterium generally regarded as safe (GRAS) was used in the study and glycerol was used as the renewable carbon source. Glycerol is a favorable substrate for many propionibacteria due to its more reduced state in comparison with traditional carbohydrates. Glycerol is formed as a side product in processes like biodiesel production from oil. Use of biodiesel as renewable fuel has led to the increase in the availability of glycerol and its market size is expected to exceed USD 3 billion in 2022. While glycerol finds a number of applications in cosmetics, chemicals and pharmaceutical industries, its potential use

as substrate in fermentation has been investigated with promising results. The main products obtained by glycerol fermentation are glyceric-, propionic- and 3-hydroxypropionic acids, all of them produced by *Propionibacteria*. The main advantage of using glycerol as the sole carbon source for production of propionic acid is the absence of acetic acid as byproduct, a desired feature that certainly reduces costs of product recovery.

A fermentation process normally involves inoculating a growth medium with a bacterial culture, growing the cells until the cells stop growing or when maximum amount of product is formed, and is followed by separating the cells and the product. Usually the product formed leads to inhibition of cell growth as well as process efficiency. The fermentation time can be reduced by retaining the bacterial cells in the bioreactor, e.g. by collecting the cells after the fermentation, returning them to the bioreactor for using in subsequent fermentation cycles. A strategy frequently used to retain the cells in the bioreactors is immobilization on solid materials called supports or carriers. This allows cells to remain in the bioreactor in a "ready to work" state for several rounds of production instead of starting every fermentation with new inoculum. Different ways of immobilization have been developed with the use of different materials, polymers and chemicals that mediate the adhesion of the cells to a support. A polymer that is highly effective in attaching the cells to a solid surface is the positively charged polyethyleneimine (PEI) through attraction to the negatively charged cell surface. However, chemical mediated immobilization, besides adding to the cost, may also result in an undesired inhibitory effect on the cells. On the other hand, several bacteria have the natural capacity to produce extracellular matrix polymers, also called biofilm, that aids the cells in binding to solid surfaces. Since *Propionibacterium acidipropionici* was not previously reported as a biofilm forming bacteria, stressful conditions for growing the bacteria were applied to induce the formation of extracellular matrix. Citric acid and sodium chloride were found as good stress factors for the cells to induce the biofilm formation.

Solid materials based on plastic (AnoxKaldnes®) and recycled glass (Poraver®) were used as supports for cell immobilization, either by biofilm formation or by the treatment with PEI. The cells immobilized through PEI gave higher product yields and production rate. As an alternative way to establish high cell density fermentations, cell-retention by the use of a ceramic membrane filter was also studied. Operating the reactor in a continuous mode while recycling the cells retained by the membrane, relieved the inhibitory effect of the propionic acid product to some extent. The membrane reactor was operated continuously for a period of two months.

Finally, the possibility of developing a *P. acidipropionici* strain capable of tolerating higher concentration of propionic acid and acidic conditions was investigated by adapting the wild type bacteria in a culture medium with increasing concentrations of propionic acid. A strain that was adapted to grow with 20 g/L of propionic acid

added to the medium showed good tolerance and enhanced rate of product formation. The tolerant strain immobilized to PEI-treated Poraver® could convert glycerol at concentration as high as 160 g/L to propionic acid. The strain was also active at pH down to 5. The robust system with the tolerant strain was run for more than two months with stable performance in the repeated fermentations.

As a result of the stressful conditions applied to induce the biofilm formation by *P. acidipropionici*, and for developing the tolerant strain at higher propionic acid concentration, the bacteria were found to express genes related to the production of trehalose, a sugar known for increasing the stability of cells and biomolecules. This study thus provides basis for further work on investigating the changes in *P. acidipropionici* cells at the genetic level when exposed to the stress environments and also for continuing further work on efficient bioprocess for propionic acid production.

Populär sammanfattning

Propionsyra är en kortkedjig fettsyra som för närvarande produceras via kemisk syntes från fossilbaserade resurser. På grund av dess hämmande effekt på mögel och många bakterier, används den ofta för konservering av livsmedel, särskilt bröd och andra bakverk, foder och spannmål, och även som herbicid. Andra viktiga tillämpningar av propionsyra är som ingredienser i plaster och läkemedel. Den industriella produktionen av propionsyra, som uppskattas till över 400 000 ton globalt, är beroende av petroleumresurser som genomgår kemisk förädling och syntes med förfaranden som inte är miljövänliga. Dess tillväxt till 2026 förväntas vara över 500 000 ton.

Propionsyra produceras också av mikroorganismer i tarmarna som jäser den svårsmälta maten; de kortkedjiga fettsyrorna som bildas har positiva effekter på mag-tarmhälsa. Flera mikroorganismer producerar propionsyra via fermentering av olika sockerarter som glukos, xylos och maltos. Bland de viktiga är mejeripropionibakterier som används som startkulturer för att mogna ostar av schweizisk typ. Propionibakterier väcker också uppmärksamhet för sina lovande probiotiska egenskaper, som delvis tillskrivs produktionen av propionsyra. Att använda propionibakterier för industriell produktion av propionsyra från förnybara resurser skulle därmed utgöra ett miljövänligt alternativ till den nuvarande fossilbaserade produktionen. Men för att detta ska förverkligas finns det ett behov av att övervinna flaskhalsen som propionsyrans hämmande effekt på bakterierna utgör, speciellt vid höga koncentrationer, vilket resulterar i låga skördar och produktivitet och höga kostnader. Detta är möjligt att uppnå genom att utveckla en robust process baserad på användningen av stora mängder celler som kan återvinnas och även utveckla cellerna genom anpassning till hög propionsyrakoncentration.

Detta examensarbete studerar olika sätt för de så kallade högcellstäthetsfermentationerna med hjälp av vildtyp såväl som anpassade bakterier för propionsyraproduktion. Propionibacterium acidipropionici, en bakterie som allmänt anses vara säker (GRAS) användes i studien och glycerol användes som den förnybara kolkällan. Glycerol är ett gynnsamt substrat för många propionibakterier på grund av dess mer reducerade tillstånd jämfört med traditionella kolhydrater. Glycerol bildas som en biprodukt i processer som biodieselproduktion från olja. Användning av biodiesel som förnybart bränsle har lett till ökad tillgång på glycerol och dess marknadsstorlek förväntas överstiga 3 miljarder USD 2022. Medan glycerol hittar ett antal tillämpningar inom kosmetika-, kemi- och

läkemedelsindustrin, är dess potentiella användning som substrat i fermentering har undersökts med lovande resultat. De viktigaste produkterna som erhålls genom glyceroljäsning är glycerol-, propion- och 3-hydroxipropionsyror, alla producerade av Propionibacteria. Den största fördelen med att använda glycerol som den enda kolkällan för produktion av propionsyra är frånvaron av ättiksyra som biprodukt, en önskad egenskap som säkerligen minskar kostnaderna för produktåtervinning.

En jäsningsprocess innefattar normalt inokulering av ett tillväxtmedium med en bakteriekultur, odling av cellerna tills cellerna slutar växa eller när maximal mängd produkt bildas, och följs av separering av cellerna och produkten. Vanligtvis leder den bildade produkten till hämning av celltillväxt såväl som processeffektivitet. Fermentationstiden kan minskas genom att bakteriecellerna kvarhålls i bioreaktorn, t.ex. genom att samla upp cellerna efter fermenteringen, återföra dem till bioreaktorn för användning i efterföljande fermenteringscykler. En strategi som ofta används för att hålla kvar cellerna i bioreaktorerna är immobilisering på fasta material som kallas stöd eller bärare. Detta gör att cellerna kan förbli i bioreaktorn i ett "redo att arbeta" tillstånd under flera produktionsomgångar istället för att starta varje fermentering med nytt inokulum. Olika sätt för immobilisering har utvecklats med användning av olika material, polymerer och kemikalier som medierar vidhäftningen av cellerna till ett underlag. En polymer som är mycket effektiv för att fästa cellerna till en fast yta är den positivt laddade polyetyleniminen (PEI) genom attraktion till den negativt laddade cellytan. Emellertid kan kemisk förmedlad immobilisering, förutom att öka kostnaden, också resultera i en oönskad hämmande effekt på cellerna. Å andra sidan har flera bakterier den naturliga förmågan att producera extracellulära matrispolymerer, även kallade biofilm, som hjälper cellerna att binda till fasta ytor. Eftersom Propionibacterium acidipropionici inte tidigare rapporterats som en biofilmbildande bakterie, användes stressiga förhållanden för att odla bakterierna för att inducera bildningen av extracellulär matris. Citronsyra och natriumklorid visade sig vara bra stressfaktorer för cellerna att inducera biofilmbildning.

Fasta material baserade på plast (AnoxKaldnes®) och återvunnet glas (Poraver®) användes som stöd för cellimmobilisering, antingen genom biofilmbildning eller genom behandling med PEI. Cellerna immobiliserade genom PEI gav högre produktutbyten och produktionshastighet. Som ett alternativt sätt att etablera fermentationer med hög celldensitet studerades även cellretention med användning av ett keramiskt membranfilter. Att driva reaktorn i ett kontinuerligt tillstånd samtidigt som de celler som kvarhålls av membranet återfördes, lindrade i viss utsträckning den hämmande effekten av propionsyraprodukten. Membranreaktorn drevs kontinuerligt under en period av två månader.

Slutligen undersöktes möjligheten att utveckla en P. acidipropionici-stam som kan tolerera högre koncentrationer av propionsyra och sura förhållanden genom att anpassa vildtypsbakterierna i ett odlingsmedium med ökande koncentrationer av propionsyra. En stam som var anpassad att växa med 20 g/L propionsyra tillsatt till

mediet visade god tolerans och ökad hastighet för produktbildning. Den toleranta stammen immobiliserad till PEI-behandlad Poraver® kunde omvandla glycerol i koncentrationer så höga som 160 g/L till propionsyra. Stammen var också aktiv vid pH ner till 5. Det robusta systemet med den toleranta stammen kördes i mer än två månader med stabil prestanda i de upprepade fermentationerna.

Som ett resultat av de stressande förhållanden som används för att inducera biofilmbildning av P. acidipropionici, och för att utveckla den toleranta stammen vid högre propionsyrakoncentration, visade sig bakterierna uttrycka gener relaterade till produktionen av trehalos, ett socker känt för att öka stabilitet hos celler och biomolekyler. Denna studie ger således underlag för fortsatt arbete med att undersöka förändringar i P. acidipropionici-celler på genetisk nivå när de utsätts för stressmiljöer och även för att fortsätta arbetet med effektiv bioprocess för propionsyraproduktion.

Resumen popular

El ácido propiónico es un ácido graso de cadena corta que actualmente se produce mediante síntesis química a partir de recursos fósiles. Debido a su efecto inhibidor sobre mohos y muchas bacterias se usa comúnmente para la preservación de alimentos, especialmente pan y otros productos horneados, forraje y cereales, así también como herbicida. Otras aplicaciones importantes del ácido propiónico incluyen su uso ingrediente en la producción de plásticos y productos farmacéuticos. La producción industrial de ácido propiónico, estimada en más de 400 000 toneladas métricas a nivel mundial, depende de los recursos petrolíferos que se someten a refinamiento químico y síntesis con procedimientos que no son amigables con el medio ambiente. Se espera que su crecimiento para 2026 supere las 500 000 toneladas métricas.

El ácido propiónico también es producido por microorganismos que fermentan los alimentos no digeribles en los intestinos; los ácidos grasos de cadena corta formados poseen efectos positivos sobre la salud gastrointestinal. Varios microorganismos producen ácido propiónico a través de la fermentación de diferentes azúcares como glucosa, xilosa y maltosa. Entre los más importantes se encuentran las propionibacterias lácteas que se utilizan como cultivos iniciadores (*starters*) para la maduración de quesos tipo suizo. Las propionibacterias también están captando atención por sus prometedoras propiedades probióticas, mismas que se atribuyen en parte a la producción de ácido propiónico. El uso de propionibacterias para la producción industrial de ácido propiónico a partir de recursos renovables proporcionaría una alternativa amigable con el medio ambiente a la actual producción basada en fósiles. Sin embargo, para que esto se realice, existe la necesidad de superar las dificultades debidas al efecto inhibidor del ácido propiónico sobre las bacterias, especialmente a altas concentraciones, que da como resultado bajos rendimientos y productividad, así como también altos costos. Esto es posible de lograr mediante el desarrollo de un proceso robusto basado en el uso de una gran cantidad de células que pueden reciclarse y también evolucionar mediante adaptación a alta concentración de ácido propiónico.

Esta tesis estudia diferentes formas de las denominadas fermentaciones de alta densidad celular utilizando bacterias tanto del tipo silvestre como adaptadas para la producción de ácido propiónico. En el presente estudio se utilizó *Propionibacterium acidipropionici*, una bacteria generalmente considerada como segura (GRAS), y como fuente de carbono renovable se utilizó glicerol. El glicerol es un sustrato

favorable para muchas propionibacterias debido a su mayor estado de reducción en comparación con los carbohidratos tradicionales. El glicerol se forma como producto secundario en procesos como la producción de biodiésel a partir de aceites vegetales. El uso de biodiésel como combustible renovable ha llevado a un aumento en la disponibilidad de glicerol y se espera que su mercado supere los USD 3 mil millones en 2022. Si bien el glicerol encuentra una serie de aplicaciones en las industrias cosmética, química y farmacéutica, su uso potencial como sustrato en la fermentación se ha investigado con resultados prometedores. Los principales productos obtenidos de la fermentación del glicerol son los ácidos glicérico, propiónico y 3-hidroxipropiónico, todos ellos producidos por propionibacterias. La principal ventaja de usar glicerol como única fuente de carbono para la producción de ácido propiónico es la ausencia de ácido acético como subproducto, una característica deseada ya que reduce los costos de recuperación del producto.

Un proceso de fermentación implica normalmente la inoculación de un medio de cultivo con un cultivo bacteriano, dejar crecer las células hasta que estas dejen de hacerlo o hasta que se forme la máxima cantidad de producto deseado, seguido de la separación de las células y el producto. Normalmente, el producto formado provoca la inhibición del crecimiento bacteriano y así también la eficiencia del proceso se ve reducida. El tiempo de fermentación puede reducirse reteniendo las células bacterianas en el biorreactor, por ejemplo: recogiendo las células después de la fermentación y devolviéndolas al biorreactor para su uso en ciclos de fermentación posteriores. Una estrategia frecuentemente utilizada para retener las células en los biorreactores es la inmovilización sobre materiales sólidos llamados soportes o carriers. Esto permite que las células permanezcan en el biorreactor en un estado "listas para trabajar" durante varios ciclos de producción en lugar de comenzar cada nuevo ciclo de fermentación con un nuevo inóculo. Se han desarrollado diferentes formas de inmovilización con el uso de diferentes materiales, polímeros y productos químicos que median la adhesión de las células a un soporte. Un polímero que es muy eficaz para unir las células a una superficie sólida es la polietilenimina cargada positivamente (PEI) a través de la atracción a la superficie celular cargada negativamente. Sin embargo, la inmovilización mediada por productos químicos, además de aumentar los costos, también puede dar como resultado un efecto inhibidor no deseado sobre las células. Por otro lado, varias bacterias tienen la capacidad natural de producir polímeros de matriz extracelular, también llamados biopelículas o biofilms, mismos que ayudan a las células a unirse a superficies sólidas. Dado que *Propionibacterium acidipropionici* no fue previamente reportado como una bacteria formadora de biofilm, se aplicaron condiciones no óptimas de cultivo, denominadas condiciones de estrés, para el crecimiento de las bacterias y así inducir la formación de matriz extracelular o biofilm. Se encontró que el ácido cítrico y el cloruro de sodio son buenos factores de estrés para que las células induzcan la formación de biofims.

Se utilizaron materiales sólidos a base de plástico (AnoxKaldnes®) y vidrio reciclado (Poraver®) como soportes para la inmovilización celular, ya sea por formación de biofilm o por tratamiento con PEI. Las células inmovilizadas a través de PEI dieron mayores rendimientos de producto y productividad. Como una forma alternativa de establecer fermentaciones de alta densidad celular, también se estudió la retención celular mediante el uso de un filtro de membrana de cerámica. Hacer funcionar el reactor en modo continuo mientras se reciclan las células, reteniéndolas mediante la membrana, alivió en cierta medida el efecto inhibidor del producto de ácido propiónico. El reactor de membrana se hizo funcionar continuamente durante un período de dos meses.

Finalmente, se investigó la posibilidad de desarrollar una cepa de *P. acidipropionici* capaz de tolerar mayores concentraciones de ácido propiónico y condiciones ácidas adaptando la bacteria del tipo silvestre en un medio de cultivo con concentraciones crecientes de ácido propiónico. Una cepa que se adaptó para crecer con 20 g/L de ácido propiónico añadido al medio mostró una buena tolerancia y productividad mejorada. La cepa tolerante inmovilizada en Poraver® tratado con PEI logró convertir hasta 160 g/L de glicerol en ácido propiónico. La cepa también fue activa a pH por debajo de 5. Este sistema robusto con la cepa tolerante funcionó durante más de dos meses con un rendimiento estable en fermentaciones repetidas.

Como resultado de las condiciones de estrés aplicadas para inducir la formación de biofilms por *P. acidipropionici*, y para desarrollar la cepa tolerante a una mayor concentración de ácido propiónico, se encontró que las bacterias expresaban genes relacionados con la producción de trehalosa, un azúcar conocido por aumentar la estabilidad de células y biomoléculas. Por lo tanto, este estudio proporciona bases para seguir investigando los cambios nivel genético en las células de *P. acidipropionici* cuando se exponen a entornos de estrés y también para continuar investigando bioprocesos eficientes para la producción de ácido propiónico.

List of Papers and Manuscripts

The thesis is based on the following papers and a manuscript, listed in roman numerals:

I. Dishisha T., Ibrahim M.H.A., **Cavero V.H**., Alvarez M.T., Hatti-Kaul R. (2015). Improved propionic acid production from glycerol: combining cyclic batch- and sequential batch fermentations with optimal nutrient composition. *Bioresource Technology* 176:80–87.

II. **Cavero-Olguin V.H.**, Hatti-Kaul R., Cardenas Oscar V., Romero Danitza X., Alvarez-Aliaga M.T. (2019). Stress induced biofilm formation in *Propionibacterium acidipropionici* and use in propionic acid production. *World Journal of Microbiology and Biotechnology* 35 101. https://doi.org/10.1007/s11274-019-2679-9.

III. **Cavero-Olguin V.H.**, Rahimpour F., Dishisha T., Alvarez-Aliaga M.T., Hatti-Kaul R. (2021). Propionic acid production from glycerol in immobilized cell bioreactor using an acid-tolerant strain of *Propionibacterium acidipropionici* obtained by adaptive evolution. *Process Biochemistry* 110, 223–230.

IV. **Cavero-Olguin V.H.**, Dishisha T., Hatti-Kaul R. (2022). Membrane-based continuous fermentation with cell recycling for propionic acid production from glycerol by *Propionibacterium acidipropionici*. *MANUSCRIPT*.

My contribution to the papers

Paper l: I carried out experiments about optimization of the medium in batch cultures without pH control and analyzed the results for graph presentations in the paper.

Paper ll: I designed most of the experiments, analyzed most of the data and wrote the first draft of the manuscript and was involved in the revision of the manuscript. I am the corresponding author and participated actively in the corrections demanded by the reviewers and editor.

Paper lII: I carried out most of the experiments, designed half of the experiments, analyzed data and wrote the first draft of the manuscript. Participated actively in the corrections and responses to the reviewers and editor.

Paper IV: I set up the reactor and performed the complete experiment, analyzed data and wrote the first draft of the manuscript, and was involved in revising the manuscript.

List of abbreviations

1,2-PDO	1,2-Propanediol
AA	Acetic acid
ADP-ACS	ADP-forming acetyl-CoA synthetase
AI2	Autoinducer 2
ALE	Adaptive laboratory evolution
ASD	Autism spectrum disorder
CSTR	Constant stirred tank reactor
CoA	Co-enzyme A
DCCP	Dichlorprop
DEAE	Diethylaminoethyl-cellulose
DHA	Dihydroxy acetone
DHAP	Dihydroxyacetone phosphate
ECM	Enzymatically treated corn mash
eDNA	Extracellular DNA
EMP	Embden-Meyerhof-Parnas glycolytic pathway
EPS	Exopolysaccharides
FBB	Fibrous-bed bioreactor
FDA	Food and Drug Administration
FUM	Fumarate dehydrogenase
G3-P	Glyceraldehyde 3-phosphate
GABA	γ-aminobutyric acid
GASP	Growth advantageous stationary phase
GDH	Glycerol dehydrogenase
GDH	Glycerol dehydrogenase
GHG	Greenhouse gas
GRAS	Generally regarded as safe
HCD	High cell density
HRT	Hydraulic retention time
LA	Lactic acid
LAB	Lactic acid bacteria

MCPP	Mecoprop
MDH	Malate dehydrogenase
MFB	Multipoint fibrous bed bioreactor
MMC	Methylmalonyl-CoA carboxytransferase
MMD	Methylmalonyl decarboxylase
MRS	Mismatch repair system
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
OtsA	Trehalose 6-phosphate synthase
OtsB	Trehalose 6-phosphate phosphatase
PA	Propionic acid
PEI	Polyethyleneimine
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxytransphosphorylase
PPC	Phosphoenolpyruvate carboxylase
PYC	Pyruvate decarboxylase
Pyr	Pyruvate
QPS	Qualified Presumption of Safety
RpoS	RNA polymerase sigma S
SA	Succinic acid
SCFA	Short Chain Fatty Acids
SDH	Succinate dehydrogenase
Suc-CoA	Succinyl co-enzyme A
TCA	Tricarboxylic cycle acids
TreH	Trehalase enzyme
TreY	Maltooligosyl-trehalose synthase
TreZ	Maltooligosyl-trehalose trehalohydrolase
YY	Dityrosine

Chapter 1. Introduction

Our over-dependence on fossil resources during the past century, to meet our needs for energy, chemicals and materials has led to the critical situation we face today, with rising global temperatures and the resulting climate change. This is likely to have a dramatic impact on humanity and ecosystems, the signs of which we have already started to observe and feel. There has thus been an increasing interest during the past decades to decouple industrial production from fossil feedstock and move to a more sustainable circular bioeconomy. Development of an economical and competitive bioeconomy requires learning from the structure of the petrochemical refineries to integrate the production of several products in a biorefinery. Moreover, this has to be achieved without competition with food and feed, which implies that biomass residues from agriculture, forestry and industrial side-streams become the valuable renewable resources. This would not only imply a major reduction of greenhouse gases (GHG) emissions, but also reduction of waste, and increase the material value of our resources. The production of bio-based chemicals and materials, in addition to bioenergy and biofuels, is estimated to generate a revenue of 10 billion US dollars for the global chemical industry (IEA 2020).

Transition to a biomass-based feedstock implies development of different chemistries and technologies to make the products providing useful functions. Organic acids constitute a group of valuable chemicals, with varying carbon chain and properties, that can be used on their own, as a component in polymers, or as a platform for making other chemicals (Becker et al., 2015). Industrial biotechnology has turned out to be a key enabling technology for the production of organic acids and other products from renewable feedstock, some important commercial examples of organic acids being lactic acid and succinic acid with global productions of 400 000 tons/year and ca. 36 600 tons/year, respectively (Becker et al., 2015). Different microorganisms possess the metabolic capacity to convert sugars, alcohols, polyols and carbon dioxide to different organic acids. For industrial production, it is necessary to exploit this ability of the microorganisms to use substrates at high concentrations and convert them efficiently to products in high yields. For this, the metabolic bottlenecks and inhibitory effects of the process environment and the product need to be overcome, which may be achieved e.g. by genetic modification of the microorganism and by applying different process engineering strategies.

Microbial production of a vast number of molecules and the industrial implementation by means of fermentation technology is the focus of a lot of research and investment nowadays, as it provides a more environmentally friendly route since the processes used are generally carried out under mild conditions that have lesser undesired effects on the environment. While the first production processes for acetic and citric acid date back to 1823 and 1913, respectively (Becker et al., 2015), the search of natural products from microorganisms began with the discovery of penicillin by Alexander Fleming in 1928, starting the era of natural compounds as antimicrobial agents produced in fermentation cultures. Biotechnological processes like microbial fermentations offer nowadays greener alternatives for the production of a large variety of materials which are still traditionally obtained from petroleum-based feedstock.

Propionic acid is an important chemical for different industries, ranging from food, feed, pharmaceutical, to cosmetics and plastics. The production of propionic acid by microbial fermentation has been increasingly studied using different substrates, and there is still need for more research to make bioproduction of propionic acid able to be competitive in the market. One of the most useful microorganisms for this production is *Propionibacterium acidipropionici*, which is generally regarded as safe (GRAS) bacteria, and is vastly used in Swiss cheese ripening in the food industry. This aerotolerant microorganism anaerobically metabolizes different sugars and glycerol as carbon sources for growth and produces propionic acid as its main fermentation product. Residual streams from agriculture and different industries can be used as carbon sources for fermentations, as is the case of the biodiesel industry, which generates glycerol-rich side-stream. Glycerol, a C-3 chemical with a high reduction state, is an ideal substrate for propionic acid fermentation (Boyaval et al., 1994).

1.1. Scope of the thesis

The aim of the thesis was to investigate propionic acid fermentation using *Propionibacterium acidipropionici* by applying different strategies of high cell density fermentations including recycling of free cells and immobilization of cells. For the latter, immobilization was facilitated either by chemical modification of the solid matrix or by stressing the bacteria to trigger their ability for biofilm formation. Glycerol was used as the carbon source all along the work.

The thesis is based on 4 papers, three of which are published:

In **Paper l**, cyclic and sequential batch modes of high cell density fermentations were evaluated for propionic acid fermentation. Optimal concentrations of glycerol and yeast extract as carbon and nitrogen source, respectively, were first determined for use in the fermentations.

Paper ll presents a study on inducing the *P. acidipropionici* to form biofilm by chemical stress factors including sodium chloride and citric acid, and evaluating the immobilization of the bacteria to AnoxKaldness® and Poraver® supports for propionic acid production. The possible correlation with the synthesis of trehalose produced under the selected stress conditions was also studied.

In **Paper lII,** a propionic acid-tolerant strain of *Propionibacterium acidipropionici* was obtained by adaptive evolution and was used in two immobilized cell bioreactors using PEI-treated Poraver® as support, for production of propionic acid with increasing concentrations of glycerol and decreasing pH, respectively. The expression of trehalose production related genes was also evaluated.

Paper IV presents the results from a study on propionic acid production by membrane-based cell recycle fermentation by using a ceramic membrane filter.

The following chapters in this thesis provide description of propionic acid and routes for its production (Chapter 2), Propionibacteria species and in particular *Propionibacterium acidipropionici* and the metabolic pathways for propionic acid and trehalose production (Chapter 3), different ways for laboratory evolution of the bacteria for enhanced propionic acid production (Chapter 4), strategies for high cell density fermentations including recycling cells, immobilization, biofilm formation and cell retention (Chapter 5) and the results obtained (Chapter 6). The thesis ends with conclusions and outlook (Chapter 7).

Chapter 2. Propionic acid

Propionic acid (PA) is a short chain fatty acid with a chemical formula CH_3CH_2COOH, that occurs naturally in apples, grains, cheese, strawberries, and even in human sweat (Ahmadi et al., 2017). Its commercial production so far relies mainly on the fossil resources. The largest PA producer globally is BASF in Germany, with 176 million pounds (ca. 80 000 kilotons) per year of dedicated capacity; other companies producing PA include Chemische Werke Hüls (Germany), Distillers Company (Great Britain), Celanese Chemical Company (USA), Eastman Chemical Company (USA), and others (Liu et al., 2012). Since 2007, the global production of PA increased from around 38 kilotons to 400 kilotons in 2013 (Es et al., 2017). The global PA volume in 2020 was estimated at 430 780 metric tons and is expected to grow to 534 170 metric tons by 2026 (https://www.statista.com/statistics/1245247/propionic-acid-market-volume-worldwide/).

2.1. Properties

Propionic acid is an organic acid with a characteristic pungent odor and is totally miscible with water. Its main physico-chemical properties are summarized in Table 2.1. Its carboxylic group reacts with alcohols, esters, bases and organic salts to form esters, amides, anhydrides, and chloride derivatives, respectively (Figure 2.1). Addition of Na^+, K^+, or Ca^{2+} salts leads to its precipitation as propionate salts (Ahmadi et al., 2017).

Table 2.1. Chemical and physical properties of propionic acid

IUPAC name	Propanoic acid
Other names	Propionic acid, ethanecarboxylic acid, carboxyethane, ethylformic acid, metacetonic acid and methylacetic acid
CAS number	79-09-4
Molecular formula	CH_3CH_2COOH
Molar mass	74.07854 g/mol
Appearance	Colorless liquid
Odor	Slightly rancid
Melting point	-21 ˚C
Boiling point	141 ˚C
Density	0.99 g/mL
Solubility in water	Miscible
pK_a	4.87
Viscosity	10 mPa s

2.2. Applications of propionic acid

Applications of PA are summarized in Figure 2.1.

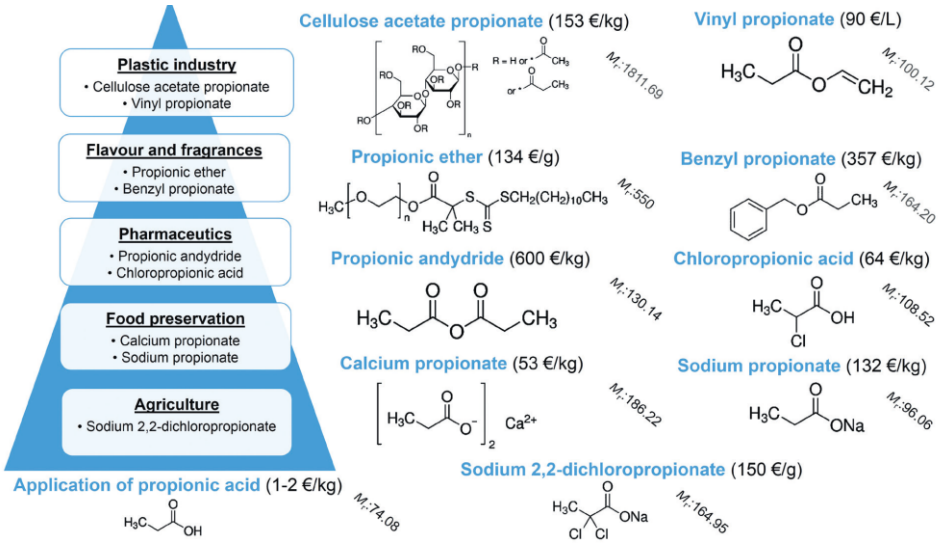


Figure 2.1 Chemical structure, relative molecular mass (M_r) and approximate price (in €) of propionic acid derivatives in different fields of application (from Ranaei et al. 2020)

Propionic acid inhibits the growth of bacteria and molds, when used at low concentration. Hence, its main applications have been as preservative for animal feed, grains and foods. Use of PA in preventing undesired fermentation in barley silage has also been reported (Mills and Kung, 2002). Around half of global production of PA goes for animal feed and grain preservation applications (Dishisha et al., 2013; Killerby et al., 2022), while preservation of baked goods is another major application. Its use in poultry feed reduces the risk of infections by *Salmonella* species (Mani-López et al., 2012). Food industry needs to preserve food for long periods of time and under several physical conditions, some related to weather and some to storage conditions needed for the product. To provide people with good quality products it is of utmost importance to have good and safe preservatives like PA and its Na^+, K^+ and Ca^{2+} salts, which are recognised to be Generally Regarded as Safe (GRAS) by the Food and Drug Administration (FDA) in USA. PA can be useful in preventing the infection by many harmful microorganisms including fungi in monocotyledonous and dicotyledonous plants with no harm for human health (Ranaei et al., 2020). As a result of agricultural activity in rural and urban areas, certain propionic acid-based herbicides as the phenoxypropionate herbicides (*RS*)-2-(2,4-dichlorophenoxy) propanoic acid (known as DCPP or dichlorprop) and (*RS*)-2-(4-chloro-2-methylphenoxy) propanoic acid (known as MCPP or mecoprop) can be found in groundwater that is used for human consumption. The threshold limit concentrations for pesticides were set, by the EU, to 0.1 g/L and 0.5 g/L for any single pesticide and for a total concentration of mixed pesticides, respectively, in drinking water. *Sphingomonas herbicidovorans* was found to completely degrade MCPP (Zipper et al., 1996) while still more environmental bacterial degraders of these compounds are currently being investigated to reduce and eliminate the health risk they may have (Feld et al., 2016). Propionic acid as herbicide can meet demands of specificity and low hazard for human health standards to eliminate the organisms that destroy certain cultivars, besides being decomposed naturally to acetic acid, formic acid, and finally to CO_2 and H_2O, the characteristics that make PA not harmful for the environment (Ranaei et al., 2020).

The antimicrobial effect of PA is attributed to its diffusion into the cells facilitated by hydrophobicity of the protonated form of propionic acid and the cellular membrane, and its dissociation into propionate and protons inside the cells creating instability in proteins. H^+-ATPase activity in the cells counteracts the excess of protons but at the expense of consuming ATP, which affects the normal metabolism of the cells. Contrary to the use of antibiotics, PA does not provoke resistance; nonetheless, possible acid adaptation by bacteria should be kept in consideration to find the proper doses of PA (Mani-López et al., 2012).

An interesting action of PA when given as a food additive to animals was its hypocholesterolemic effect, besides the fat-lowering effect in liver and plasma. In humans, however, only the fat-lowering effect seems to be similar to that observed in animals whereas there are no conclusive results for the hypocholesterolemic

effect (Al-Lahham et al., 2010). These characteristics give PA a good profile for its use in human health as dietary supplement.

Development of effective anti-inflammatory compounds involving PA has been shown to be possible. PA-based derivatives like 2-aryl propionic acid derivatives are frequently prescribed as anti-inflammatory agents (Al-Khateeb et al., 2021). PA has a moderate inhibitory action on cyclooxygenase enzyme involved in the production of pro-inflammatory cell-signaling eicosanoids. Some PA-based anti-inflammatory compounds showed ulcerogenic effects which were eliminated with the incorporation of 2-2-fluoro-4-(2-oxocyclopentyl)methyl]phenyl}propionic acid (Ranaei et al., 2020). The antibacterial action of PA prevents the infestation of the colon by pathogens like *Salmonella typhimurium*, in turn preventing inflammation (Al-Lahham et al., 2010). Lymphocyte proliferation was inhibited by the action of PA, and in a similar way PA inhibited the production of the pro-inflammatory cytokine, resistin, by human adipose tissue (Al-Lahham et al., 2010). It is suggested that the anti-inflammatory action of PA may well depend on its effect on inflammation causing bacteria, cytokines, adipokines, fatty acids or other agents (Al-Lahham et al., 2010).

Several advantages of PA range from its positive effects on gut microbiota to organoleptic qualities. PA, like other short chain fatty acids (SCFA), stimulates the release of the dipeptide dityrosine (YY) in the human gut and also causes the activation of SCFA receptors, leading in this way to a reduction of lipogenesis and enhancing the feeling of satiety (Ahmadi et al., 2017; Ranaei et al., 2020). PA is naturally produced by the microbiota in the gut, and is the main SCFA absorbed by epithelial cells in the colon. After absorption, PA is transported by blood to be metabolized mainly in the liver where it enters the Krebs cycle at the succinyl-CoA (Suc-CoA) level to be converted to oxaloacetate, which is finally converted to glucose, for which biotin and vitamin B12 are essential. PA is a major source of glucose in ruminants; in the absence of biotin and vitamin B12 the blood levels of PA were found to be increased (Al-Lahham et al., 2010). Although PA is regarded as safe and even the daily intake for humans is reported as unlimited (Mani-López et al., 2012), recent studies have related autism spectrum disorder (ASD) to the consumption of PA in children. Since some bacteria that form the microbiota produce PA, it has the capability to enter the blood stream and trespass the blood-brain barrier having some neuroactive effects (Ranaei et al., 2020).

Other value-added uses of PA include its use as an intermediate in the synthesis of chemicals and polymers like cellulose propionate, a useful thermoplastic, and vinyl propionate, a precursor for pesticides and pharmaceuticals. PA is also used in the manufacture of ester solvents, fruit flavors, in the enhancement of the scorching resistance of butyl rubber and as an esterifying agent in thermoplastics industry (Colomban et al., 1993; Boyaval et al., 1994; Himmi et al., 2000). 2,2-Bis(hydroxymethyl) propionic acid based cyclic carbonate monomers and their (co)polymers are used as advanced materials for biomedical applications (Ansari et

al., 2021) and certain other anti-arthritic drugs and perfume bases (Colomban et al., 1993).

2.3. Traditional petrochemical production

The commercial production of propionic acid mainly depends on petrochemistry involving the use of ethylene, ethanol, acrylonitrile, acrylic acid or lactic acid (Fig. 2.2). Three of the most common chemical processes for production of PA are the following:

- The Reppe process, developed in Germany, based on hydrocarboxylation of ethylene using CO and steam.
- The Larson process involving the use of ethanol, CO and BF_3 as catalyst.
- The Fischer-Tropsch process involving aerobic oxidation of liquid-phase propane or propionaldehyde.

These methods rely on non-renewable petrochemical feedstock that mostly cause environmental pollution (Es et al., 2017).

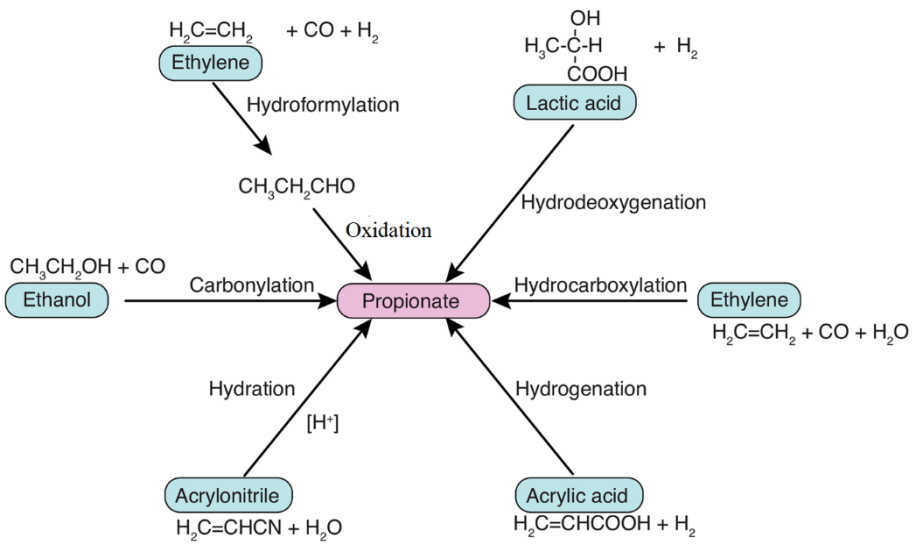


Figure 2.2 Traditional petrochemistry-based production of propionic acid (adapted from Es et al., 2017)

2.4. Microbial production of propionic acid

Production of PA by microorganisms occurs via three fermentative pathways including dicarboxylic-, 1,2-propanediol (1,2-PDO)-, and acrylic acid routes (Fig. 2.3). The dicarboxylic route, called Wood-Werkman cycle is the most preferred in propionic acid bioproduction and is described in Chapter 3.

Certain bacteria in the rumen of ruminant animals have the capacity to form 1,2-propanediol (1,2-PDO) that is used by other bacteria to form PA. There are also some bacteria like *Salmonella typhimurium* that can both produce and use this chemical. The biosynthesis of 1,2-PDO is achieved by catabolism of deoxy sugars (methyl pentoses) like rhamnose and fucose, which after being phosphorylated, are cleaved by an aldolase to s-lactaldehyde and di-hydroxyacetone phosphate (DHAP), the former being finally reduced to 1,2-PDO (Figure 2.3). Using genetically engineered *E. coli* and other microorganisms it has been possible to produce 1,2-PDO from a common sugar like glucose (Saxena et al., 2010). Another route for the formation of 1,2-PDO found in *Thermoanaerobacterium thermosaccarolyticum* and *Clostridium sphenoides*, is via reduction of methylglyoxal formed from DHAP to be subsequently reduced to 1,2-PDO by an aldose reductase and glycerol dehydrogenase (Bennett and San, 2001). The degradation of lactic acid to produce 1,2-PDO was reported for *Lactobacillus brevis* and *L. buchneri* using glycerol as the electron acceptor (Saxena et al., 2010).

The conversion of 1,2-PDO to PA occurs by the combined actions of diol dehydratase and two promiscuous enzymes, usually associated with acetate metabolism, CoA-dependent aldehyde dehydrogenase, phosphotransacylase and acetate kinase in a two-step metabolic reaction, forming propionyl-CoA as the intermediate and with the generation of one ATP molecule. 1,2-PDO is a valuable chemical and used in several applications, and its use for production of PA is not economically attractive (Gonzales-Garcia et al., 2017).

Only certain microorganisms like *Clostridium propionicum*, *Prevotella ruminicola* and *Megasphaera elsdenii* use the acrylic pathway, which involves the conversion of lactate to propionate with the intermediate formation of acryloyl-CoA which is converted to propionyl-CoA through acrylyc-CoA reductase and the consumption of NADH (Figure 2.3). Lactate, propionyl-CoA and acrylate follow the same pathway by propionyl-CoA transferase resulting in lactoyl-CoA, propionate and acryloyl-CoA, respectively (Prabhu et al., 2012). In these bacteria, with the exception of glucose, several substrates as serine, alanine, lactate and ethanol have shown to be useful for PA production. Glucose seems not to trigger the expression of lactate racemase, which is needed to trigger the acrylic acid route (Gonzales-Garcia et al., 2017).

In contrast to the price of US dollar/kg of the petrochemically produced PA, the product obtained via biotechnology ranges between 1.5-2 USD/kg (Liu et al,. 2012;

Es et al., 2017). An economic and environmental assessment estimated that PA volumetric productivities of 2 g/L/h and yields of 0.6 g/g (PA/sugar) and titer of 100 g/L are needed for bioproduction of PA to be economically viable (Anh Do Quynh et al., 2003; Gonzales-Garcia et al., 2017).

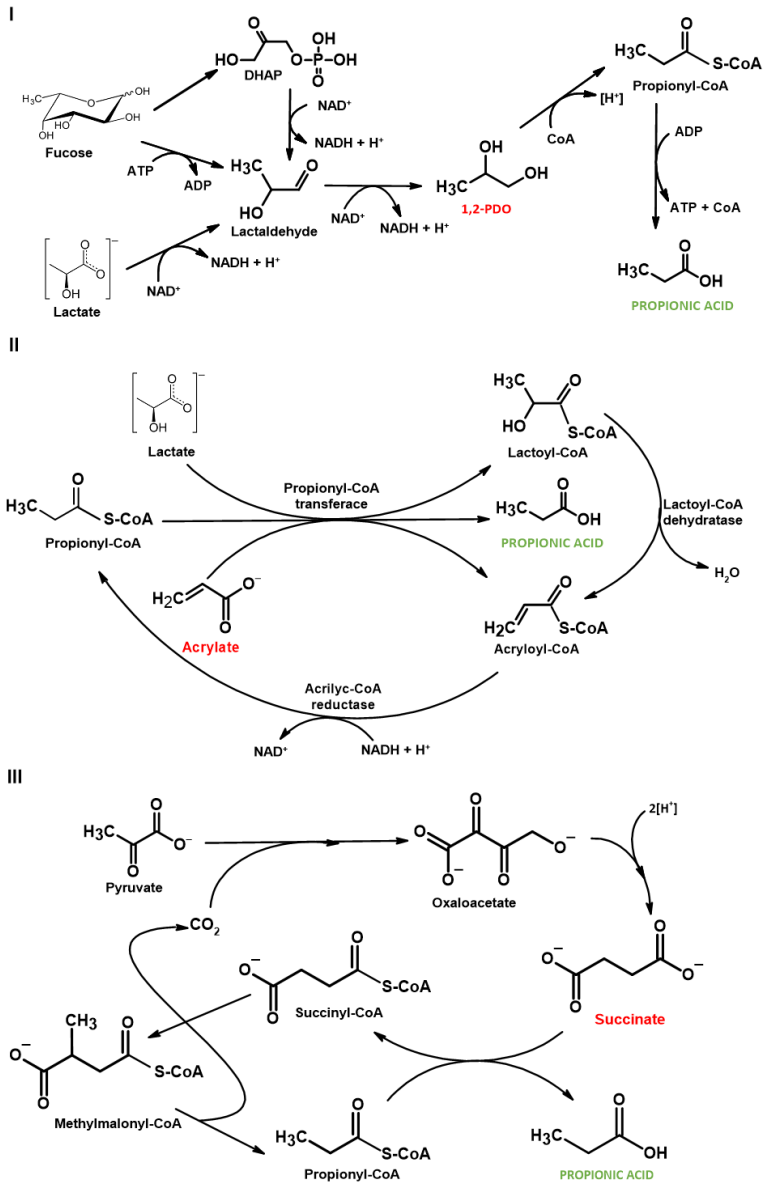


Figure 2.3 Fermentative metabolic pathways to produce propionic acid (**I**. 1,2-propanediol pathway, **II**. acrylate pathway, **III**. Wood-Werkman cycle.). Abreviations: DHAP, dihydroxyacetone phosphate

Chapter 3. Propionibacteria species as a source of propionic acid and other valuable products

3.1. Propionibacteria

Propionibacterium is a genus of bacteria known for their unique metabolism that results in the production of propionic acid. The genus belongs to the family of *Propionibacteriaceae* in the order of *Actinomycetales* within the class of *Actinobacteria*. Propionibacteria are gram-positive, non-motile, slow-growing, catalase-positive, non-spore forming, rod-shaped or small cocci, that are arranged in pairs, short chains or clusters, and are anaerobic to aerotolerant bacteria. These bacteria are ubiquitous and commensals of humans and other animals. They have been mainly isolated from soil, silage, fermenting olives, intestine of ruminants (known as classical propionibacteria), and dairy products (Moslemi et al., 2016). In the rumen, they are responsible for urea breakdown and ammonia release.

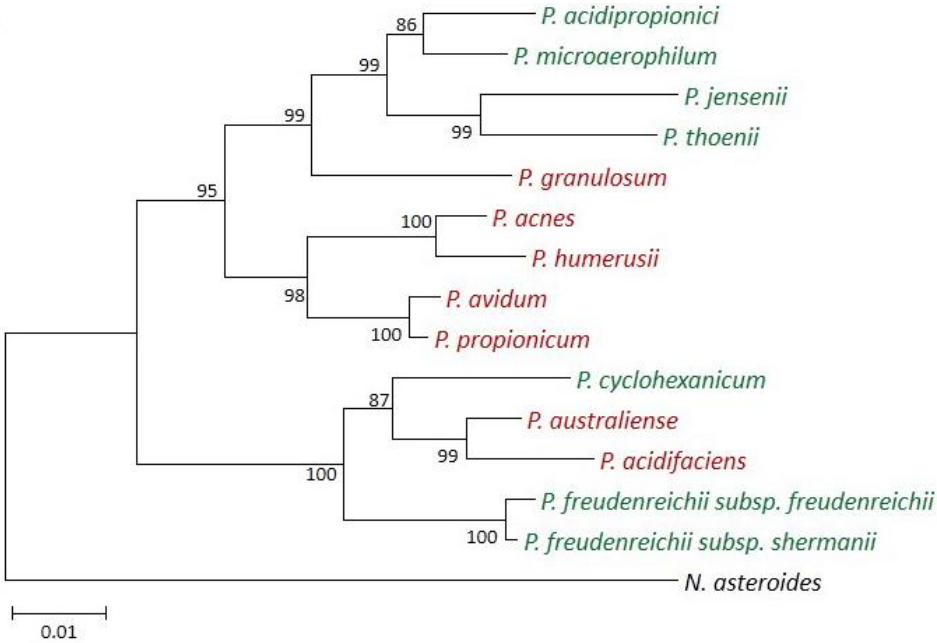
Based on the source of isolation, *Propioniacterium* genus is classically divided into cutaneous and dairy species. Cutaneous propionibacteria include *P. acnes, P. avidum and P. granulosum* which are usually recognized as pathogens, the most known example being *P. acnes* that causes acne and other opportunistic skin infections. This bacterium is divided into three phylogenetic types: l, ll and lll (Scholz and Kilian 2016). Dairy propionibacteria are recognized to be safe, and include species like *P. acidipropionici* and *P. freudenreichii* spp. *shermanii* and spp. *freudenreichii* that have been extensively studied due to their ability to produce propionic acid, vitamin B12, as probiotic, and for their role in Swiss type cheese production (Ahmadi et al., 2017; Rabah et al., 2017). Dairy propionibacteria are also the only food grade bacteria that have metabolic capacity for trehalose (a low-calorie sugar) biosynthesis (Moslemi et al., 2016).

Figure 3.1 shows the phylogenetic relationship among different species of the *Propioniacterium* genus. Highly advanced analyses of phylogenetic relations and sequence data were used to obtain improved resolution of the phylogenetic relationships within this genus leading to a reclassification for several species into the proposed genera *Acidipropionibacterium*, *Cutibacterium* and

Pseudopropionibacterum (Scholz and Kilian 2016). Nonetheless, there is some resistance to the general adoption of this proposal (McCubbin et al., 2020). The dairy propionibacteria and also *P. thoenii*, *P. jensenii*, *P. zeae* and *P. beijingense*, have been studied for biotechnological purposes due to their products and other characteristics like the ability to adhere to the epithelium in the intestine and to endure digestive stress conditions, an important requirement for being considered as probiotic. There is strong evidence of the versatility of *P. freudenreichii* and *P. acidipropionici* in using different carbon sources to produce not only PA but also certain metabolites that are interesting for human health (Rabah et al., 2017). Propionibacteria have a wide spectrum of antimicrobial activity for inhibiting the growth of gram-positive and some gram-negative bacteria as well as some yeasts and molds. The bacteriocin production by these bacteria has been studied to a less extent, however these products are undoubtedly important for their role as probiotics (Moslemi et al., 2016).

The optimum temperature and pH for growth of propionibacteria are 30–37 °C and 6–7, respectively; pH values lower than 4.5 stop their growth and propionic acid production (Ahmadi et al., 2017).

A



B

Dairy (classical) propionibacteria No pathogenicity	**Cutaneous propionibacteria** Opportunistic pathogens
P. acidipropionici	*P. acidifaciens*
P. cyclohexanicum	*P. acnes*
P. thoenii	*P. australiense*
P. jensenii	*P. avidum*
P. microaerophilum	*P. granulosum*
P. freudenreichii subsp. *shemanii*	*P. propionicum*
P. freudenreichii subsp. *freudenreichii*	*P. humerusii*

Figure 3.1 (**A**) Propionibacteria minimum evolution phylogenetic tree based on 16S rDNA sequences. The 16S rDNA sequence of the Actinomycetale *Nocardia asteroides* was used as a distant outgroup to root the tree. (**B**) Distribution of *Propionibacterium* species in two distinct groups, diary and cutaneous (From Rabah et al., 2017)

3.2. Propionic acid production by propionibacteria

Propionic acid production by means of fermentation has been studied since 1923. The first description of *Propionibacterium* species and their capability to produce propionic acid from certain sugars was from 1878 by Albert Fitz, who showed that 3 moles of lactic acid would produce 2 moles of propionic acid, one mole of acetic acid, one mole of CO_2 and one mole of H_2O (Gonzales-Garcia et al., 2017). Microorganisms from the *Propionibaterium* genus are regarded as the best propionic acid producers, the most known being *P. thoenii, P. freudenreichii, P. shermanii, P. acidipropionici*, and *P. beijingense* (Liu et al., 2012). Besides *Propionibacteria*, other gram-negative anaerobic bacteria like *Selenomonas ruminantium, Anaerovibrio lipolytica, Clostridium*, *Veillonella* spp., *Propionispira arboris, Bacteroides fragilis* and *Fusobacterium* spp. are also capable of producing propionic acid (Ahmadi et al., 2017). While several microorganisms producing propionic acid happen to be pathogens, *P. acidipropionici* and *P. freudenreichii*, (subsp. *shermanii* and subsp. *freudenreichii*) are generally regarded as safe (GRAS) and are included in the Qualified Presumption of Safety (QPS) list. They are extensively investigated in human health-related research as probiotic products from dairy industry (Zarate et al., 2016; Rabah et al., 2017; Moslemi et al., 2016; Thiel et al., 2004).

3.3. Biosynthesis of propionic acid

Figure 3.2 shows the metabolic routes for the production of PA from sugars and glycerol.

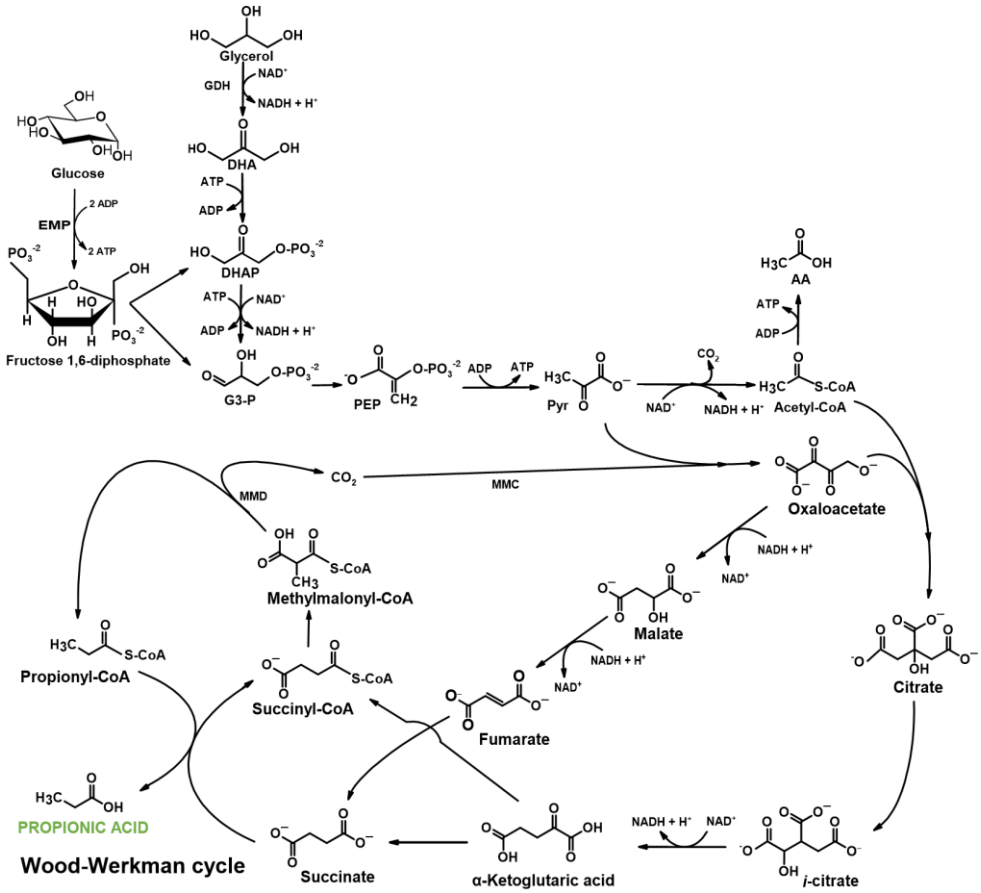


Figure 3.2 Biosynthetic pathways of propionic acid from glycerol and glucose in Propionibacteria. Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; G3-P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AA, acetic acid; GDH, glycerol dehydrogenase; MMC, methylmalonyl-CoA carboxytransferase; MMD, methylmalonyl-CoA decarboxylase

Glucose is catabolized by Embden–Meyerhof–Parnas (EMP) pathway (glycolysis) to pyruvate (Pyr) as the final product. Pyr is then converted to oxaloacetate by the action of pyruvate dehydrogenase, accompanied by consumption of a CO_2 molecule. Phosphoenolpyruvate (PEP), the precursor of Pyr, could be directly converted to oxaloacetate by the action of phosphoenolpyruvate carboxylase (PPC) in a recombinant strain of *P. jensenii* in which the gene *ppc* was overexpressed (Liu et al., 2016). This direct conversion is also catalyzed by phosphoenolpyruvate

carboxytransphosphorylase (PEPC) which is a widely distributed enzyme in plants, algae, protozoa, cyanobacteria and bacteria including propionibacteria; nonetheless, it was not present in *P. acidipropionici*, *P. acnes*, *P. Freudenreichii* and *M. phosphovorus* as shown by genome sequencing and comparative analysis (Parizzi et al., 2012). The dissimilation of glycerol starts with its oxidation by the action of glycerol dehydrogenase (GLDA) using NAD^+ as cofactor to form dihydroxyacetone (DHA), which is then phosphorylated to dihydroxyacetone phosphate (DHAP), the intermediate in the EMP pathway.

Oxaloacetate is converted to malate and then to fumarate with the consumption of one molecule of NADH in each reaction, to then produce succinic acid (SA), which is the first step in the Wood-Werkman cycle. SA is converted to succinyl co-enzymeA (Suc-CoA) that is further transformed to methylmalonyl-CoA. The latter is decarboxylated by methylmalonyl-CoA decarboxylase (MMD) to propionyl-CoA, with the release of CO_2 that is assimilated by PEP or Pyr through the action of methylmalonyl-CoA carboxytransferase (MMC) to form oxaloacetate (Figure 3.2). Finally, the release of the CoA by propionyl-CoA allows PA to be accumulated as the only final product of the cycle.

The accumulation of SA during PA production was evidence of the involvement of dicarboxylic acid cycle, typical in propionibacteria (Vivek et al., 2017). Biotin is known to control the activity of phosphoenolpyruvate carboxylase (PPC), methylmalonyl-CoA carboxytransferase (MMC) and methylmalonyl-CoA decarboxylase (MMD), the three carboxylases that control the carbon flux towards the dicarboxylic acid cycle (Ranaei et al., 2020). Although SA is the starting point of Wood-Werkman cycle to produce PA, its accumulation in fermentations is also investigated due to commercial interest.

The degree of reduction of propionic acid is the same as that of glycerol, hence one mole of PA requires one single mole of glycerol (i), while that is not the case for glucose and lactic acid (ii and iii, respectively).

i. $CH_2OHCHOHCH_2OH \rightarrow CH_3CH_2COOH + H_2O$

ii. $1.5\ C_6H_{12}O_6 \rightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$

iii. $3CH_3CHOHCOOH \rightarrow CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$

The synthesis of one mole of glyceraldehyde 3-phosphate (G3-P) from one mole of glycerol produces two moles of NADH while the formation of SA from oxaloacetate consumes two moles of NADH, implying the redox state during glycerol dissimilation is balanced inside the cell (Vivek et al., 2017). The conversion of oxaloacetate to succinate in *P. acidipropionici* occurs through the formation of the intermediates malate and fumarate by malate dehydrogenase (MDH) and fumarate dehydrogenase (FUM), respectively, and succinate formation by succinate dehydrogenase (SDH) (Parizzi et al., 2012; Vivek et al., 2017). However, according to Ranaei et al. (2020), glycerol may lead to redox imbalance if it is used as the sole

carbon source. In contrast, with glucose as substrate the difference in the degree of reduction provokes the formation of other metabolites to maintain a balanced redox state inside the cell.

According to Gonzales-Garcia et al. (2017), the maximum theoretical yield of PA from glucose via Wood-Werkman cycle is 1.71 mol/mol, when the reduced cofactors can be produced by an alternative pathway such as pentose phosphate pathway where the achievable yield of pyruvate would be 0.6 g/g glucose. Barbirato et al. (1997) reported PA yields of 1.44 mol/mol, 0.75 mol/mol and 0.84 mol/mol from glucose, lactic acid and glycerol, respectively, which is close to the theoretical yield mentioned above, Zhang et al. (2015) showed that addition of CO_2 increased the volumetric productivity of PA from 1.56 g/L/h to 2.94 g/L/h, but the effect was not the same when glucose was the carbon source.

3.4. Factors influencing PA fermentation

Besides the microbial strain, a number of fermentation parameters such as the carbon and nitrogen source, pH and temperature, influence the level and efficiency of propionic acid production.

3.4.1. Carbon source

Propionibacteria are able to use different carbon sources including glucose, fructose, xylose, maltose, lactose, lactic acid and glycerol. Glycolysis, gluconeogenesis, Wood-Werkman cycle and pentose phosphate pathway are present in all genomes of *Propionibacterium* with the exception of the latter in *P. avidum* (Parizzi et al., 2012; McCubbin et al., 2020). The tricarboxylic acids cycle (TCA) was seen to be complete with the genes corresponding to the aerobic and anaerobic respiration detected, the path from 2-oxoglutarate to succinate was seen to be carried out via glyoxylate shunt by three alternative paths, either through succinyl-CoA ligase, through 2-oxoglutarate decarboxylase followed by succinate semialdehyde dehydrogenase or through a complete γ-aminobutyric acid (GABA) shunt, all present in *P. acidipriopionici* (McCubbin et al., 2020). Since the formation of succinic acid is the starting point for the Wood-Werkman cycle, increase in its production needs high amount of reducing equivalents (NADH) for the reductive reactions (reverse of the cycle) in TCA from oxaloacetate to malate, fumarate and succinate that consume 2 NADH (see Fig. 3.2). In this sense, the use of sugars like glucose or xylose can drive the fermentation process to a shortage of reducing equivalents since their use only produces half amount of reducing equivalents in comparison with glycerol (Yazdani and Gonzalez, 2007).

PA production is primarily investigated using propionibacteria monocultures where the low amount of NADH favors the decarboxylation of pyruvate (Pyr) to form acetyl-CoA and NADH followed by the action of ADP-forming acetyl-CoA synthetase (ADP-ACS) which cleaves acetyl-CoA to acetate and CoA with a concomitant ATP synthesis. Therefore, PA production is a cyclic process which involves the reduction of pyruvate to fumarate and to succinate to initiate the Wood-Werkman cycle and the oxidation of pyruvate to acetate (Parizzi et al., 2012). *P. adidipropionici* was established to be the most promising microorganism for PA production from glycerol among three species of *Propionibacteria* (Barbirato et al., 1997; da Silva et al., 2009). The use of sugars or lactic acid as carbon sources benefits the formation of acetic acid, and one carbon is lost as carbon dioxide (Liu et al., 2012). Using lactose in cultivations of *P. acidipropionici* at different pH values gave an increase in PA yield ranging from ca 0.033 g/g to 0.063 g/g as the pH decreased from 6.1-7.1 to 4.5-5.0, while production of AA was not greatly affected and its yield remained between 0.009 - 0.012 g/g at all pH values tested (Hsu and Yung, 1991). Comparison of glucose, lactose and lactic acid as carbon sources with immobilized *P. acidipropionici* in continuous cultures, showed lactic acid was the most beneficial for PA production but not for growth (Lewis and Yung, 1992), most likely due to the similar reduction state of lactic acid as PA compared with other more oxidized carbon source like glucose or lactose.

Some studies on PA production using co-cultures have also been reported. In co-cultures of *P. shermanii* with *Lactobacillus xylosus* using glucose and xylose as carbon sources, lactate produced by *L. xylosus* was used as the substrate by *P. shermanii* for PA production (Tyree et al., 1991). The synergy of propionibacteria, and other propionic acid-producing bacteria, with lactic acid bacteria (LAB) was also studied using co-cultures of *Lactobacillus zeae* with *Veillonella cricetid*, and *Lactobacillus acidophilus* with *P. shermanii*, showing significant increase in PA yield in comparison with the monocultures of *P. shermanii* and *V. cricetid*, respectively (Es et al., 2017; Ahmadi et al., 2017). The lactate produced by LAB served as substrate for the propionic acid-producing bacteria. Nonetheless, during co-culture of *P. shermanii* and *V. cricetid* for production of PA, *V. cricetid* was inhibited by lactic acid concentrations higher than 10 g/L (Sabra et al., 2013). Co-fermentation of glycerol and glucose was also observed to give higher fermentation yields (Vivek et al., 2017).

Some residual materials from industry, agriculture and municipal services have been investigated as cheap carbon-source alternatives in PA production with the aim to lower the production costs. Glycerol formed as a by-product in the production of the biofuels, biodiesel and bioethanol, forms an attractive raw material for PA production (Clomburg and Gonzalez, 2013). The use of hydrolyzed whey as raw material in propionibacteria cultures enhanced PA and decreased vitamin B12 production by 30 % and 50 %, respectively (Czaczyk et al., 1996). Cultures of *P. freudenreichii* subsp. *shermanii* grown in whey permeate containing ca 45 g/L of

lactose supplemented with lactic acid gave better results than with whey alone for the production of biomass and organic acids like PA and AA (Marcoux et al., 1992). Use of corn steep liquor in combination with glucose in fed-batch cultivation of *Propionibacterium freudenreichii* resulted in PA titer of 52.5 g/L and yield of 0.66 g/g (Wang et al., 2012). Jerusalem artichoke hydrolysate, composed of 20 g/L glucose and 40 g/L fructose, was used for PA production by *P. adidipropionici* in fibrous-bed bioreactor (FBB) giving productivity, yield and titer values of 1.55 g/L/h, 0.43 g/g and 68.5 g/L, respectively; subsequent recycling batch fermentations led to enhanced productivity of 3.69 g/L/h (Liang et al., 2012). The feasibility to use lignocellulosic corn stover hydrolysate as carbon source for *P. adidipropionici* showed enhancement in PA production when going from batch fermentation to fed-batch fermentation, roughly doubling the final titer and productivity from 32.1 g/L and 0.31 g/L/h to 64.7 g/L and 0.77 g/L/h, respectively (Wang et al., 2017).

The relation between PA formation and glycerol consumption varies depending on the microorganism consuming this carbon source. A direct relation between the consumption of glycerol and PA formation was observed for *P. acidpropionici*, contrary to *P. freudenreichii* ssp. *shermanii* fermentation where the substrate consumption persisted even when the product formation ceased, showing the tendency of this bacterium to produce other secondary metabolites (Himmi et al., 2000). The PA/AA molar ratios for cultivations of *P. acidpropionici* and *P. freudenreichii* ssp. *shermanii* with glycerol were 5.7 and 3.8, respectively, while with glucose the ratios were 1.7 and 1.3, respectively. With the use of glycerol the yields were 0.79 and 0.58 mol/mol for *P. acidpropionici* and *P. freudenreichii* ssp. *shermanii*, respectively, which were 45 % and 49 %, respectively, higher than the yields obtained with glucose. These data indicate the homofermentative tendency of glycerol consumption and the advantage of using *P. acidpropionici* in propionic acid production (Himmi et al., 2000). PA fermentation with glycerol as carbon source without acetic acid formation was also reported for *P. thoenii* (Boyaval et al., 1994).

3.4.2. Nitrogen source

Complex nitrogen-containing mixtures like yeast extract, peptone and corn steep liquor are regarded as good nitrogen sources for enhancing PA production by *Propionibacterium* spp. (Ahmadi et al., 2017; Ranaei et al., 2020). Carbon: nitrogen ratio (C:N) is a usual indicator when following the effects of the carbon and nitrogen sources used in fermentation. **Paper I** revealed the rate of PA production to be significantly influenced by yeast extract concentration in batch cultivations. PA production by *P. acidipropionici* was enhanced after reducing C:N ratio by doubling the yeast extract concentration from 10 to 20 g/L at 60 g/L glycerol, increasing in this way PA productivity and yield by >1.5 fold from 0.21 to 0.37 g/L/h, and from 0.48 to 0.73 mol/mol (**Paper III**).

3.4.3. pH of the culture medium

Propionibacteria are sensitive to pH shifts, the optimal range for growth being 6.5-7. Acidic conditions below pH 5 inhibit the growth but the bacteria can still be metabolically active and produce propionic acid. pH control is of utmost importance during fermentations for reducing the inhibitory effect on the cell growth and product formation. pH between 5 and 6 was found to be beneficial for the production of PA by *P. acidipropionici*, enhancing PA/AA ratio, productivity and yield, while growth rate was benefited at pH higher than 6, as reported earlier (Ahmadi et al., 2017; Ranaei et al., 2020). PA/AA of 5.4:1 and productivity of 0.11 g/L/h were found in fermentations with *P. acidipropionici* at pH 5, while at pH 6.5 PA/AA decreased to 2.5:1 and productivity increase to 0.23 g/L/h (Seshadri and Mukhopadhyay, 1993). pH shift from 6.5 to 6.0 in *P. freudenreichii* fermentations enhanced PA titer from 14.6 g/L to 19.2 g/L and yield from 0.36 g/g to 0.48 g/g, respectively (Feng et al., 2010). Lactate exhibited better buffering effect compared with glycerol and molasses leading to slower drop in pH (Coral et al., 2008). A PA-tolerant strain of *P. acidipropionici*, obtained by adaptive evolution, was able to produce 10.9 g/L PA at pH 5 at a rate of 0.23 g/L/h while at pH 5.5 and 6 the growing strain produced similar amounts of PA at rates of 0.16 and 0.14 g/L/h, respectively (**Paper lII)**.

3.4.4. Cultivation temperature

Different temperatures for growth ranging from 14 °C to 40 °C and for PA production from 30 °C to 40 °C, have been investigated. Production of propionic acid is sensitive to temperature and despite few studies that stated the optimal temperature for *P. acidipropionici* to be 37 °C, it is now generally agreed that the optimal temperature for growth and propionic acid production is between 30-32 °C.

3.4.5. Substrate and product inhibition

Among the various organic acids examined, propionic acid was found to be the most inhibitory for the growth and survival of propionibacteria species (Rehberger and Glatz, 1998; Mosche and Jordening, 1999). The mathematical expression $\mu = \mu_{max} \times C/(C + K_M)$ developed by Monod in 1942 relates the growth of a microorganism (μ) with its half-saturation constant (K_M) for a given limiting growth factor as a carbon source (C). High concentrations of glycerol, lactate or glucose have been shown to affect the cell growth leading to low productivity and yield, decreasing the ratio of PA to other byproducts (Liu et al., 2012).

The effects of PA on cultivations of *Propionibacterium thoenii* were studied with increasing concentrations of the organic acid from 2.77 g/L to 30.41 g/L resulting in decreased microbial growth, productivity and substrate consumption rate (Gu et

al., 1998). The inhibitory effect of PA is explained by the protonated form of this organic acid which is lipophilic, and able to penetrate the cell membrane. Once inside the cell, protons are released from PA that lower the pH inside the cells leading to adverse osmolarity, instability of the metabolic machinery, inhibition of enzyme activity as well as destabilization and denaturation of protein structures. This inhibition is mainly detrimental for productivity and growth, but also for product recovery since other metabolites are formed at low pH and add to the separation costs. Nonetheless, pH shift strategy was used successfully for PA production where after acquiring a good biomass growth at pH 6.5, the culture was shifted to pH 6.0 in order to stimulate only the production of PA (Feng et al., 2010).

3.5. Fermentation modes for propionic acid production using propionibacteria

The main obstacles to be overcome in propionic acid production are the slow growth and end-product inhibition, that result in low productivity and product yields. The end-product inhibition can be addressed by different strategies e.g. by varying the modes of fermentation. Conventional batch fermentations are the most used mode of operation for PA production because the control of the process is relatively easy. The main limitations of this mode of operation are the time it takes for every production (two weeks), usually leading to low titers (<40 g/L), low yields (<0.5 g/g), and low productivities (<1 g/L/h) (Ahmadi et al., 2017). Batch cultivations of *P. acidipropionici* with corn steep liquor as carbon source gave high product yield and low productivity of 0.79 g/g and 0.005 g/L/h, respectively (Teles et al., 2019). One of the simplest designs for the batch fermentations is the Constant-Stirred Tank Reactor (CSTR). Among the first reported studies on PA production with *P. acidipropionici* with the use of a CSTR in continuous fermentation reported productivity and titer of about 0.125 g/L/h and 3.74 g/L, respectively (Clausen and Gaddy, 1984). Several years later, PA productivity and titer of 0.88 g/L/h and 13.9 g/L was reported in CSTR with immobilized bacteria (Wallenius et al., 2015).

Batch cultivations are often useful to find optimal substrates or cultivation parameters such as pH and temperature. Comparative batch fermentations of *P. acidipropionici* with glycerol and glucose showed a higher yield of 0.475 g/g with glycerol in contrast to 0.303 g/g obtained with glucose, while 0.572 g/g yield was obtained when both substrates were mixed for co-fermentation. The optimized use of the co-substrates with glycerol:glucose ratio of 4:1 (mol/mol) gave PA titer of 21.9 g/L in batch, which was ultimately enhanced to 29.2 g/L in fed-batch cultivation (Liu et al., 2011). Batch co-fermentations of glucose and glycerol mixtures with *Propionibacterium freudenreichii* subsp. *shermanii* gave higher

yields in the range of 0.54-0.65 g/g in comparison to the use of either carbon source alone (Wang and Yang, 2013).

Certain complex agriculture carbon sources have been tried in batch fermentations as well. Batch fermentation with corn mash sugar as carbon source reached PA yield of 0.55 g/g, which was considered nearly competitive in the market since the media costs would be reduced to less than $1.00 per kg (Rodriguez et al., 2014). One of the best applications of batch cultivations was achieved using enzymatically treated corn mash (ECM) with cyanocobalamin, giving productivity and yield of 0.5 g/L/h and 0.60 g/g, respectively. The low cost of the media would thus narrow the gap between biotechnology and petrochemical production of PA, which was estimated at 0.82 USD/kg in 2017 (Stowers et al., 2014).

To overcome the effect of substrate inhibition, a good strategy is to run the cultivations in fed-batch or continuous mode. The use of wild type *P. acidipropionici* with lignocellulosic biomass as carbon source showed promising results; when cultivated with corn stover hydrolysate, PA titer and productivity were enhanced from 32.1 g/L and 0.31 g/L/h in batch to 62.7 g/L and 0.45 g/L/h, respectively, in fed-batch fermentation (Wang et al., 2017). The evaluation of glucose, xylose and arabinose as the main components of hemicellulose for PA production by *P. acidipropionici* in batch cultivations gave titers of 13.4, 13.3 and 13.8 g/L, respectively (Liu et al., 2012). Fed-batch fermentation with xylose, the major component of hemicellulose, gave an enhanced titer of 53.2 g/L, and further use of corncob molasses (a byproduct from xylitol production) as representative of hemicellulose hydrolysate with xylose as the major component (~ 450 g/L) in fed-batch cultivation gave 71.8 g/L PA (Liu et al., 2012). Similar improvements have been obtained using developed strains, as an engineered *P. acidipropionici* cultivated with glycerol as carbon source increased the PA titer from 19 to 106 g/L when going from batch to fed-batch cultivation, while maintaining the product yield of 0.55 g/g in both modes of operation (Zhang and Yang, 2009). Similar phenomenon was obtained when an acid-tolerant strain of *P. acidipropionici* showed an increase in PA titer from 28.5 to 44.6 g/L from batch to fed-batch cultures, while the productivity and yield remained practically the same (Zhu et al., 2010).

A high cell density (HCD) fermentation with an acid-tolerant strain of *P. acidipropionici* showed an improvement of PA titer from ~ 40 g/L in batch to > 55 g/L with a productivity of 2.23 g/L/h (Wang et al., 2015). Despite the promising results obtained at bench scale, no significant enhancement was achieved when an acid-tolerant strain of *P. acidipropionici* was used in a scaled-up fermentation of 10 000 L, yielding a PA titer of 47.28 g/L after 240 h of fed-batch mode with glycerol (30 g/L).

Few studies have been conducted on PA production using continuous fermentation systems (Ahmadi et al., 2017). A drop in PA titer from 22.9 to 7.3 g/L while an

enhancement in productivity from 0.24 to 0.42 g/L/h were obtained for fermentations with *P. acidipropionici* in batch and continuous mode cultivation, respectively, using glucose and xylose at a ratio of 3:1 (Carrondo et al., 1988). In another study, both yield and productivity were enhanced from 51 % and 0.312 g/L/h to 54 % and 0.715 g/L/h, respectively, in batch and continuous fermentations using cheese whey for PA production by *P. acidipropionici* (Gupta et al., 2001). The continuous cultures also need more control over long periods of time.

Table 3.1 summarizes PA production in batch, fed-batch and continuous fermentations reported in literature. Batch mode of operation was demonstrated to be good for PA production with moderate concentrations of carbon sources. High loads of glycerol, lactate or glucose affected the cell growth, leading to production parameters being affected as well (Liu et al., 2012). The ratio between PA and byproducts increases as concentration of PA becomes higher. The more the byproducts produced the more compromised is the PA yield.

Table 3.1 Comparative results from the literature about PA production by *P. acidipropionici* and other propionibacteria in batch, fed-batch and continuous modes of operation

Strain	Mode of operation	Carbon source	pH	Qp (g/L.h)	Ymol/mol	Titer (g/L)	Reference
P. acidipropionici	Batch	Corn stover hydrolysate	6.0	0.31	0.47 g/g	32.1	Wang et al., 2017
	Fed-batch			0.20	0.45	62.7	
	High-cell density fed-batch			0.50	0.77	64.7	
P. acidipropionici	Batch	Lactose	6.5	0.23	0.435	20.75	Goswami and Srivastava, 2000
	Fed-batch			-	0.364	26.3	
P. acidipropionici	Batch	Wood hydrolysates	6.0	0.24	55%	22.9	Carrondo et al., 1988
	Continuous			0.42	-	7.3	
P. acidipropionici	Batch	Corn steep liquor	6.5	0.005	0.79	-	Teles et al., 1999
Engineered *P. jensenii*	Fed-batch	Lactate	6.5	0.146	-	34.93	Liu et al., 2016
Engineered *P. acidipropionici*	Batch	Glycerol	7.0	0.026	0.55	19.3	Zhang and Yang, 2009
	Fed-batch in FBB			-	0.56	106	
P. acidipropionici	Sequential batch	Sorbitol	6.5	0.5-0.6	1.29-1.67	34.4-39.5	Duarte et al., 2015
P. acidipropionici	Sequential batch	Glycerol	6.5	0.88	0.84	43.8	Dishisha et al., 2013
P. acidipropionici	Batch	Glycerol	6.5	0.108	0.475	18.1	Liu et al., 2011
		Glucose		0.068	0.303	11.5	
P. acidipropionici	Batch	Arabinose		-	-	13.8	Liu et al., 2012
	Fed-batch	Xylose	6.0	0.23	-	53.2	
		Corncob molasses		0.28	-	71.8	
Propionibacterium freudenreichii subsp. *shermanii*	Batch	Glucose	6.5	0.19	0.39	-	Wang and Yang, 2013
		Glycerol		0.11	0.65	-	
		Glycerol/glucose (1-1/5)		0.19-0.25	0.54-0.65	-	
Acid-tolerant *P. acidipropionici*	Batch	Glycerol	7.0	0.19	0.57	28.53	Zhu et al., 2010
	Fed-batch			0.20	0.56	44.62	
P. acidipropionici	Batch	Soy molasses	6.5	0.35	0.39	21.9	Yang et al., 2018
	Sequential batch			0.81	0.42	-	
P. acidipropionici	Batch	Lactate	-	0.11	0.44	15.06	Coral et al., 2008
		Glycerol		0.051	0.72	6.77	
P. acidipropionici	Batch	Glycerol	6.8	0.11	0.68	13.6	Kośmider et al., 2010
P. acidipropionici	Immobilized cell Recycle batch	Glycerol	6.5	0.35	0.59	35.2	Dishisha et al., 2012
P. acidipropionici	Continuous	Glycerol		0.82	-	-	Wallenius et al., 2015

3.6. Biosynthesis of trehalose in propionibacteria

Propionibacteria are also known for the production of trehalose, a non-reducing disaccharide composed of two molecules of glucose (IUPAC name: α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) (Figure 3.3). The 1-1 α-bond is responsible for certain peculiar characteristics like resistance to high temperatures and acidic conditions. It creates closed structures with high-water retention capacity that protect proteins and other biomolecules in drought conditions.

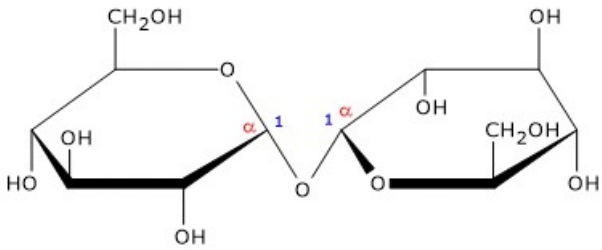


Figure 3.3 Trehalose structure

There are six known metabolic routes for the biosynthesis of trehalose, of which three are present in bacteria (Fig. 3.4). OtsA-OtsB is a two-step route, which starts from nucleoside phosphate-activated glucose (UDP-glucose, ADP-glucose, GDP-glucose or TDP-glucose) and glucose-6-phosphate, producing trehalose-6-phosphate (while releasing the nucleoside phosphate-activated glucose), which is further converted to trehalose and inorganic phosphate by the action of trehalose 6-phosphate synthase (OtsA) and trehalose 6-phosphate phosphatase (OtsB), respectively. Route TreY-TreZ makes use of maltodextrins, starch-, glycogen- or maltooligosaccharides, to form maltooligosyl-trehalose, and then trehalose by the action of maltooligosyl-trehalose synthase (TreY) and maltooligosyl-trehalose trehalohydrolase (TreZ), respectively. Trehalose synthesis routes TreS, TreP and TreT are out of the scope of this thesis. Certain microorganisms that are able to use trehalose as carbon source, have trehalase enzyme (TreH) to cleave trehalose into two glucose molecules. The presence of more than only one pathway for trehalose synthesis may respond to the requirement of accumulating a carbon source for a carbon-limited unfavorable condition (Boch et al., 2006).

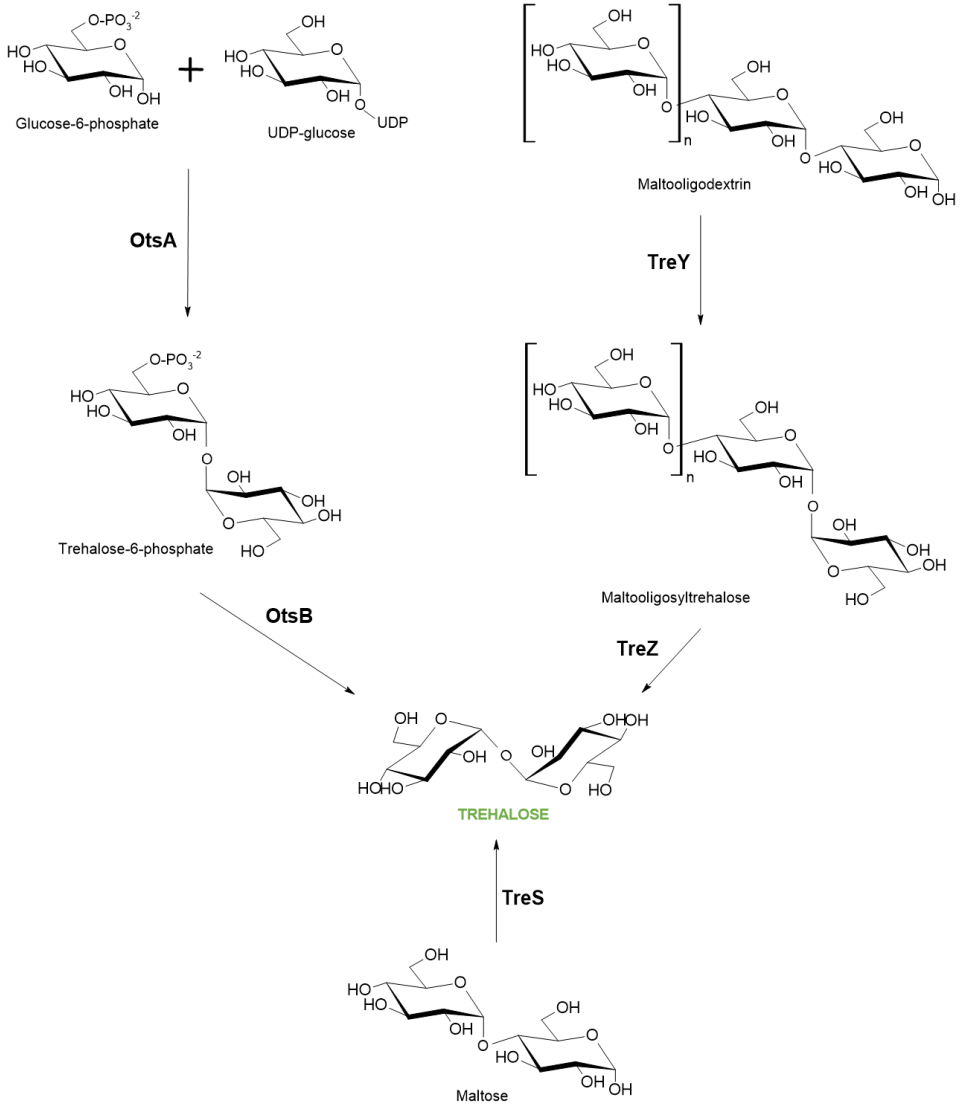


Figure 3.4 Bacterial trehalose synthesis metabolic pathways. Trehalose is synthesized either from glycolytic intermediates via the OtsA-OtsB pathway, from alpha glucans via the TreY-TreZ pathway or via isomerase, trehalose synthase TreS. (Adapted from Kalscheuer and Koliwer-brandl 2014)

Trehalose production is widely distributed in several species of *Propionibacterium* genus. Cultivations of *Propionibacterium freudenreichii* ssp. *shermanii* showed OtsA-OtsB and TreS to be responsible for trehalose biosynthesis. Use of lactose was more beneficial than lactate for the synthesis of this disaccharide, which was more advantageous for cell growth. Also, certain suboptimal conditions like low pH and

osmotic stress triggered trehalose accumulation (Cardoso et al., 2004; 2007). The ability of several strains belonging to *Propionibacterium* genus to synthesize trehalose from glycerol has been confirmed. *P. freudenreichii* ssp. *shermanii* was able to produce 195 mg/L of trehalose when grown in a medium with 8 % glycerol (Pawlicka-Kaczorowska and Czaczyk, 2017). Production of trehalose and the expression of *otsA* and *treY* were studied as a response to stress factors like NaCl and citric acid (**Paper ll**) and in the PA tolerant strain obtained by adaptive evolution cultivated with PA-containing medium (**Paper lll**).

Chapter 4. Laboratory evolution of propionibacteria for propionic acid fermentation

Natural selection is the evolutionary process through which the fittest organism survive in a certain environment and becomes dominant in the population. Natural selection Darwinism considers environmental pressure as the only driving force for selection of the best phenotype.

The need for developing strains for biotechnological purposes is addressed through different approaches to manipulate the culture conditions in which microorganisms grow and develop desired profiles under laboratory-controlled conditions. Evolution experiments usually select the mutants with the most increased growth kinetics in comparison to their parental strains, whereas for real applications that is not always the case since high yields, for instance, are needed more than the simple high growth rate (Lenski, 2017). Clonality and periodic selection were thought to be the only mechanisms driving adaptation, evolution and speciation in prokaryotes. Nonetheless, with the advances in molecular tools it became possible to adapt an organism to different conditions e.g. through mutagenesis. In this way, the way certain proteins evolve as response to selective pressure has been better understood (Gogarten et al., 2002). Hence, evolutionary theory, phylogenetic reconstruction, structural information, and protein engineering, along with information obtained from the metabolism, physiology, and ecology, are all needed to understand adaptation at the molecular level (Golding and Dean, 1998).

4.1. Adaptive Laboratory Evolution (ALE)

Laboratory defined and controlled conditions aiming to select a certain strain with an improved trait/phenotype through serial batch cultivations is called adapted laboratory evolution (ALE). Analyses of evolutionary phenomena both at molecular and phenotypic levels are followed sequentially as the culture conditions are changed during the approach to obtain a desired strain. The evolved phenotypic changes in ALE are associated with the changed growth environment which leads

to proper selection of a mutant strain with the desired enhanced traits. During the exposure to a certain selective pressure the culture is not homogeneous at any point and several clones compete for dominance in the bacterial population. ALE relies on the adaptation a strain develops during thousands of generations throughout several serial cultivations in which the laboratory-imposed condition is the selective pressure for the fittest mutants to become the dominant cells in the population. ALE does not require *a priori* knowledge of the genotype-phenotype relationship, as needed in rational strain engineering, and is a useful complement to metabolic engineering work (Dragosits and Mattanovich, 2013; Godara and Kao, 2020).

Prolonged periods of cultivation ranging from weeks to even years mean hundreds to thousands of generations pass before the selection of an improved phenotype. The withdrawal of cells should be done while they are still in exponential growth, with a highly active metabolism, when the adaptation is directly related to nutrient consumption, but also could be done in the stationary phase when the adaptation does not depend on nutrients and a phenomenon called growth advantageous stationary phase (GASP) is taken into consideration. In principle, dependence between growth and product formation is required for ALE to be useful to isolate an enhanced phenotype for further strain improvement. This growth-coupled phenotype includes the consumption of certain substrate and tolerance to inhibitors (Godara and Kao, 2020), as in the case of end-product inhibition provoked by PA. Protective molecules developed during cultivations in stressful environments can be enhanced with environmental engineering as part of ALE where the selective pressure conditions of cultivation are set during the long time of serial cultivations (Godara and Kao, 2020). **Paper lII** involves the adaptation of *P. acidipropionici* to high PA concentrations added to the culture medium.

Adaptive evolution was, for a long time, regarded as the postmutational sorting process of natural selection where the changes in mutation rate could be either environment-related or heritable (Metzgar and Wills, 2000). When a microorganism is cultivated in non-optimal or stressful conditions, its DNA undergoes mutations as a result of damage. There are different mechanisms to protect and correct DNA mutations caused under stressful circumstances. Interestingly, some of these mechanisms are known to increase the mutagenesis rate at the same time. These mechanisms found in *Escherichia coli* and other bacteria include the SOS response regulation, which is an evolved inducible control system that promotes multiple proteins to maintain the integrity of the DNA. SOS response is known to be induced by accumulation of single-stranded DNA during the replication of damaged DNA, also induced by base-dimers like pyrimidine dimers and even by other endogenous mechanisms that damage DNA, triggering in these ways the SOS response regulation. The adaptation to new environments with various types of stress conditions involves response pathways as the control of SOS factors increasing the mutation rates (Tenaillon et al., 2000; Maslowska et al., 2019).

Other stresses like starvation, high osmolarity, low pH and low temperature induce the RNA polymerase sigma S (RpoS) regulon which activates the anti-mutator mismatch repair system (MRS) and the expression of *dinB* coding for translesion polymerase synthesis that tolerates DNA lesions, being in this way an error-prone DNA polymerase (Tenaillon et al., 2000; Kai and Wang, 2003), contributing in this way to the generation of stress-induced mutations. Stresses have also been shown to induce the mobility of transposons and insertion sequences, leading in this way to gene inactivation or activation (Tenaillon et al., 2000). Under stress conditions, these systems increase the mutagenesis rate due to a low fidelity in pairing affinity, as of the error-prone DNA polymerase, meaning they are a source of genetic variability which enhance the adaptation of the cells in a stressful environment (Tenaillon et al., 2000; Metzgar and Wills, 2000). Therefore, a new phenotype is produced not only by the mutational events caused by external aggressions but also by a differential regulation of gene expression (Massey and Buckling, 2002).

Mutagenesis under stress conditions is adaptive (Tenaillon et al., 2004) and the constant exposure to stressful environments drives the mechanisms underlying the generation of phenotypic variations, increasing in this way the probability of a successful phenotype. The range and repeatability of the exposure critically influence the molecular mechanisms, including the repairing mutational events (as described above) and differential regulation of gene expression (Massey and Buckling, 2002). Adaptive mutations can affect the physicochemical properties of the protein products of the genes affected, but on the other hand the level of expression of proteins is another alternative consequence of the adaptive mutations (Babu and Aravind, 2006). The PA-tolerant strain of *P. acidipropionici* obtained by adaptive evolution showed an increased expression of the trehalose-metabolism involved gene *treY* of 82.32-fold compared with the 16S rRNA gene under the same conditions (**Paper lII)**.

4.2. PA production by tolerant strains

Depending on the strategy to adapt propionibacteria, few studies have shown the feasibility of using adapted strains to high PA concentrations, making them promising resources/systems/strategies to improve PA production (Woskow and Glatz, 1991; Paik and Glatz, 1994; Suwannakham and Yang, 2005; Zhu et al., 2010). Sequential batch cultivations of *P. acidipropionici* were carried out during 1 year with stepwise increased PA concentrations from 0.5 % to 5 % to obtain an acid-tolerant strain. Such a tolerant strain was used in PA production with a maximum enhanced titer of 47 g/L in semicontinuous fermentation (Woskow and Glatz, 1991). The same tolerant strain was also immobilized in calcium alginate beads for batch, fed-batch and continuous PA production with a higher titer of 57 g/L PA in fed-batch fermentation (Paik and Glatz, 1994). Zhu et al. (2010) used the method

described by Woksow and Glatz (1991) to obtain a *P. acidipropionici* tolerant strain with the ability to grow in the presence of 20 g/L PA with productivity and titer of 0.20 g/L/h and 44.62 g/L, respectively, to be further used in a 10 000 L bioreactor in which PA concentration of 47 g/L was reached. After about 900 h of fermentation in a fibrous bed bioreactor (FBB), an adapted strain of *P. acidipropionici* was isolated, showing a reduced growth rate to 50 % when cultivated in 10 g/L PA-containing medium while in similar conditions of cultivation the wild type showed a 70 % reduction in the growth rate (Suwannakham and Yang, 2005). The reason behind the enhanced PA tolerance was found to be the mutations in two key enzymes in PA production, oxaloacetate transcarboxylase and propionyl CoA: succinyl CoA transferase. Both enzymes showed higher specific activity and lower sensitivity to PA in the adapted strain (Suwannakham and Yang, 2005).

Knocking out genes involved in certain pathways is a common practice in engineered strains when one related pathway needs to be favored. In this way, acetate formation was blocked by the inactivation of the acetate kinase gene (*ack*) in a *P. acidipropionici* mutant (ACK-Tet), favoring in this way the production of PA against acetate formation (Suwannakham et al., 2006: Zhang and Yang, 2009). After fed-batch cultivation of the ACK-Tet mutant immobilized in a FBB for 4 months, PA titer reached 106 g/L, meaning the tolerance to PA was enhanced as the ACK-Tet mutant was adapted. Such adapted ACK-Tet mutant in free-cell cultivation showed higher growth rate, PA titer and productivity of 0.16 h^{-1} vs. 0.05 h^{-1}, 26 g/L vs. 19 g/L and 0.10 g/L/h vs. 0.03 g/L/h in comparison with the non-adapted ACK-Tet mutant, respectively. Interestingly, the production of acetic acid was seen to have increased again, although not to a high level, still leaving PA as the main product with a 3-fold higher productivity (Zhang and Yang, 2009).

Acetic acid synthesis is coupled with ATP formation which supplies energy for biomass and the antiport action by H^+-dependent ATPase for proton pumping, in this way keeping normal intracellular pH gradient, counteracting the increased concentration of protons inside the cell (Zhang and Yang, 2009). The biosynthesis of H^+-dependent ATPase was increased in an acid-tolerant *P. acidipropionici* strain, decreasing the intracellular protons and leading to an increase of NAD^+/NADH (Guan et al., 2013). A slightly lower membrane-bound ATPase activity together with higher and lower levels of long-saturated chains of fatty acids and unsaturated fatty acids, respectively, was observed by Suwannakham and Yang (2005), decreasing in this way the membrane fluidity as a response by an acid-tolerant *P. acidipropionici*. The addition of arginine, aspartic acid and glutamic acid was also suggested to help acidic tolerance by consuming more protons and generating more ATP (Guan et al., 2013). Deamination and decarboxylation in amino acid catabolism have been identified in *Propionibacterium* spp. as part of acid tolerance mechanisms, *P. acidipropionici* contains both mechanisms without antiporters which suggests a less specialized acid tolerance mechanism associated with Gram-positive bacteria (Guan et al., 2013; Guan and Liu, 2020). The engineered *P.*

acidipropionici acid-tolerant strain previously used by Zhang and Yang (2009) was serially adapted in FBB to obtain a PA tolerant strain which maintained ~ 60 % of its specific growth rate (0.15 vs. 0.24 h^{-1}) when cultivated with 20 g/L PA, while the specific growth rate of the wild type decreased from 0.19 to 0.04 h^{-1}, meaning ~ 80 % growth rate loss at the same PA concentration (Wang et al., 2015). Sequential batch high cell-density fermentations of this adapted strain at pH 6.5 produced 40 g/L of PA at a rate of 2.98 g/L/h with a yield of 0.44 g/g, while at lower pH values of 5.0 and 5.5 the yield increased to 0.53 and 0.62 g/g, respectively, with a decrease of productivity to 1.28 g/L/h. This adapted strain was finally used in a three-stage simulated fed-batch in serum bottles, with 30 g/L of glucose in each stage, obtaining an increase of PA titer from 20 g/L to 40 g/L, and finally to 50 g/L. The overall titer, yield, and productivity of 49.2 g/L, 0.53 g/g and 0.66 g/L/h, respectively, were among the highest values obtained in free-cell PA production (Wang et al., 2015). Similarly, after increasing the cell density of *P. acidipropionici* in a FBB by repeated batch cultivations, fed-batch mode was applied by pulse-feeding to keep glucose concentration right above zero, while the PA concentration became stable, with titer and productivity of 51.2 g/L and 0.71 g/L/h, respectively (Zhu et al., 2012). A summary of the use of PA adapted strains of *P. acidipropionici* is presented in Table 4.1.

Table 4.1 Adapted strains of *Propionibacterium* isolated from adaptive evolution-related procedures

Strain	Time spent for adaptation	Max. PA concentration tolerance	Method of adaptation	Q (g/L/h)	Yield (g/g)	PA titer (g/L)	Reference
P. acidipropionici	1 year	0.5 g/L [(1)]	Sequential increase of PA concentration in stepwise batch cultivations	-	-	47	Woskow and Glatz, 1991
						57	Paik and Glatz, 1994
	-	20 g/L [(1)]		0.20	-	44.62 (fed-batch)	Zhu et al., 2010
	ca 15 weeks	20 g/L [(1)]		0.52	-	16.8	(**Paper III**)
	ca 900 h	10 g/L [(1)]	Adaptation in FBB	-	-	-	Suwannakham and Yang, 2005
	4 months	106 g/L [(2)]		0.10	-	26	Zhang and Yang, 2009
P. acidipropionici (Engineered tol-strain)	120 h	20 g/L [(1)]		1.28	0.62	-	Wang et al., 2015
				0.66	0.53	49.2	
	>200 h	51.2 [(2)]		0.71	-	51.2	Zhu et al., 2012

[(1)] initial concentration
[(2)] final concentration or titer while being adapted

4.3. Genetic- and metabolic engineering

Molecular tools like genetic and metabolic engineering are used to enhance productivity and yield of PA, parallelly to PA-tolerant strains obtained by ALE that exhibit enhanced production due to low sensitivity to end-product inhibition.

The key enzyme for the use of glycerol as carbon source is glycerol dehydrogenase (GDH), while malate dehydrogenase (MDH) and fumarate dehydrogenase (FUM) are the key enzymes more closely related to the Wood-Werkman cycle. The overexpression of other enzymes in the Wood-Werkman cycle demonstrated increase in the production of PA from glycerol and other carbon sources. The biotin-dependent enzymes pyruvate decarboxylase (PYC), methylmalonyl-CoA carboxytransferase (MMC) and methylmalonyl-CoA decarboxylase (MMD) isolated from *P. acidipropionici* were overexpressed in *P. freudenreichii* ssp. *shermani* showing enhanced propionic acid production (Wang et al., 2015). These *P. freudenreichii* ssp. *shermani* mutants overexpressing MMC and MMD favored PA production by 14 % and 17 % increase in yield and productivity, respectively, while the mutants that overexpressed PYC produced more succinate, and the PA productivity was lowered by 12 %. These experiments, carried out with glucose and mixtures of glucose-glycerol, demonstrated *mmc* and *mmd* genes as the clue targets for metabolic engineering of propionibacteria in order to enhance PA production (Wang et al., 2015).

Combined strategy of metabolic engineering and adaptive evolution was carried out by Liu et al. (2020) to obtain a mutant strain of *P. acidipropionici* with *mmc* gene overexpressed. Such a mutant strain exhibited increase in growth rate up to 3.5-fold, titer and productivity of PA increased by 37.1 % and 37.8 %, respectively, in comparison with the wild type bacteria. The regeneration rates of the key cofactors involved in the production of PA, NAD^+/NADH, ATP/ADP and CoA, determine the rate of PA production and is the focus of metabolic engineering for enhancement of such production (Liu et al., 2012). Increasing the expression of genes involved in the production of pyruvate decarboxylase, succinate-CoA or propionyl CoA transferase, the enzymes involved in the Wood-Werkman cycle, gave promising results in terms of directing the carbon flux towards a more efficient conversion of the substrate to the biosynthesis of PA by propionibacteria (Es et al., 2017).

Acetic acid (AA) and succinic acid (SA) are the two byproducts that can be reduced during PA production. Two *ack* knocked-out mutants of *P. acidipropionici*, obtained through disruption of acetate kinase gene (*ack*), showed increased propionic acid yield to 0.45 g/g from 0.40 in wild type strain cultivation. Although *ack* disruption alone did not completely eliminate acetate production, the propionate yield was increased by ca. 13 % and acetate yield reduced by ca 14% (Suwannakham et al., 2006).

Propionibacterium jensenii was demonstrated to be a good candidate for PA production after being transformed with a *P. jensenii -E. coli* shuttle vector constructed with the isolated plasmid pZGX01 from *P. acidipropionici*, the *E. coli* pUC18 plasmid and a chloramphenicol resistance gene. Overexpressing the glycerol dehydrogenase-encoding gene (*gldA*) from *Klebsiella pneumoniae* in the *P. jensenii -E. coli* shuttle vector increased the PA production to 28 g/L in comparison from 22 g/L in the wild type (Zhuge at al., 2013). Later, *ppc* gene from *K. pneumoniae* was overexpressed in *P. jensenii* accessing in this way the direct synthesis of oxaloacetate from PEP, and avoiding the Pyr intermediate formation. Also, genes encoding lactate dehydrogenase (*ldh*) and pyruvate oxidase (*poxB*) were deleted to block the synthesis of the byproducts lactic and acetic acid, respectively. The PA titer was increased from ca 27 to 33.21 g/L in the *ppc* overexpressed strain, and to 30 g/L in the strain with *ldh* deletion, whereas *poxB* deletion decreased it (Liu et al., 2016).

Chapter 5. High Cell Density Fermentations for Propionic Acid Production

Microbial metabolism is a highly regulated process, e.g. at any given time only the necessary enzymes are made, and once a sufficient quantity of a compound is made, synthesis of the enzymes involved is stopped and the activities of the enzymes already formed are reduced by a number of specific regulatory mechanisms such as feedback inhibition. Some metabolites have also other inhibitory effects e.g. through lowering the pH or affecting the membrane integrity leading to lower or no cell growth. Moreover, anaerobic microorganisms are slow growers, as the fermentation in the absence of oxygen yields only limited amount of energy as a result of internally balanced oxidation-reduction reaction. Hence, for overproduction of microbial metabolites, strategies to overcome the inhibitory effects need to be implemented. The concept of high cell density fermentation is well known, which involves developing high cell mass by a certain mechanism that can be repeatedly used for the production of target metabolites, without having to start from a new inoculum and avoiding the long lag phase. High cell density cultures also have the advantage of reducing the risk of contamination, and tolerance of the microorganism is enhanced towards end products and other chemical components that may cause inhibition in a normal free cell cultivation. Both free cells and immobilized cells can be used to perform high cell density fermentations.

5.1. Sequential and Cyclic Cultivations

Sequential batch cultivation with cell recycle simply involves recovery of cell biomass after a batch cultivation process and its use for the subsequent cultivation in a fresh medium. This was nicely demonstrated in case of propionic acid production from glycerol with *P. acidiporopionici* in a medium with potato juice as the nitrogen source; the cells were separated by centrifugation under sterile conditions and recycled 11 times using glycerol and potato juice as carbon and nitrogen/vitamin source, respectively (Dishisha et al., 2013). During the first batch cultivation with glycerol at 50 g/L, the PA titer obtained after more than 120 h of

cultivation was ~ 28 g/L of which nearly 50 % was produced during stationary phase, implying that the acid production was partially uncoupled from the cell growth. In subsequent batches, the amount of cells and fermentation rates increased from batch to batch. After 9 batches, cell concentration was increased 215 times to 21.5 g/L from 0.1 g/L in the first batch, and PA productivity was increased 6-fold to 1.35 g/L/h. Sequential batch fermentations were also performed with stepwise increase in glycerol : yeast extract concentrations, from 60:20 g/L to 150:50 (**Paper I**).

Cyclic batch fermentation with cell recycle is similar to the sequential batch fermentation except that about 90 % of the culture broth is replaced after batch fermentation finishes with an equal volume of fresh medium to start a new batch cultivation. This is useful for removal of the inhibitory products and retaining cells that are already adapted to the fermentation conditions and would result in higher fermentation rates. The withdrawn culture broth can be used for product recovery. Cyclic batch fermentation was carried out for PA production from glycerol with *P. acidiporopionici* (**Paper 1**).

5.2. Immobilized cells

Immobilization is the physical confinement of viable microbial cells on the surface and/or inside the pores of a solid carrier; the bound cells exhibit different hydrodynamic characteristics from the planktonic cells of the surrounding environment (Zur et al., 2016). Cell immobilization for microbial fermentations started with vinegar production in 1823 by Scheutzenbach, according to Dervakos and Webb (1991). During the second half of the twentieth century, immobilization of enzymes and whole cells turned into a very active field and number of processes were developed to industrial scale. The first polymers used for immobilization were polyacrylamide and diethylaminoethyl-cellulose (DEAE), the latter being first industrialized by Spezyme® as a carrier for beer industry used by Sinebrychoff brewery in Finland for maturation and by the Bavaria brewery in Holland for alcohol-free beer production (Norton and Damore, 1994), although Chibata and Tosa (1980) state the first industrial application of immobilized microbial cells made use of polyacrylamide for the production of L-aspartic acid by Tanabe Seiyaku Co. Ltd, of Osaka, Japan. The current major applications of immobilized cell systems include the production of metabolites like antibiotics, organic acids, amino acids, alcohol, acrylamide, etc. (Zur et al., 2016). Nonetheless, there are limitations caused by immobilization that can produce some negative effects in the metabolism as changes in the cell morphology, disturbances in growth and production pattern (Dervakos and Webb, 1991). Other phenomena like surface tension or osmotic stress, decreased water activity, altered membrane permeability and diffusion are to be considered when choosing the matrix for immobilization.

5.2.1. Mechanism of immobilization and support carriers

General techniques used for immobilization include flocculation, adsorption on surfaces, covalent bonding to carriers, cross-linking of cells, entrapment, encapsulation and nanocoating (Zur et al., 2016) (Figure 5.1). Cells are commonly immobilized by entrapment in polymer gels e.g. calcium alginate or carrageenan, and adsorption on an inert support e.g. positively charged materials (Norton and Damore 1994; Paper III). In principle, the hydrodynamics of the immobilized cells in a matrix is different from the surrounding media (Dervakos and Webb, 1991). Depending on the support, the environment of the immobilized cells can strongly be affected by mass transfer limitation, producing different microenvironments with different concentrations of substrate and products, leading in this way to different growth and production kinetics (Walsh and Malone, 1995). Low water activity inside the matrix could limit the growth rate but at the same time the maintenance metabolism can be improved to ideally reach a state where cells are product factories using the energy and matter almost exclusively for the production of the desired metabolite (Zur et al. 2016).

Factors affecting cell immobilization can be divided according to the support surface characteristics such as roughness, porosity, hydrophobicity, functional groups on the surface and toxicity, and the microorganism including the culture environment e.g. pH, temperature, presence of ions, flow of the medium, nutrients, rheology of the culture medium and dissolved oxygen, and the cell characteristics as EPS production, physiological state and surface proteins (Zur et al., 2016). For industrial scale use, inexpensive immobilization materials as well as bioreactor systems are needed (Liu et al., 2012).

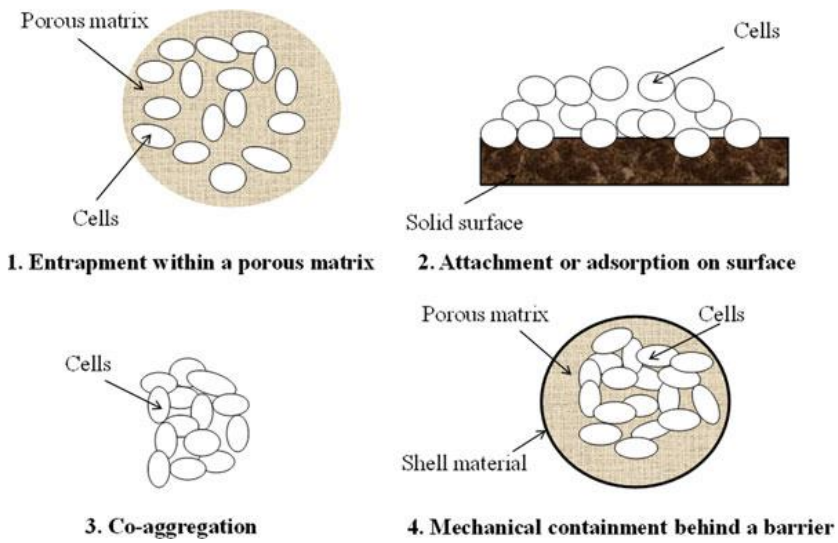


Figure 5.1 Different methods for cell immobilization (Adapted from: Gallo et al., 2016)

The most common materials for immobilization include calcium alginate, calcium polygalacturonate and cotton fiber (Liu et al., 2012; Chen et al., 2013). Gel beads containing cells could become too weak and easily damaged when operating in large plug flow bioreactors and for long periods in stirred bioreactors (Colomban et al., 1993). Different types of bagasse have been tried as supports, e.g. sorghum bagasse for immobilization of *S. cerevisiae* to produce ethanol (Yu et al., 2007; 2010), sugarcane bagasse used in a plant fibrous-bed bioreactor, for PA production by *Propionibacterium freudenreichii* (Chen et al., 2013). In the latter case, the fiber surface was even considered as a carbon source for the bacteria, which showed increment in the fluxes of PA biosynthesis and pentose phosphate pathway by 84.6 and 227.6 %, respectively, and enhanced the growth rate to produce the high titer of 136 g/L in fed-batch (Chen et al., 2013). Supports should be of high porosity and wide surface to allow high loading of cells, leading to efficient immobilization that would resist any kind of acceptable flow of the medium. *P. acidipropionici* immobilized in a xylan hydrogel matrix, reaching dry cell density of 99.7 g/L at high dilution rates with PA productivity of 0.88 g/L/h (Ranaei et al., 2020). According to Chen et al. (2013), cotton fiber does not have those qualities, making it not very useful to immobilize bacteria, nonetheless, fibrous-bed bioreactor (FBB) and multipoint fibrous-bed bioreactor (MFB) were constructed by packing spiral cotton towel into a glass column and used for immobilizing propionibacteria for PA production (Suwannakham and Yang, 2005; Feng et al., 2010). Supports for fibrous-bed bioreactors have been made of different materials ranging from synthetic inorganic polymers to plant-based bagasse.

In **Paper II** and **III**, Poraver® (6 to 8 mm in diameter), obtained from Dennert Poraver (Postbauer-Heng, Germany), and AnoxKaldnes® carriers for water treatment (Veolia Water Technologies) were used as materials with wide surface and highly porous areas, respectively, qualities that enhance the attachment of the cells for long periods of time (Figure 5.2). Poraver® is a highly porous light material made of post consumer glass treated at high temperature, its surface roughness, porosity and the amount of surface Mg^{2+} were found to be determinant for anaerobic bacterial consortium immobilization, nonetheless mass transfer limitations were seen to be originated by biomass accumulation (Pereira et al., 2000). Poraver® was used for the immobilization of sulphate reducing bacteria from a sludge environmental sample for sulphide production in anaerobic packed bed reactor obtaining good operation stability (Alvarez et al., 2006). AnoxKaldnes® carriers are designed for organic matter removal, (de)nitrification and detoxification by different microorganisms, ranging from bacteria to ciliates and rotifers, including consortia that are established in biofilms in wastewater treatment.

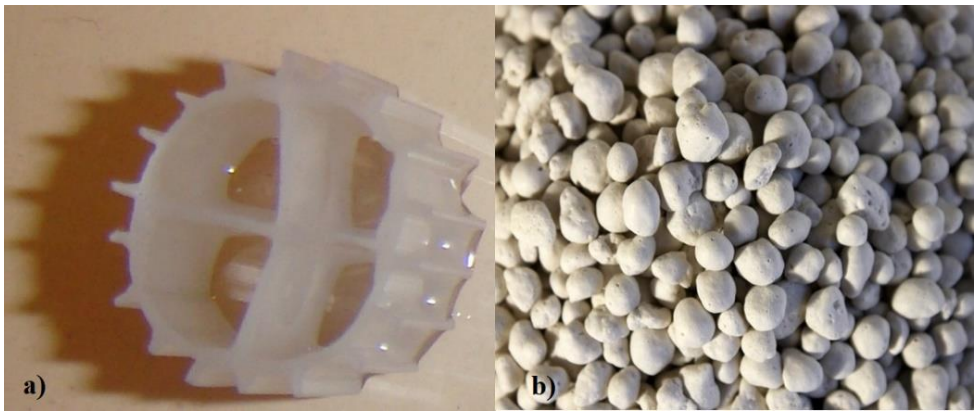


Figure 5.2 Supports used for the different experiments with immobilized cell bioreactors. a) AnoxKaldnes® (photo: Rossoni L.) and b) Poraver® beads (taken from https://materials.soa.utexas.edu/search/materials/details/t/product/id/3604)

Immobilization can be mediated by natural events or by the addition of certain chemicals to make cells interact with the surface of the support. The direct interaction of the cells with the support is defined as adsorption, and in principle, only cell monolayers are considered as adsorbed onto a support material (Norton and Damore, 1994). Adsorption may be achieved without addition of any chemical to mediate the interaction between the cells and the support; natural adsorption is popularly utilized in wastewater treatment (Groboillot et al., 1994). Porous materials with large surface areas as support materials enhance the adsorption of cells. The time needed for the cells to be adsorbed onto the support is an important factor; longer the time the more aged and dead cells are accumulated, which is detrimental for bioprocesses (Es et al., 2017). In the same way, packed-bed and membrane bioreactors tend to gradually accumulate dead cells leading to a decrease in their metabolic activity.

Immobilized cells are known to be more effective for bioremediation and biotransformation, which is attributed to the adsorption of toxic and pollutant molecules to the matrix, and the close vicinity to the immobilized cells facilitates adaptation of the cells and degradation of the compounds, contrary to what happens with planktonic cells (Zur et al., 2016). Certain chemicals and polymers mediate immobilization e.g. polyethyleneimine (PEI), a positively charged polymer, mediates immobilization of bacterial cells since the negatively charged cell surface interacts with the polymer (Kilonzo et al., 2011**)**, see Figure 5.3. PEI-treated Poraver® was used to immobilize *Lactobacillus casei* for the production of lactic acid in packed-bed and stirred tank reactors with promising results. It was observed that high concentrations of PEI used for immobilization did not affect the bacterial metabolism (Dong et al., 1992). The small size (2–4 mm) of Poraver beads influenced the *L. casei* to grow in filamentous shapes clumping the beads together,

leading ultimately to block the packed-bed recycle batch reactor used for lactic acid production, while the support size of 4–8 mm enhanced the system avoiding the formation of filaments even after 20 batches (Senthuran et al., 1999).

The yeast *Saccharomyces cerevisiae* was immobilized on cotton, polyester, nylon, polyurethane and cellulose treated with PEI and glutaraldehyde obtaining enhanced adhesion (Kilonzo et al., 2011). The yeast was also immobilized on PEI-treated collagen fiber for batch and continuous fermentations for ethanol production, which was enhanced in comparison with the use of other supports like glass and porous ceramic (Zhu et al., 2018). *Propionibacterium acidipropionici* was immobilized in Poraver® and Luffa supports, both treated independently with PEI for propionic acid production using glycerol as carbon source. PEI-Poraver® demonstrated to have immobilized 31 times higher amounts of cells than PEI-luffa (Dishisha et al., 2012). In a bioreactor with PEI-treated 3-D nylon support for PA production from glucose by *P. freudenreichii*, productivity was decreased inspite of efficient cell immobilization, attributed to the inhibitory effect of PEI (Belgrano et al., 2018). Binding is usually achieved by ionic or even covalent interactions between the cells and the support. Recycle batch fermentations were carried out in a bioreactor with a PA-tolerant strain of *P. acidipropionici* immobilized on PEI-treated Poraver® in which glycerol concentration was sequentially increased and pH was lowered, respectively (**Paper lll**), and PA production was obtained at high glycerol concentrations and low pH values.

Although PEI-treatment is usually applied to the supports, a direct treatment of the bacterial cells with PEI was carried out on an ureolytic *Acetobacter* sp., giving flocs that were used for immobilization in cotton fiber for continuous cultivation with the aim to hydrolyze urea (Kamath and D'Souza, 1991). Cross-linking agents can promote cell flocculation and attachment to a support, however covalently bound cells could have decreased activity (Dervakos and Webb, 1991) which generally represents a disadvantage since the metabolism might be negatively affected and the nature of the carrier may provoke mass transfer limitations (Groboillot et al., 1994).

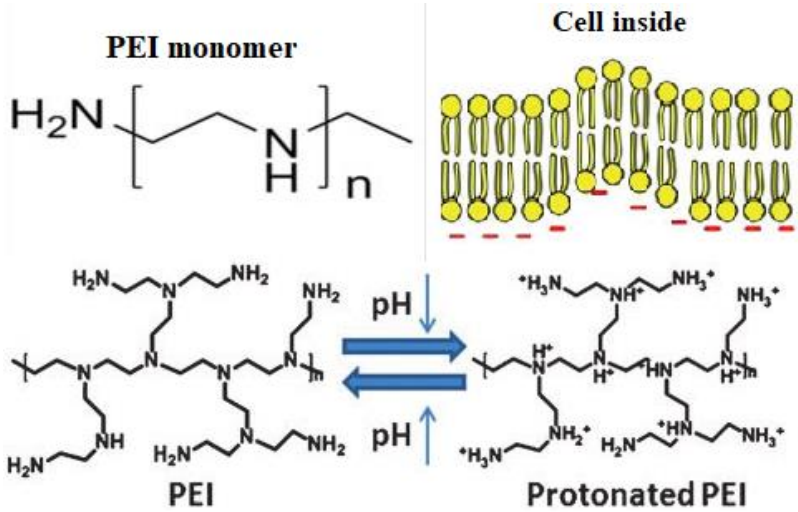


Figure 5.3 PEI mediated immobilization. Charge of the polymer depends on the pH. Positively charged polymer attracts bacterial cells due to their negative charged cell surface. Partially adapted from Yuan and Li, 2010

Table 5.1 provides examples of the chemical/polymer-assisted immobilization of bacteria on different supports.

TABLE 5.1 Distinct chemicals and supports used for immobilization of microbial cells for different applications

Chemical used for immobilization	**Support used**	**Microorganism**	**Product/Application**	**Reference**
PEI	Poraver® beads	*Lactobacillus casei*	Lactic acid	Dong et al., 1992; Senthuran et al., 1999
PEI Glutaraldehyde	Cotton, polyester, nylon, polyurethane and cellulose	*Saccharomyces cerevisiae*	Immobilization [(1)]	Kilonzo et al., 2011
PEI	Collagen fiber	*Saccharomyces cerevisiae*	Ethanol	Zhu et al., 2018
PEI	Poraver® Luffa	*Propionibacterium acidipropionici*	Propionic acid	Dishisha et al., 2012
PEI	3-D nylon support	*P. freudenreichii*	Propionic acid	Belgrano et al., 2018
PEI	Poraver®	Adapted *Propionibacterium acidipropionici*	Porpionic acid	(**Paper III**)
PEI	Cotton fiber	*Acetobacter* sp.	Urease activity	Kamath and D'Sousa 1991
PEI	poly-ε-caprolacton nanofibers	*Gluconobacter oxydans*	Whole-cell sensor	Gordegir et al., 2019
Polyetherimide Steric acid	Linen	*Clostridium acetobutyclium*	Butanol	Zhuang et al., 2017 [(1)]
Concanavalin A	Magnetic nanoparticles	*E. coli*	1.3 dihydroxyacetone	Zhuang et al., 2017 [(2)]
Mannose-functionalized nanoparticles	Nanocarrier Fe_2O_3	*E. coli*	1,3-dihydroxyacetone	Li et al., 2019

[(1)] Immobilization of recombinant *S. cerevisiae* C468/pGAC9 strain (ATCC # 20690) and non-recombinant *S. cerevisiae* C468 strain (ATCC # 26599) by adhesion on fibrous matrices

5.2.2. PA production in immobilized bioreactors

The first work on propionic acid fermentation using immobilized cells was carried out by Lewis and Yang in 1992, in which *P. acidipropionici* was immobilized through natural attachment on cotton and used for continuous fermentation with lactate as carbon source. The productivity was enhanced four times compared with the standard batch fermentation and the maximum cell concentration reached 37 g/L. Huang et al. (2002) reported a yield of 58 % with productivity of 2.12 g/L/h by *P. acidipropionici* immobilized in a FBB, attributing the high values to the extra nutrients of the corn meal hydrolysate. Besides, the study revealed that more carbon was channeled to PA formation than to biomass, as a result of the immobilization of *P. acidipropionici* in FBB in comparison with free cell cultivation. The procedure described in **Papers ll** and **lll** was to first allow the cells to be immobilized on a selected support either by the formation of biofilm (**Paper ll**) or by attachment to PEI-treated support in a bioreactor (**Paper lll**) to be then emptied and refilled with fresh medium for propionic acid fermentation in a recycle batch mode.

Cotton fiber was shown to be a good support for immobilization by Suwannakham and Yang (2005) and Feng et al. (2010) for PA production with *P. acidipropionici* and *P. freudenreichii*, respectively. PA titers of 71.8 and 52.2 g/L were reported for the immobilized adapted strain and wild type, respectively, in comparison to the titer of 51.5 g/L when the adapted strain was cultivated in planktonic state, hence clearly showing the advantage of immobilized cells. *Propionibacterium freudenreichii* immobilized on cotton fiber pieces packed to a tube shape and affixed to the baffles of the bioreactor in a so-called multipoint fibrous bed bioreactor (MFB) depleted glucose concentrations to produce 32.65 g/L and 20.41 g/L of PA with 80 g/L and 40 g/L of glucose, respectively, in comparison with 14.58 g/L PA obtained from free cell cultivation with 40 g/L glucose (Feng et al., 2010). The same system operated in fed-batch with 155 g/L glucose produced 67.05 g/L PA showing the increase in the PA titers were not proportional with the increase of the glucose in the medium.

Batch cultivation of *P. freudenreichii* immobilized in a plant fibrous bed bioreactor, with sugar cane bagasse as support for immobilization, produced 41.20 g/L PA from 80 g/L glucose, whereas only 34.03 g/L was achieved in free cell fermentation (Chen et al., 2013); PA concentration of 136.23 g/L was achieved after continuous fed-batch fermentation using the same set up. A novel xylan-based disulfide-crosslinked hydrogel matrix reinforced with nanocrystals of cellulose was found to be a good support material for immobilization of *P. acidpropionici* in continuous cultivation for PA production in a packed bed reactor (Wallenius et al., 2015). At dilution rates of 0.11 h^{-1} productivity and yield of 0.88 g/L/h and 0.56-0.58 g/g, respectively, were achieved. Poultry sludge was used as inoculum for PA production in a fluidized bed bioreactor made of grounded tire particles as support for immobilization which was carried out for 8 days in a recycling mode, and then crude

glycerol was added for starting the fermentation at varying hydraulic retention times (HRT) (Nazareth et al., 2018). The maximum productivity reached was 4.09 g/L/h at 0.5 h HRT with 5 g/L crude glycerol. Nylon 3-D printed support was used for immobilization of *P. acidiporopionici* in a recycling immobilized cell bioreactor with glucose as carbon source for PA fermentation where time was shortened in comparison with the free cell fermentations, giving maximum productivity and propionic acid titer of 0.46 g/L/h and 25.8 g/L, respectively (Belgrano et al., 2018). *P. acidipropionici* immobilized on sorghum bagasse gave an increased productivity of 1.17 g/L/h, which was six-fold higher than the free cell fermentation (Castro et al., 2020), but no significant enhancements in titer and yield were obtained. A summary of the main published investigations of immobilized bioreactors for PA production is presented in Table 5.2.

Application of new immobilization techniques can be efficiently used with bioreactor systems and can bring significant economic advantage for PA production (Ranaei et al., 2020). Metabolic flux analyses of the FBB compared with cell free fermentation of *P. freudenreichii* showed that PA synthesis and pentose phosphate pathway were increased by ~ 85 and ~ 228 %, respectively, demonstrating the advantage of immobilizing cells for PA and biomass production (Chen et al., 2013).

5.3. Biofilm bioreactors

The natural adhesion of microbial cells on a surface can be mediated by different weak interactions like hydrogen bonds, Van der Waals forces and ionic interactions; the cells in planktonic state have similar interactions with the medium (Kilonzo et al., 2011**).** The general mechanism of biofilm formation involves adherence of the planktonic cells to a surface followed by physical changes that involve upregulated production of an exopolysaccharide-rich extracellular matrix and maturation into a film (Fig. 5.4). Multiplication of the cells in the biofilm results in the release of planktonic cells in the medium.

Table 5.2 Propionic acid production in immobilized bioreactors

Immobilization principle	Support used	Microorganism	Carbon source	Mode of operation	Production parameters			Reference
					Q_p (g/L/h)	$Y_{P/S}$ (g/g)	PA (g/L)	
Natural attachment	Cotton	*P. acidipropionici*	Lactic acid					Lewis and Yang (1992)
Mechanical entrapment by slow recirculation through FBB	Matrix of corn fibers	*P. acidipropionici*	Corn meal hydrolysate	-	2.12	0.58	-	Huang et al., 2002
Adhesion by filtration effect	Spiral cotton wound towel	*P. acidipropionici*	Glucose	Fed-batch	c.a 0.2	0.4 – 0.65	71.8	Suwannakham and Yang 2005
Adhesion	Multi-point cotton fibrous-bed	*P. freudenreichii*	Glucose 80 g/L	Batch	0.19	0.41	32.65	Feng et al., 2010
			Glucose 155 g/L	Fed-batch	0.14	0.43	67.05	
Adhesion	Sugar cane bagasse	*P. freudenreichii*	Glucose	Constant fed-batch	0.57	-	136.23	Chen et al., 2013
				Intermittent fed-batch	0.47	-	118.26	
Adhesion	Poraver®	*P. acidipropionici*	Glycerol	Continuous	1.40	0.86 (mol/mol)	15	Dishisha et al., 2012
Adsorption	Xylan-based hydrogel matrix	*P. acidipropionici*	Glycerol	Continuous (0.11 h^{-1})	0.88	0.56 – 0.58	-	Wallenius et al., 2015
Adhesion	Tire particles	Poultry (1)	Glycerol	Continuous	4.09	-	-	Nazareth et al., 2018
Adsorption	Nylon 3-D printed supports	*P. acidiporopionici*	Glucose	Recycle batch	0.46		25.8	Belgrano et al., 2018
Biofilm formation	Poraver®	*P. acidiporopionici*	Glycerol	Recycle batch	0.78	0.56	11.73	Cavero-Olguin et al., 2019 (**Paper II**)
	AnoxKaldnes®				0.43	0.59	11.51	
Adhesion	Sorghum bagasse	*P. acidipropionici*	Cellulose and hemicellulose	Batch	1.17	0.46	35.3	Castro et al., 2020
Adsorption	Poraver®	PA-tolerant strain of *P. acidiporopionici*	Glycerol	Recycle batch	0.28	0.64 (mol/mol)	58	Cavero-Olguin et al., 2021 (**Paper III**)

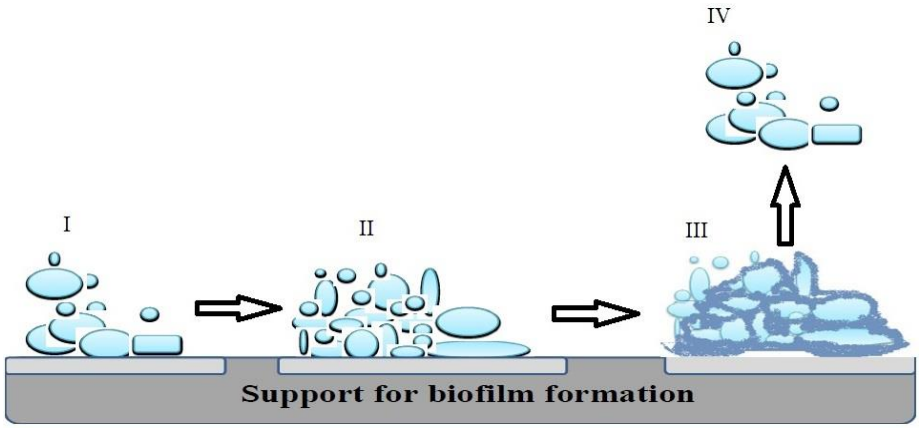


Figure 5.4 Biofilm formation process. Initial attachment (I); Irreversible adhesion, proliferation and maturation of biofilm (II); bacterial cells enshrouded in exopolysaccharide matrix (III); dispersal of planktonic bacterial cells (IV). Adapted from Sonkusale and Tale (2015)

Several genes are involved in the process of biofilm formation as in case of *Bacillus subtilis* (Branda et al., 2004). High molecular weight biopolymers are formed by the cells leading to their entrapment into the matrix of polymers. Different components as polysaccharides, proteins, lipids, extracellular DNA (eDNA), humic substances are present in EPS (Zur et al., 2016). The polymeric substances secreted in a biofilm can be divided into three groups, those secreted after the stimulation of the interaction between the cell and the support material and the environment, the ones formed by the metabolism of the nutrients, and finally the substances from cell lysis or biodegradation of cell components (Zur et al., 2016). According to the degree of attachment to the membrane, the EPS could be capsular, released or loosely bound, tightly bound and slimy. EPS production is involved in protection against environmental stresses like osmotic stress, extreme temperatures, non-optimal pH, etc. EPS is also involved in cell-cell communication, and adhesion to surfaces (Belgrano et al., 2018). The nature and proportion of the components of EPS determine the quality of the biofilm; polysaccharides and proteins are the most abundant components, having similar functions as adhesion to biotic of abiotic surfaces and cell aggregation.

For the bacteria to successfully survive in complex and coordinated biofilms, coordinated mechanisms of cell–cell communication may be an asset (Hardie and Heurlier, 2008). Natural biofilms have unique qualities, a good example is electrical conductivity in *Geobacter* biofilms which make them potential candidates for several applications (Wang et al., 2019). Inducing microorganisms to enhance or to trigger their self-capacity to form the extracellular matrix for immobilization purposes could eliminate the need for chemical aids for cell attachment to surfaces. Bagasse was considered to be a good material for immobilization of

propionibacteria since the cells were naturally adsorbed after 48 h and the opened surfaces, formed by cutting the material, favored nutrient transfer and also PA production (Es et al., 2017).

Production of EPS was demonstrated to be useful for immobilization and PA production by *Propionibacterium freudenreichii* (Belgrano et al., 2018). Immobilization on non-treated Poraver® support demonstrated better results for PA production, regarding productivity, yield, and final product titer. The EPS concentration produced by *P. freudenreichii* increased from 376 mg/L in flask fermentation without pH control to 617 mg/L in the immobilized cell bioreactor at pH 6.5 (Belgrano et al., 2018). In cultivations of *P. acidipropionici* DSM 4900 in milk microfiltrate at 23 °C the EPS production was reported to be composed of galactose, mannose, and glucosamine, with traces of glucose, galactosamine and phosphate, in two major polysaccharides with different composition, molecular weight and charge (Gorret et al., 2003). The polymer behavior in aqueous solution was of a polyelectrolyte nature, with galactose-containing fraction accounting for more than 78 % of the total polysaccharide content. The findings from the present thesis open up the possibility to use the capacity of *P. acidipropionici* to produce a biofilm as a sole immobilization procedure, reducing costs for chemicals and polymers. Production of natural biofilm by *P. acidipropionici* DSM 4900 using chemical stress factors is presented in **Paper ll**.

5.3.1. Relationship between biofilm formation and trehalose synthesis in *Propionibacterium acidipropionici*

Certain molecules are produced in response to non-optimal conditions such as high-salt, high-acid concentrations, low pH, sub-optimal temperatures and other factors during cultivation, some of them used for biofilm formation in this work (**Paper ll**). Metabolic adaptation and drastic metabolic changes are the two strategies microorganisms have evolved to face abiotic stress conditions. The former implies that metabolic capabilities such as enzymatic activities are modified or membrane stability is changed when exposed to high temperature or salinity. The latter implies more evolved biosynthetic pathways to counteract stress conditions by producing osmotically active compounds, cryoprotectants or thermoprotectants, to enable survival of the cells. Among the compounds synthesized to counteract non-optimal growing conditions are polyols such as mannitol, sorbitol, some amino acids like proline and glutamic acid, quaternary ammonium salts as glycine betaine, and disaccharides as sucrose and trehalose (Avonce et al., 2006; Boch et al., 1996).

Direct relation between trehalose synthesis and tolerance to radiation and heavy metal stress were found in a novel strain of *Aureobasidium subglaciale*, isolated form soils polluted by radiation and heavy metals (Liu et al., 2017). Genes encoding DNA repair proteins, oxidases, oxidative stress response factors, and transported

proteins were found to be up-regulated dramatically when trehalose was overproduced in mutant strains of *A. subglaciale* having a 3-fold increased trehalose content, significantly improving its tolerance level. Trehalose was also accumulated in a toluene-tolerant *Pseudomonas* sp., and trehalose-biosynthetic genes were found to be expressed on exposure of the cells to toluene (Park et al., 2007). Several studies on trehalose have revealed its importance as a protective agent against diverse chemical and physical stress factors such as non-optimal temperatures, dehydration, desiccation, oxygen radicals, high osmolarity and nutrient starvation (Wu et al., 2017; McDougall et al., 1993). Trehalose forms a glass-like matrix structure upon dehydration (Gibson et al., 2002) and is also known for its protective action in dehydrated environments.

5.3.2. Role of *luxS* in biofilm formation

Since biofilms are abundant in nature the communication between microorganisms is mediated by several chemical signals. A common signaling molecule named autoinducer 2 (AI2) produced by *luxS* is known to be shared by Gram-positive and Gram-negative bacteria (Hardie and Heurlier, 2008; Dagher et al., 2010). The role of *luxS* in biofilm formation is not clear, since it enhances the biofilm formation in many microorganisms and not in others. The effect of AI2 was studied in three strains of *E. coli* obtaining a 30-fold increased biofilm mass, and it was concluded that AI2 directly stimulated the biofilm formation through regulation of the cellular motility (Barrios et al., 2006). On the other hand, a *luxS*-deficient strain of the biofilm forming *Listeria monocytogenes* formed an even more robust biofilm than the parental strain and the addition of exogenous AI2 showed no effect on the biofilm formation (Sela et al., 2006). Belval et al. (2006) suggested *S*-ribosyl homocysteine, the precursor of AI2, is directly related to biofilm formation and cell to cell communication in *L. monocytogenes*. When 32 isolated strains of *L. monocytogenes* were taken from different foods and food-related sources every strain was able to form biofilm even though 7 strains did not possess the *luxS* meaning there might be other mechanisms for biofilm development (Bonsaglia et al., 2014). In the present work, the expression of *luxS* was detected by FISH during *P. acidipropionici* cultivation with stress factors NaCl and citric acid, while no expression was detected in the absence of such chemicals (**Paper II**).

5.4. Cell retention in membrane bioreactor

Cell recycling by retention on semi-permeable membranes represents a promising strategy of recycling of free cells and combines the advantages of both free cell and immobilized cell processes for high cell density fermentations, and could even be simpler than immobilization. It can be run in both batch and continuous modes; the

latter allows removal of the inhibitory product during fermentation. Cell recycling is best applied to slow-growing microorganisms to avoid long lag phases (de Assis et al., 2022), although operational complexity should be considered together with the extra costs the filtration/cell retention unit would mean and the mechanical stress faced by the cells during long fermentations.

An automated system for the control of cell concentration was applied to membrane filtration for lactic acid production by *Bacillus coagulans*, with increasing concentrations of glucose and dilution rates, doubling the overall productivity of batch fermentations (Fan et al., 2017), and demonstrating the feasibility of the system to fermentation processes characterized by product-inhibition. The use of membrane modules has been applied widely in biohydrogen production (Lee et al., 2008, 2014; Aslam et al., 2018; Jabbari et al., 2019), but its application to other fermentation processes is not widely investigated. The presence of extrapolymeric substances led to decrease in the permeate flux in a constant stirred tank reactor for biohydrogen production, resulting in membrane clogging and severe membrane fouling (Lee et al., 2008). The effect of the viscous extrapolymeric substance on reducing the membrane performance was also seen in an axial-microfilter fermenter during aerobic cultivation of *S. cerevisiae* (Naja et al., 2006). A simulation model developed based on results from penicillin, ethanol and lactic acid production showed multistage continuous high-cell density cultivations as the most promising system to adopt for several fermentation products (Chang et al., 2011).

Use of a microfiltration unit to recycle cells during PA fermentation was first reported by Boyaval and Corre (1987), when *P. acidiporopionici* cells were retained in recycled mode during fermentation with sweet cheese whey in a 3 L reactor. Interestingly, specific productivity decreased from 0.4 to 0.17 g/g/h while the cell dry weight increased from 10 to 80 g/L when the dilution rate was increased during continuous mode of operation. Similar reduction of PA productivity at higher cell densities was controlled by bleeding-out the system to keep cell concentration of *P. acidiporopionici* at approximately 40 g/L in the ultrafiltration unit, obtaining an average PA titer and yield of 35 g/L and 0.54 g/g, respectively, and volumetric productivity between 0.31-0.65 g/L/h during 15 fermentation cycles using the whey medium (Colomban et al., 1993).

Chapter 6. Results (Papers I-IV)

Different ways of high cell density fermentations have been investigated for the production of propionic acid from glycerol using *Propionibacteria acidipropionici* DSM4900 within the framework of this thesis. These included sequential and cyclic batch fermentation, immobilization of cells, and cell recycling by membrane retention. Besides, the bacteria were induced for biofilm formation and subjected to adaptive evolution for enhanced acid tolerance, respectively.

6.1. Cyclic and sequential batch fermentations with optimal nutrient composition (Paper I)

In this study, initially the effect of carbon and nitrogen concentrations in the culture medium on the cell growth of *P. acidipropionici* DSM 4900 and propionic acid production in batch cultivation was studied using three-way ANOVA analysis. Nine experiments planned by a factorial design (3^2) had glycerol and yeast extract concentrations as variables, at three levels each (30, 60 and 90 g/L for glycerol and 10, 20 and 30 g/L for yeast extract). The obtained data revealed the concentration of yeast extract was statistically significant ($P < 0.05$) with respect to propionic acid volumetric production rate. The highest impact of the yeast extract increment by 10 g/L was obtained at 30 g/L glycerol, which led to an increase in maximum cell density, volumetric productivity and titer by 0.55 g_{CDW}/L, 0.02 g/L/h and 1.65 g/L, respectively. On the other hand, at 60 or 90 g/L glycerol, these parameters were increased by 0.37 g_{CDW}/L, 0.01 g/L/h and 1.10 g/L, respectively.

Batch pH-controlled fermentations were carried at various C:N ratios. At glycerol concentration of 90 g/L, a 3-fold increase in yeast extract concentration from 10 to 30 g/L increased the productivity from 0.16 g/L/h to 0.35 g/L/h, product concentration from 32.1 g/L to 43.4 g/L, and yield from 0.67 mol/mol to 0.77 mol/mol, while the fermentation time was lowered from 141 h to 103 h. The increased yield was accompanied by decrease in the formation of co-products, succinic acid, acetic acid and propanol. Yeast extract is a complex nitrogen source and can also provide vitamins and other cofactors needed for cell growth and metabolism.

In order to test cell recycling by cyclic and sequential batch fermentations, glycerol:yeast extract concentration ratio was maintained at 3:1 with initial concentrations of 60 and 20 g/L, respectively, which were subsequently increased.

Two parallel systems were carried out during the cyclic batch fermentations (CBF) by replacing 90 % of the culture broth at the end of the fermentation with the same amount of fresh new autoclaved medium. System 1 was carried out with glycerol and yeast extract ratio of 60:20 (g:g) during three cyclic cultivations, while the second system was carried out with 60:20 and 90:30 during one and two cyclic cultivations, respectively. The second batch of System 1 showed 11 h lag phase while during the first batch the lag phase was 24 h, both systems showed shortened lag phases in comparison with batch cultivation with the same medium composition. The productivity in System 1 remained between 0.37 and 0.42 g/L/h during the three cyclic batches, while in System 2 the highest productivity and yield were 0.53 g/L/h and 0.93 mol/mol, respectively, when glycerol:yeast extract ratio was of 90:30 g/L in the third batch (Table 6.1).

Table 6.1 Cyclic batch fermentation (CBF) and sequential batch fermentation with cell recycle (SBF) (adapted from paper I)

Glycerol (g/L)	Yeast extract (g/L)	Qp (g/L/h)	$Y_{P/S}$ (mol/mol)
System 1 (CBF)			
60	20	0.42	0.71
60	20	0.37	0.74
60	20	0.43	0.64
System 2 (CBF)			
60	20	0.42	0.73
90	30	0.43	0.61
90	30	0.53	0.93
Sequential batch fermentation (SBF)			
60	20	1.19	0.74
60	20	1.36	0.70
90	30	1.47	0.71
90	30	1.63	0.73
120	40	1.13	0.71
120	40	1.12	0.71
150	50	0.30	0.74

Abbreviations: Qp – volumetric productivity; Y – Yield

In sequential batch fermentation with cell recycling (SBF), the cells were harvested by centrifugation under sterile conditions and suspended in fresh medium to start a new batch of fermentation. The product yield varied between 0.7-0.74 mol/mol while the productivity exceeded 1 g/L/h up to glycerol concentration of 120 g/L (Figure 6.1). Maximum productivity of 1.63 g/L/h was achieved at 90 g/L glycerol, which is the highest reported for propionate production from glycerol in a batch mode of fermentation. Utilization of the entire glycerol amount was achieved at all

combinations of glycerol and yeast extract, with the exception of the highest concentration of 150:50 when only 86% of the initial glycerol amount was consumed.

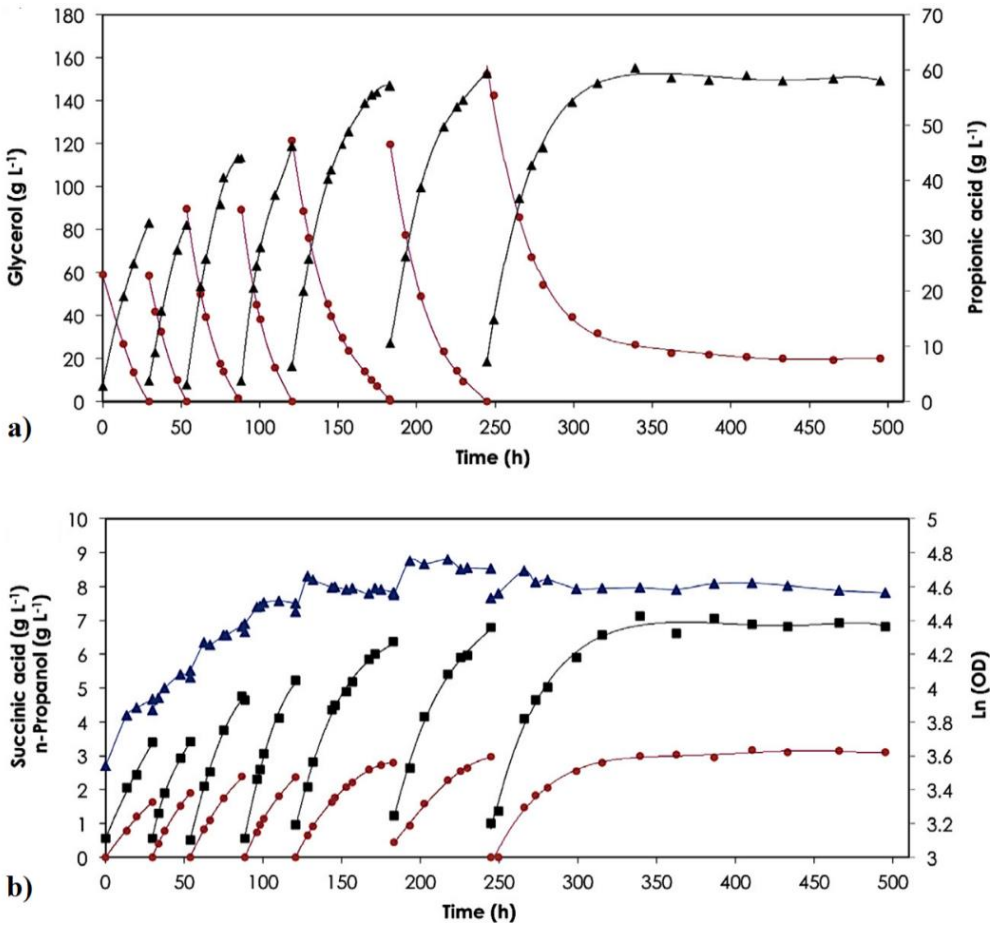


Figure 6.1 Production of propionic acid by sequential batch fermentation (SBF) using *P. acidipropionici* DSM 4900 with cell recycle showing the concentrations of: **a)** glycerol (●) and propionic acid (▲), **b)** succinic acid (■), n-propanol (●), and biomass (▲). The initial concentrations of glycerol and yeast extract (g/L) were 60:20 for batches 1 and 2, 90:30 for batches 3 and 4, 120:40 for batches 5 and 6, and 150:50 for batch 7. (Taken from **Paper I**)

6.2. Inducing biofilm formation in *P. acidipropionici* for immobilization (Paper II)

With the aim to facilitate immobilization of *P. acidipropionici* to solid surfaces for use in propionic acid fermentation, the ability of the bacteria to form biofilm was investigated by exposing the culture to several stress factors. These included different concentrations of acetic acid, citric acid, ethanol, hydrogen peroxide, sodium chloride, and sodium hypochlorite, respectively.

Cell growth was observed only in cultures incubated for two weeks with sodium chloride, citric acid and acetic acid, respectively. Exopolysaccharide (EPS) production was slightly increased from 0.169 g/L produced in the control culture to 0.178 and to 0.187 g/L in NaCl- and citric acid-containing culture, respectively (Table 6.2). Highest biofilm forming capacity (BFC) index were found to be 0.644 and 0.230 during log phase and lag phase of the bacterial cultures with 0.8 M NaCl and 25 mM citric acid, respectively. The production of cell-bound capsular and released polysaccharides differed according to the stress factor used. While 77.38 mg/L capsular polysaccharides and 55.87 mg/L of released polysaccharides were found in the control cultivation in base culture medium, 162.85 and 71.57 mg/L were produced as capsular and released polysaccharides, respectively, in cultivations with citric acid. Only capsular polysaccharides of 93.08 mg/L were found to be elevated in cultures with NaCl as stress factor, while the released polysaccharide content was lowered to 50.64 mg/L.

Table 6.2 Exopolysaccharide content (EPS) obtained in the media containing stress factors during cell growth in serum bottles (adapted from paper II)

Stress factors	EPS (g/L)
Control [a]	0.169 ± 0.04
NaCl (0.8 M)	0.178 ± 0.06
Citric acid (25 mM)	0.187 ± 0.06
Acetic acid (80 mM)	0.126 ± 0.02

(a) Control included no stress factor in the medium.

Scanning electron microscopy (SEM) was used to reveal the different shapes the cells acquired in the presence of the stress factors (Figure 6.2). More spherical shape at the time of being tightly packed together was observed in cells from citric acid containing medium, while a clear extracellular matrix formed around the cells could be seen in the cells from the NaCl containing medium, typical of biofilm structures.

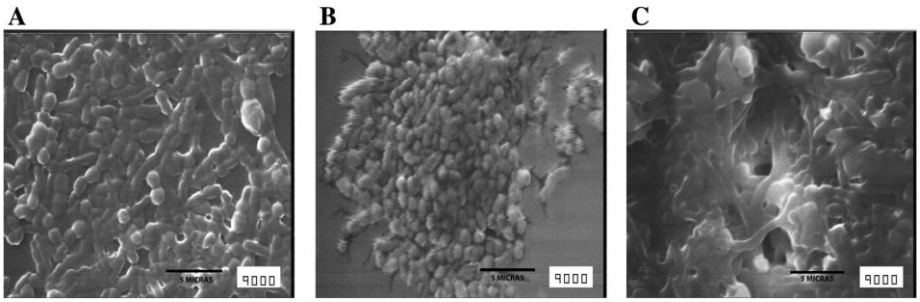


Figure 6.2 Scanning electron micrographs of *P. acidipropionici cells* from different cultures at × 5000 magnification. (**A)** Cells in planktonic state when cultivated in basal medium, **(B)** cells aggregated in culture with citric acid as stress factor, **(C)** extracellular polymeric substance in culture with NaCl as stress factor, cells can be seen embedded into the matrix formed by the polymeric substance (**Taken from paper II**)

The amount of trehalose accumulated by the cells with these stress factors was followed as the disaccharide is known to be produced under non optimal conditions in different microorganisms. Trehalose increased from 2.7 g/L in the control culture to 6.8 and to 6.3 g/L in citric acid- and NaCl containing cultures, respectively. Corresponding increase in the expression of the *treY* gene encoding maltooligosyl-trehalose synthase was observed by RT-qPCR only in citric acid containing two-week cultures in serum bottles. Expression of the biofilm formation-related gene *luxS* and *treY* in biofilm formation was observed by fluorescent *in situ* hybridization (FISH) analysis with both citric acid and sodium chloride containing cultures.

The stress factors were then used for developing biofilm on two different support materials, Poraver®- recycled glass beads and AnoxKaldnes® carriers, followed by recycle batch fermentations for propionic acid production using 20 g/L glycerol as carbon source. In Poraver® reactors, the first batch during the production stage showed improvement of growth rate from 0.04 to 1.85 g_{CDW}/L/h and from 0.08 to 1.86 g_{CDW}/L/h in citric acid- and NaCl-containing media, respectively. Product yield, titer and productivity were significantly increased as well. In AnoxKaldnes® reactors, the highest growth rates during production stages were 1.06 and 0.27 g_{CDW}/L/h with citric acid and NaCl, respectively. Highest productivities of 0.7 and 0.78 g/L/h were obtained in Poraver® reactors while 0.39 and 0.43 g/L/h in AnoxKaldnes® reactors with citric acid and NaCl, respectively. More efficient biofilm formation and propionic acid production were achieved in Poraver® reactors.

6.3. Adaptive evolution of *P. acidipropionici* and propionic acid fermentation in an immobilized cell bioreactor (Paper III)

P. acidipropionici DSM 4900 was subjected to adaptive evolution with the aim to increase its resistance to high propionic acid concentration and low pH as a way to lower product inhibition during the production process. For this, the parental *P. acidipropionici* was subjected to adaptive laboratory evolution (ALE) by stepwise increasing propionic acid concentration up to 40 g/L (with increments of 10 g/L), in batch cultivations. Three sequential cultivations were carried out at each concentration prior to a next increase of propionic acid content in the medium; the cultivations at each concentration lasted between 1 to 3 weeks. No growth was observed when the parental strain was cultivated with 20 g/L PA, while under the same conditions the adapted strain showed enhanced titer and productivity of 16.8 g/L and 0.52 g/L/h, respectively, in comparison with titer and productivity of 8.72 g/L and 0.17 g/L/h, respectively, obtained when the parental strain was cultivated without the addition of PA. The adapted *P. acidipropionici* cells exhibited significant increase in *treY* expression as in the previous study (Paper II).

The adapted culture was then immobilized to Poraver® beads coated with the polycation polyethyleneimine (PEI) and used for propionic acid production by recycle batch fermentation in two reactors where increasing glycerol concentration and decreasing pH were investigated, respectively (Figure 6.3 and Table 6.3). Glycerol at 100 g/L was completely consumed at pH 6.5 to give about 58 g/L of propionic acid at yield and productivity of 0.64 mol/mol and 0.28 g/L/h, respectively. In comparison, the sequential batch cultivation using free cells gave much higher productivity even with 120 g/L glycerol feed (Paper I). Interestingly, the adapted strain was able to produce propionic acid at pH down to 5.0 at a higher rate than at pH 6.

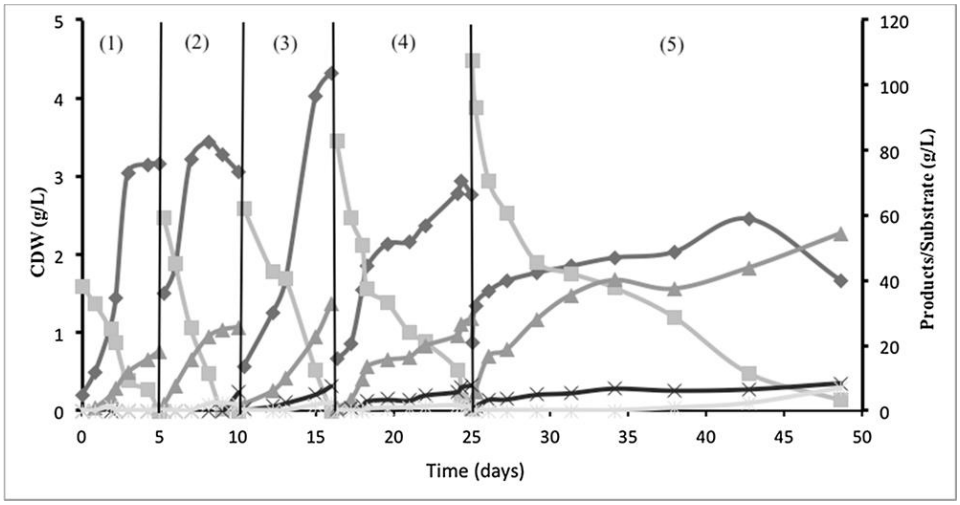


Figure 6.3 Profiles of growth in terms of CDW (◆), glycerol consumption (■), and production of propionic acid (▲) and succinic acid (×) by the adapted strain of *P. acidipropionici* in the immobilized cell bioreactor during consecutive recycle batch cultivations in the medium with glycerol: yeast extract concentration ratios of (1) 40:10, (2) 60:10, (3) 60:20, (4) 80:10 and (5) 100:20, respectively separated by vertical bars. The high biomass at the beginning of the last batch was caused by a short previous batch of five days, which was aborted due to technical problems (not shown). The figure is adapted from Paper III

Table 6.3 Production of propionic acid by adapted *P. acidipropionici* cells immobilized on PEI treated Poraver® beads during fermentations controlled at different pH values

pH	Q_x (CDWg/L/h)	Q_p (g/L/h)	$Y_{p/s}$ (g/g)	Propionic acid max (g/L)
6.0	0.03	0.14 ± 0.07	0.68 ± 0.01	10.04 ± 0.06
5.5	0.01	0.16 ± 0.11	0.78 ± 0.00	10.95 ± 0.31
5.0	0.00	0.23 ± 0.15	0.63 ± 0.00	10.88 ± 0.15

6.4. Cell recycling by membrane retention (Paper IV)

A tubular ceramic membrane filter was connected to a bioreactor (300 mL) for cell recycling while the propionic acid fermentation was carried out in a continuous mode. Right after inoculation of the reactor a batch fermentation was run with glycerol depletion in less than 70 h and ~ 6 g_{CDW}/L of biomass formation. The system was then switched to continous mode of operation at D = 0.05 1/h with cell recycling by the ceramic membrane, which led to 32.6 g_{CDW}/L at glycerol and yeast extract concentration of 20 and 10 g/L, respectively. The set up was operated continuously with carbon : nitrogen ratio variations by increasing glycerol concentrations from 20 to 70 g/L and varying yeast extract concentrations between 10 and 20 g/L. Following increase in glycerol concentration to 40, 50 and 60 g/L resulted in 58.9, 61.57 and 49.12 g_{CDW}/L, respectively. Productivity of 1.23 g/L/h

and product yield of 0.48 g/g substrate were obtained with 50 g/L glycerol at yeast extract concentration of 10 g/L.

Increasing the glycerol and yeast extract concentration to 60 g/L and 20 g/L, respectively, led to increase in propionic acid productivity, product yield and concentration to 2.35 g/L/h, 0.78 g/g and 47.03 g/L, respectively. Under these conditions, the highest cell density of 90.09 g_{CDW}/L was obtained. Nonetheless, complete glycerol depletion was obtained only at initial concentration up to 50 g/L, while only 83.5 % glycerol was consumed when used at a concentration of 60 g/L. Further increase in yeast extract concentration was not considered due to potential increase in material costs for the fermentation. Instead, lower D = 0.025 1/h was applied with 60:20, 60:10 and 70:10 g/L glycerol: yeast extract concentrations, which maintained the cell density between 63.56 and 77.49 g_{CDW}/L but without any enhancement in any of the production parameters.

Since the cells were constantly subjected to mechanical and chemical stresses due to the high pressures imposed by the constant shearing through the pumping and high concentrations of glycerol and propionic acid, the isolation of an adapted strain was tested. Cells were withdrawn form the reactor at the end of five-month long fermentation for batch cultivations in 90-mL media at low pH and then with high concentrations of propionic acid. Firstly, cultivations were carried out at pH 7, 6 and 5, respectively, after which cells from pH 5 cultivation were transferred to media with 10, 20, 30 and 40 g/L of propionic acid. Growth was obtained only in cultivations with 10 and 20 g/L. Finally, cultivations were carried out using agar plates with 10 and 20 g/L propionic-containing solid media to obtain isolated colonies at both concentrations. A colony from the solid medium with 20 g/L propionic acid was cultured in liquid medium supplemented with the same concentration of propionic acid, grown for 3 weeks to be then stored in 50 % glycerol medium at -20 °C for further use. The resulting variant was able to grow at pH 5 with 20 g/L glycerol in the presence of 20 g/L propionic acid.

Chapter 7. Conclusions and Outlook

The conclusion drawn from the studies presented in this thesis are that high cell density fermentation and increasing the bacterial resistance to high acid concentrations/low pH are needed to make the propionic acid fermentation from renewable feedstock a competitive process.

From the different modes of high cell density fermentation processes presented in this thesis, the most convenient and robust ones seem to be the ones based on sequential batch (Paper I) and immobilized cell fermentations (Paper III). The time for fermentations at industrial scale would be avoided if the cell biomass developed serves as inoculum, for starting new production batch. Avoiding contamination during the entire operation would be an important aspect, especially between different cycles when cells are withdrawn in cyclic and sequential batch fermentations or when the reactors are emptied in immobilized packed-bed reactors to be refilled with fresh new sterile media.

While immobilization is popular in fermentation technology some factors like the support materials and chemicals required for immobilization must be taken into consideration in terms of costs and stability of the system. The possibility of using the ability of bacteria to form biofilms for their immobilization could be considered as a more environmentally friendly technique besides being cheaper. However, single strains that are not known of being biofilm formers like *Propionibacterium acidipropionici* need stimuli to trigger their metabolic machinery for biofilm formation. The stress induced biofilm formation obtained in the present thesis, although resulting in enhanced production parameters, did not have a long-lasting effect as the productivity went progressively downwards (Paper II). The possible implications of using chemicals as stress factors should be studied closer to better control metabolism changes during biofilm formation and maintaining optimal metabolism for propionic acid production.

While the membrane cell recycling gave among the highest productivity and also very high accumulation of cells, the carbon source was not completely utilized beyond 50 g/L glycerol (Paper IV). Also, the process became unstable due to the high pumping rate needed for filtering the product while recycling the cells to and from the reactor. It may be possible to overcome this limitation by using an *in situ* membrane filter, however the issue of the membrane fouling would still remain. Yet another possibility is the use of a sensor for an automated control of the weight or

the level of the reactor vessel. It would of course be important to compare the performance of the process using polymeric membranes that are cheaper and relatively easy to clean.

An acid tolerant strain of *Propionibacterium acidipropionici* obtained by adaptive laboratory evolution through stepwise increasing concentration of propionic acid concentration in the culture medium was feasible in a shorter time than previously reported (Paper III). Increase in the production of trehalose as a result of exposure to stressful environment was shown, further studies would be needed to investigate further the expression of trehalose related genes as well as other enzymes.

Besides the production parameters, the techno-economic feasibility of a production process is determined by a number of factors, the most important being the cost of the carbon and nitrogen source, and downstream processing. In the thesis, the focus has been on glycerol, which is a by-product of biodiesel production. Its availability will depend a lot on how biodiesel will compete in the market as an energy carrier in the future. The processes studied here will however be applicable even to other biobased residual carbon sources. Propionibacteria require a complex nitrogen source for meeting the needs for nutrition. Use of yeast extract on large scale is expensive and will have to be replaced by a cheaper nitrogen source. Earlier studies in our laboratory have shown protein rich potato juice, obtained after extraction of starch, to be much cheaper than yeast extract but comparable as the nitrogen source for propionibacteria (Dishisha et al. 2013). There are however other sources like soybean meal/molasses, corn steep liquor and whey protein that should be compared.

Downstream processing of fermentation products often accounts for 50-80 % of the total production costs. Being a low cost product, it is important to keep the downstream costs as low as possible. Much of the cost is ascribed to the removal of the large amounts of water, hence the need for high product concentration, i.e. in the range of at least 100 g/L. Moreover, the water would need to be recycled for subsequent processes. Yet another aspect is the separation from the by-products like acetic acid and succinic acid. It would thus be ideal that the majority of the carbon content in the raw material goes to the product of interest. In an earlier report, however, succinic acid was regarded as a valuable co-product (Dishisha et al. 2013).

It would be useful to consider the economy of production in a larger perspective of a biorefinery, wherein the raw material including the residues are used to make multiple products that could be both high- and low cost products. This will enable value addition to the raw material and also make available the important chemicals and materials in the bioeconomy of the future.

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Improved propionic acid production from glycerol: Combining cyclic batch- and sequential batch fermentations with optimal nutrient composition

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HIGHLIGHTS

- Optimum glycerol:yeast extract ratio of 3:1 for maximal propionate productivity.
- Improved propionate yield and volumetric productivity using cyclic batch fermentation.
- Sequential batch fermentation yields propionate at >1 g L^{-1} h^{-1} from 120 g L^{-1} glycerol.

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ABSTRACT

Propionic acid was produced from glycerol using *Propionibacterium acidipropionici*. In this study, the impact of the concentrations of carbon and nitrogen sources, and of different modes of high cell density fermentations on process kinetics and -efficiency was investigated. Three-way ANOVA analysis and batch cultivations at varying C/N ratios at pH 6.5 revealed that propionic acid production rate is significantly influenced by yeast extract concentration. Glycerol to yeast extract ratio (w w^{-1}) of 3:1 was required for complete glycerol consumption, while maintaining the volumetric productivity. Using this optimum C/N ratio for propionic acid production in cyclic batch fermentation gave propionate yield up to 93 mol% and productivity of 0.53 g L^{-1} h^{-1}. Moreover, sequential batch fermentation with cell recycling resulted in production rates exceeding 1 g L^{-1} h^{-1} at initial glycerol up to 120 g L^{-1}, and a maximum of 1.63 g L^{-1} h^{-1} from 90 g L^{-1} glycerol.

1. Introduction

Propionic acid (PA) is a bulk chemical with an annual production capacity of 349,000 tonnes in 2006 (TranTech-Consultants, 2007). It is widely used as a preservative and is also used as ingredient in animal feed, plastics, herbicides, pharmaceuticals and perfumes (Boyaval and Corre, 1995; Kirschner, 2009; Rogers et al., 2006). Current industrial production of propionic acid is via chemical synthesis from fossil-based raw materials, mainly by oxo-synthesis route from ethylene. Its production by microbial fermentation from renewable resources has attracted increasing attention (Kirschner, 2009; Rogers et al., 2006; TranTech-Consultants, 2007). Different microorganisms can produce propionic acid as a metabolic end product, of which *Propionibacteria* have been the most investigated. These microorganisms can metabolize different carbon sources into propionate through succinate (SA) as intermediate in a so-called dicarboxylic acid pathway (Playne, 1985). Glycerol (Gly) is a more reduced carbon source as compared to sugars and lactate, and induces a homopropionate fermentation behavior generating propionate at high yield with less acetate (AA) as by-product (Barbirato et al., 1997, 2004; Coral et al., 2008; Dishisha et al., 2012, 2013; Himmi et al., 2000). Nevertheless, this production route is limited by the strong product-mediated inhibition on cell growth and metabolic activity (Blanc and Goma, 1987). This effect is caused by penetration of the undissociated propionic acid molecules from the solution through the cell membrane leading to disruption of the intracellular

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buffering system and cellular activities. The excretion of the generated protons is an ATP-dependent step. As a consequence, cell growth and metabolic activity is inhibited due to long term depletion of ATP (Theron and Lues, 2010).

The type and concentration of the nitrogen/vitamin source has earlier been reported to impact the tolerance of *Propionibacteria* to propionic acid (Quesada-Chanto et al., 1998). The availability of a suitable *N*-source as nutrient elevates the threshold concentration of propionic acid at which the specific cell growth rate (μ) and specific propionic acid production rate (q_p) become zero (Blanc and Goma, 1987; Obaya et al., 1992, 1994; Quesada-Chanto et al., 1998). The effect of nitrogen/vitamin source on propionic acid production from glycerol, a carbon source that yields lower cell density than other sugars, has not yet been investigated.

The importance of the nitrogen/vitamin source can also be noticed in batch and fed-batch fermentations. Considerable reduction in propionic acid volumetric production rate has been observed when maintaining the nitrogen/vitamin concentration constant while increasing the concentration of the carbon source in a batch operation and when feeding only a carbon source in the fed-batch mode, respectively (Barbirato et al., 1997; Boyaval and Corre, 1987; Dishisha et al., 2012, 2013; Suwannakham and Yang, 2005; Zhang and Yang, 2009a,b). Supplementation of the feeding solution with nitrogen/vitamin source enhanced/maintained the production rates (Ozadali et al., 1996; Paik and Glatz, 1994).

Besides nitrogen source, the process design also plays an important role in determining the economical feasibility of several processes. Propionic acid production was investigated in different process configurations to reach target productivity of 2–3 g L^{-1} h^{-1} required for industrialization (Dishisha et al., 2013; Werpy et al., 2004). High cell density fermentation through immobilization or cell recycling, and the semi-continuous fermentation were the most promising and resulted in considerable enhancement of propionic acid productivity, -yield and -concentration (Blanc and Goma, 1987; Boyaval and Corre, 1987; Colomban et al., 1993; Dishisha et al., 2012, 2013; Suwannakham and Yang, 2005; Woskow and Glatz, 1991; Zhang and Yang, 2009a). However, immobilized cell reactors suffered from mass transfer limitation resulting in lower specific cell productivity (Dishisha et al., 2012).

In the present study, the impact of the concentration of glycerol as a carbon source and yeast extract as a nitrogen/vitamin source on propionic acid production was investigated using statistical analysis. Subsequently, the fermentation kinetics for a chosen set of concentrations was determined under controlled pH conditions. Finally the optimized medium was used in cyclic batch fermentations (CBF) and sequential batch fermentations with cell recycle (SBF) for enhanced propionic acid production.

2. Methods

2.1. Materials

Glycerol (99%), ammonium hydroxide solution (28%) and L-cysteine HCl, anhydrous (98%) were products of Sigma–Aldrich (St Louis, MO, USA). Bacto yeast extract (YE) was procured from Difco (BD laboratories, Detroit, MI, USA) and phosphate buffer salts from Merck (NJ, USA).

2.2. Microorganism and preculture preparation

Propionibacterium acidipropionici DSM 4900 was propagated anaerobically as described earlier (Dishisha et al., 2013), where 1 mL of stock culture in 20% v v^{-1} glycerol was transferred to 20 mL of medium containing per liter: 20 g glycerol, 10 g yeast extract, 2.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 0.25 g L-cysteine HCl, (pH 7) in a 30 mL serum bottle. The culture was incubated for 4 days at 32 °C and the resulting culture was used to inoculate another 20 mL medium and incubated for 2 days. The same culture medium was used for propionic acid production experiments with the exception that glycerol and yeast extract concentrations were varied.

2.3. Evaluating the effect of yeast extract and glycerol on propionic acid production

2.3.1. Factorial design under uncontrolled pH cultivations

A 3^3 factorial design experiment was performed to determine the effect of yeast extract and glycerol on cell growth and propionic acid production. The dependent variable selected for this study was the propionic acid volumetric productivity, expressed in g L^{-1} h^{-1}, and the independent variables were the concentrations of yeast extract and glycerol. The range and the levels of these variables are given in Table S1. Fermentations were done in 100 mL serum bottles containing 90 mL fermentation medium inoculated with 4.5 mL (5% v v^{-1}) of fresh inoculum. The culture was incubated at 32 °C and samples were collected every 24 h and analyzed for cell growth and the concentrations of glycerol and propionic acid.

2.3.2. Statistical analysis

Statistica software package (Version 5.0) a product of StatSoft, was used for regression and graphical analysis.

2.3.3. Batch production of propionic acid with controlled pH

Twenty milliliters of fresh inoculum was added to 400 mL fermentation medium in a 600 mL jacketed glass bioreactor. The culture was mixed with a magnetic stirring-device at 200 rpm. Temperature was controlled at 32 °C using a circulating water bath (Haake, Germany), pH was maintained at 6.5 using a pH-electrode connected to pH controller unit (Inventron, Sweden), which controls a pump for addition of 5 N NH_4OH. For maintaining anaerobic conditions, the medium was initially bubbled with nitrogen gas and then the headspace was connected to a nitrogen gas bag. Six different combinations of glycerol and yeast extract were evaluated (40:10, 50:10, 60:10, 90:10, 60:20 and 90:30) (g L^{-1} each).

2.4. Propionic acid production in cyclic batch fermentations (CBF)

The CBF were performed in a 3 L bioreactor (Applikon, Microbial Biobundle, The Netherlands) with 1 L working volume. The stirrer speed was maintained at 200 rpm, pH at 6.5 through addition of 5 N NH_4OH, and temperature at 32 °C via a heating blanket and a cooling finger. After autoclaving, the medium was bubbled with nitrogen gas and then connected to a nitrogen gas bag to keep the overhead space saturated with nitrogen. The fermentation was started by addition of 50 mL freshly prepared inoculum (5% v v^{-1}) and was run until the glycerol concentration reached 5 g L^{-1}. The subsequent batch was started by replacing 90% of the fermentation broth with an equal volume of fresh medium. Two parallel experimental setups were performed, each composed of three consecutive cyclic batches. The concentrations of glycerol and yeast extract (g L^{-1} each) in the media in the first set were 60:20 during the three batches. In the second set, the corresponding concentrations were 60:20 for the first batch, and 90:30 for the second and third batches.

2.5. Propionic acid production in sequential batch fermentation

Fifty milliliter of freshly prepared inoculum was added to 1 L fermentation medium containing per liter 60 g glycerol, 20 g yeast

extract, 2.5 g K_2HPO_4, 1.5 KH_2PO_4 and 0.25 g L-cysteine HCl in a 3-L bioreactor (Applikon). Fermentation conditions were similar to that described in Section 2.4. The fermentation was continued for 72 h after which the broth was withdrawn and the cells were collected by centrifugation at 15,000g for 10 min and 4 °C.

The pelleted cells were resuspended in 400 mL medium to a final cell concentration of 11.56 g L^{-1} (dry weight), and fermentation was started and continued till consumption of the entire substrate. The steps of centrifugation and cell resuspension were repeated for subsequent batches. Glycerol:yeast extract concentrations (g L^{-1} each) used were as follows: 60:20 for batches 1 and 2, 90:30 for batches 3 and 4, 120:40 for batches 5 and 6, and 150:50 for batch 7. Each concentration was run twice sequentially as a way to confirm the stability of the obtained results, except for batch 7 where incomplete consumption of glycerol was observed.

2.6. Analytical methods

Cell growth expressed as units of optical density (OD) at 620 nm was measured using UV–Vis Spectrophotometer, Ultrospec 1000 (Pharmacia Biotech, Sweden) and correlated with the cell dry weight (CDW). For determination of the cell dry weight, 1 mL of fermentation broth was centrifuged at 15,000g for 2 min in weighed pre-dried tube. The cell pellet was then dried at 105 °C for 12 h. The weight difference is equivalent to the cell dry weight per milliliter.

Analyses of glycerol, propionic acid, acetic acid, succinic acid and *n*-propanol (*n*-POH) were done using an HPLC system (Jasco, Tokyo, Japan) equipped with an RI-detector (ERC, Taguchi, Japan) and chromatographic oven (Shimadzu, Tokyo, Japan). The separation was done on Aminex HPX-87H cation exclusion chromatographic column (300 × 7.8 mm and particle size 9 μm) connected to a guard column (BioRad, USA) using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. Injection volume was 50 μl and the column temperature was kept at 55 °C. Samples for HPLC were diluted to the required concentration range and then mixed with 20 μl mL^{-1} of 20% v v^{-1} sulfuric acid.

The volumetric- (Q) and specific (q) rates, and product yield ($Y_{PA/Gly}$) were calculated by taking into account the dilution of the medium as a result of base addition as follows:

$$Q_{PA}\ (g\ L^{-1}\ h^{-1}) = [(PA_{final} * \text{dilution factor}) - PA_{initial}]/[\Delta t]$$

$$q_{PA}\left(g_{PA}\ g_{CDW^{-1}}\ h^{-1}\right) = Q_{PA}/X, \text{ for propionic acid production, and}$$

$$Q_{Gly}\left(g\ L^{-1}\ h^{-1}\right) = [(Gly_{final} * \text{dilution factor}) - Gly_{initial}]/[\Delta t]$$

$$q_{Gly}\left(g_{Gly}\ g_{CDW^{-1}}\ h^{-1}\right) = Q_{Gly}/X, \text{ for glycerol consumption}$$

$$Y_{PA/Gly}(g\ g^{-1}) = [(PA_{final} * \text{dilution factor}) - PA_{initial}] / [(Gly_{final} * \text{dilution factor}) - Gly_{initial}]$$

3. Results and discussion

3.1. Medium optimization without controlling the pH

A three-way ANOVA analysis was performed to determine the effect of yeast extract and glycerol concentrations on propionic acid production in fermentations with uncontrolled pH. The minimum and maximum levels of variables used were 30–90 g L^{-1} for glycerol and 10–30 g L^{-1} for yeast extract, respectively. Three different concentrations of each component in 9 combinations were used and the cell growth, propionic acid concentration, volumetric

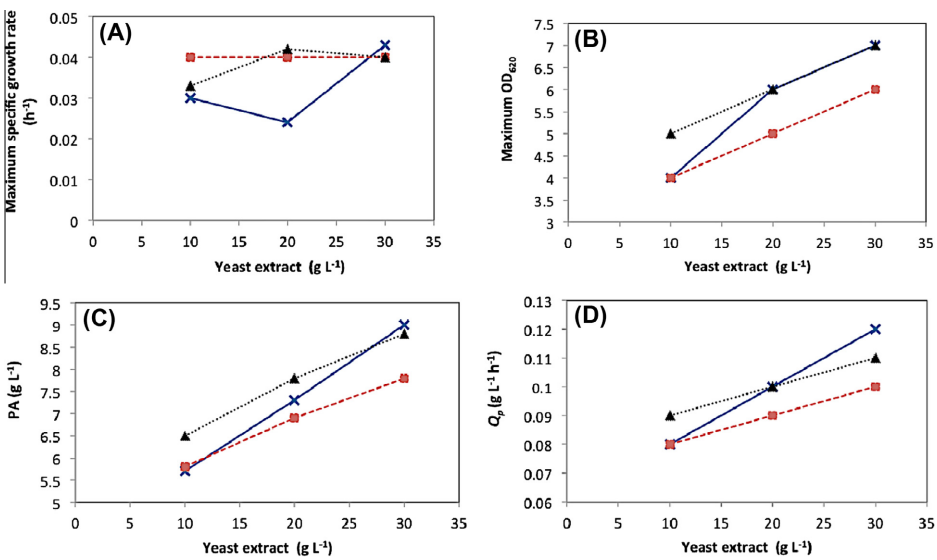

Fig. 1. Effect of yeast extract and glycerol concentrations on: (A) maximum specific growth rate of cells (μ_{max}), (B) maximum OD_{620nm}, (C) propionic acid concentration, and (D) propionic acid volumetric production rate in fermentations using *P. acidipropionici*. The concentration of glycerol in the medium was (g L^{-1}): 30 (x), 60 (■), and 90 (▲), respectively. Propionic acid production was done in serum bottles without shaking and with uncontrolled pH.

production rate and yield were determined (Fig. 1 and Table S1). The obtained data revealed that the concentration of yeast extract was statistically significant ($P < 0.05$) with respect to propionic acid volumetric production rate. The average maximum specific growth rate (μ_{max}) for the different media combinations was 0.037 ± 0.006 h^{-1} indicating minimal impact of glycerol and yeast extract concentrations on the growth rate.

The impact of yeast extract concentration was higher at 30 g L^{-1} glycerol, where increasing its concentration by 10 g L^{-1} was accompanied by increase in the maximum cell density, propionic acid concentration and its volumetric productivity, by 0.55 g_{CDW} L^{-1}, 1.65 g_{PA} L^{-1} and 0.02 g_{PA} L^{-1} h^{-1}, respectively. At 60 or 90 g L^{-1} glycerol, these rates were increased by 0.37 g_{CDW} L^{-1}, 1.10 g_{PA} L^{-1} and 0.01 g_{PA} L^{-1} h^{-1}, respectively. The maximum volumetric productivity achieved was 0.12 g L^{-1} h^{-1} and the maximum specific growth rate was 0.043 h^{-1}.

3.2. Batch production of propionic acid with controlled pH

Fermentations for the production of organic acids are characterized by a reduction in the pH which inhibits cell growth and product formation (Hsu and Yang, 1991). Consequently, increasing the concentration of glycerol from 30 to 90 g L^{-1} at constant yeast extract concentration with uncontrolled pH had limited effect on fermentation kinetics. In order to obtain the actual kinetics, controlled-pH cultivations were performed for a chosen set of glycerol and yeast extract concentrations (g L^{-1} each) of 40:10, 50:10, 60:10, 90:10, 60:20 and 90:30, respectively. The pH was maintained at 6.5 that is located between the optimum value for growth (pH 7) and that for propionic acid production (pH 6) (Hsu and Yang, 1991).

For the different media compositions, the time course for microbial growth, glycerol consumption and metabolites formation are shown in Fig. S1 and the fermentation kinetics are summarized in Table 1. The specific propionic acid production- (q_P), biomass production- (q_x) and glycerol consumption- (q_s) rates were calculated for each sample point and plotted versus propionic acid concentration at the same point (Fig. S2). This correlation was subsequently used for determination of the critical propionic acid concentrations affecting cell growth and metabolic activity as described elsewhere (Blanc and Goma, 1987; Quesada-Chanto et al., 1998). In all the combinations evaluated, consumption of the entire glycerol was achieved with the exception of glycerol: yeast extract of 90:10 where only 90% of the initial glycerol was consumed in 289 h.

Increasing the initial glycerol concentration from 40 to 90 g L^{-1}, while maintaining yeast extract at 10 g L^{-1} has led to increase in propionic acid concentration from 19.50 to 32.10 g L^{-1}, and maximum cell density from 4.39 to 7.32 g_{CDW} L^{-1}, respectively. Nevertheless, propionic acid yield was constant around 0.68 mol_{PA} mol_{Gly}^{-1}. The fermentation time was also increased from 137 h (40 g L^{-1} glycerol) to 289 h (90 g L^{-1} glycerol) as a result of strong product inhibition, which resulted in reduction of the corresponding production rate from 0.18 to 0.16 g L^{-1} h^{-1} and incomplete consumption of the supplied glycerol in the latter case. Additionally, the concentration of the by-products was increased and the molar ratio of propionic acid to by-products was decreased.

Increasing yeast extract concentrations while maintaining the glycerol concentration constant resulted in increase in final cell density from ~7.3 g_{CDW} L^{-1} at glycerol: yeast extract of 60:10 and 90:10 to 9.2 g_{CDW} L^{-1} with 60:20 and 11.7 g_{CDW} L^{-1} with 90:30. On the other hand, the maximum specific growth rate was not affected and had an average of 0.116 ± 0.006 h^{-1} (Table 1).

Increasing yeast extract concentration from 10 to 30 g L^{-1} at constant initial glycerol concentration of 90 g L^{-1} resulted in elevated propionic acid concentration and yield. The most significant outcome was the reduction in the fermentation time by 140 h, hence doubling the volumetric production rate. The amount of base added to maintain the pH was also decreased by 27%, probably a result of the increase in the buffering effect of the yeast extract components. The increased yield was accompanied by reduction in succinic acid, acetic acid and *n*-propanol production, which could be explained by the availability of co-factors required for the enzymes catalyzing the last steps in the metabolic pathway. The ratio between PA/AA, PA/SA and PA/*n*-POH reached 27.06 mol_{PA} mol_{AA}^{-1}, 12.58 mol_{PA} mol_{SA}^{-1}, 16.78 mol_{PA} mol_{nPOH}^{-1} at glycerol: yeast extract of 90:30, which are 1.6, 1.6 and 1.9-fold higher than the corresponding ratios obtained at glycerol: yeast extract of 90:10. The highest ratio of propionic acid to by-products was observed when 60:20 of glycerol: yeast extract was used, while the lowest was obtained using nutrient combination of 60:10 or 90:10.

Table 1
Effect of ratio of carbon:nitrogen source on propionic acid fermentation under pH-controlled conditions.

Parameters	Medium composition (C:N) g L^{-1} each					
	40:10	50:10	60:10	90:10	60:20	90:30
Q_P (g L^{-1} h^{-1})	0.18	0.19	0.22	0.16	0.31	0.35
Q_s (g L^{-1} h^{-1})	−0.30	−0.32	−0.42	−0.29	−0.50	−0.56
Y (mol mol^{-1})	0.68	0.73	0.66	0.67	0.78	0.77
Y (g g^{-1})	0.55	0.59	0.53	0.54	0.63	0.62
Initial OD	0.17	0.25	0.17	0.26	0.25	0.23
Final OD	10.18	12.83	16.58	12.34	21.99	25.38
Biomass (g_{CDW} L^{-1})[a]	4.39	5.86	7.25	7.32	9.15	11.71
Final PA (g L^{-1})	19.50	25.80	26.00	32.10	33.00	43.40
Final AA (g L^{-1})	0.70	0.70	1.30	1.60	0.50	1.30
Final SA (g L^{-1})	3.00	3.50	4.00	6.40	3.10	5.50
Final *n*-POH (g L^{-1})	1.70	1.80	2.80	2.90	1.50	2.10
PA/AA (mol mol^{-1})	25.67	29.88	16.21	16.26	53.50	27.06
PA/SA (mol mol^{-1})	11.65	11.75	10.36	8.00	16.97	12.58
PA/*n*POH (mol mol^{-1})	9.32	11.64	7.54	8.99	17.87	16.78
PA/by-products (mol mol^{-1})	4.14	4.89	3.44	3.36	7.48	5.68
μ_{max} (h^{-1})	0.103	0.112	0.109	0.114	0.112	0.114
Fermentation time (h)	137	150	137	288.5[b]	121	~150
Base addition (mL)	63	59	70	141	57	103
Ratio C/N	4	5	6	9	3	3

Abbreviations: Q_P – volumetric productivity; Q_S – volumetric consumption rate; Y – yield; PA – propionic acid; AA – acetic acid; SA – succinic acid; *n*-POH – *n*-propanol.
[a] Dilution factor considered.
[b] Incomplete consumption of glycerol.

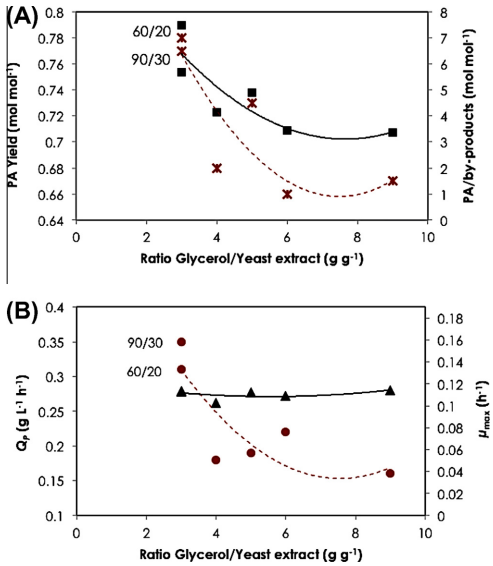

Fig. 2. Effect of glycerol to yeast extract ratio ($g\,g^{-1}$) on: (A) propionic acid yield ($mol_{PA}\,mol_{gly}^{-1}$) (x, dashed line), and propionic acid volumetric productivity ($g\,L^{-1}\,h^{-1}$) (■, solid line), and (B) molar ratio of propionic acid to by-products ($mol_{PA}\,mol_{by\text{-}products}^{-1}$) (●, dashed line), and specific growth rate of *Propionibacterium acidipropionici* (▲, solid line). Propionic acid production was performed in a batch mode of operation with controlled pH.

Going from glycerol: yeast extract of 90:10 to 90:30 increased the critical propionic acid concentration inhibiting cell growth from 25 to 32 $g\,L^{-1}$ (Fig. S2A). Additionally, critical propionic acid concentration inhibiting metabolic activity was increased from 30 to ~44 $g_{PA}\,L^{-1}$ for 90:10 and 90:30 (Fig. S2B) indicating reduced propionic acid inhibitory effect on *P. acidipropionici* cells.

The plots of propionic acid volumetric productivity, -yield and -its molar ratio to by-products, as a function of the ratio of glycerol to yeast extract (C/N; $g\,g^{-1}$) showed a general increase in these parameters with decrease in the C/N ratio (within the tested range) (Fig. 2A and B). On the other hand, the maximum specific growth rate (μ_{max}) was not affected, indicating absence of inhibitory or stimulatory effects of the carbon- and nitrogen source (Fig. 2B).

Based on these results, a ratio of glycerol to yeast extract (g:g) of 3:1 is considered essential for consumption of the entire glycerol amount with minimal effect on volumetric production rate, and was used in the subsequent studies for propionic acid production using CBF and SBF.

3.3. Cyclic batch propionic acid fermentation using P. acidipropionici

CBF is a modified repeated batch culture in which a strategy of replacing 90% of the broth at the end of the fermentation with the same volume of fresh medium is advantageous for removing most of the inhibitory products, and yielding more adapted cells which will ensure faster utilization of the substrate and production of the acid in the subsequent batches. The withdrawn broth can be subjected to downstream processing while the subsequent batch is being operated.

During CBF, the effect of well-controlled conditions gave a clear effect on the fermentation time and productivity. In comparison to the pH-controlled batch cultivation with glycerol and yeast extract concentrations of 60:20, the first cycle in both CBF cultures gave shorter batch time (75 h) and higher propionic acid productivity ($0.42\,g\,L^{-1}\,h^{-1}$) (Table 2, Fig. 3A and B). The concentrations of glycerol and yeast extract ($g\,L^{-1}$ each) in the second and third cycles were either 60:20 (System 1) or 90:30 (System 2).

The long initial glycerol-independent growth observed in the first cycle was decreased from 24 to 11 h in the second cycle 1#2, which resulted in shorter fermentation time (Fig. 3A and Table 2). The volumetric production rate and propionate yield were however close to that in the first batch. The maximum specific growth rate was reduced from $0.106\,h^{-1}$ in the first batch, to 0.073 and $0.027\,h^{-1}$ in cycles 1#2 and 1#3, respectively.

In System 2#, when higher glycerol and yeast extract concentrations were used, the volumetric productivity in cycle 2#2 and 2#3 were increased by 3.4% and 27.5%, respectively. Moreover, the propionate yield reached 0.93 $mol_{PA}\,mol_{Gly}^{-1}$ in the last cycle. A reduction in μ_{max} was also observed (Fig. 3B and Table 2).

3.4. Sequential batch fermentation with cell recycle (SBF)

High cell density fermentations under batch, fed-batch and continuous modes of operations have been reported to enhance propionic acid volumetric productivity and yield considerably (Boyaval and Corre, 1987; Colomban et al., 1993; Dishisha et al., 2013). However, increasing the initial glycerol concentration was

Table 2
Cyclic batch fermentation (CBF) and sequential batch fermentation with cell recycle (SBF).

Glycerol ($g\,L^{-1}$)	Yeast extract ($g\,L^{-1}$)	Q_P ($g\,L^{-1}\,h^{-1}$)	Q_S ($g\,L^{-1}\,h^{-1}$)	Y ($mol\,mol^{-1}$)	μ_{max} (h^{-1})	Initial CDW ($g\,L^{-1}$)
Cyclic batch fermentation (CBF) – System 1						
60	20	0.42	−0.73	0.71	0.106	0.11
60	20	0.37	−0.70	0.74	0.073	1.42
60	20	0.43	−0.79	0.64	0.027	1.21
Cyclic batch fermentation (CBF) – System 2						
60	20	0.42	−0.71	0.73	0.106	0.11
90	30	0.43	−0.74	0.61	0.056	1.52
90	30	0.53	−0.71	0.93	0.074	1.75
Sequential batch fermentation (SBF)						
60	20	1.19	−1.99	0.74		11.56
60	20	1.36	−2.43	0.70		16.09
90	30	1.47	−2.67	0.71		20.30
90	30	1.63	−2.77	0.73		26.81
120	40	1.13	−1.94	0.71		28.79
120	40	1.12	−1.94	0.71		31.86
150	50	0.30	−0.50	0.74		31.28

Abbreviations: Q_P – volumetric productivity; Q_S – volumetric consumption rate; Y – Yield; μ_{max} – maximum specific growth rate; CDW – cell dry weight.

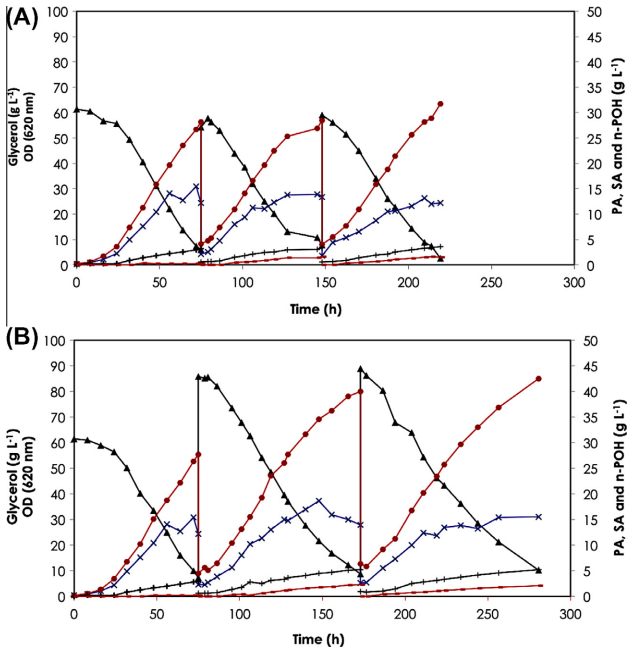

Fig. 3. Production of propionic acid by cyclic batch fermentation (CBF) using *P. acidipropionici* DSM 4900 with recycling 10% of the fermentation broth. The symbols represent the concentrations of glycerol (▲), propionic acid (●), succinic acid (+), *n*-propanol (−) and cell growth as optical density at 620 nm (x). Two experimental sets are shown: (A) three cyclic batches using 60 g L^{-1} glycerol and 20 g L^{-1} yeast extract. (B) Single batch using 60 g L^{-1} glycerol and 20 g L^{-1} yeast extract followed by two batches using 90 g L^{-1} glycerol and 30 g L^{-1} yeast extract.

accompanied with substantial reduction in production rate when heat-treated potato juice was used as nitrogen source (Dishisha et al., 2013). In the present study, four different media combinations with the C/N ratio of 3 were tested in 7 sequential batches with cell recycling. Utilization of the entire glycerol amount was achieved at all C/N combinations with the exception of the highest concentration of 150:50, where only 86% of the initial glycerol amount was consumed.

Fig. 4 shows the microbial growth, glycerol consumption and propionic acid production, and the kinetics are summarized in Table 2. The average fermentation time for each medium combination after two sequential batches was 26.5 h (60:20), 33.5 h (90:30) and 62 h (120:40) and the average corresponding propionic acid concentrations were 32.0, 46.0 and 58.0 g_{PA} L^{-1}, respectively. Propionic acid yield was highly stable between 0.70–0.74 mol_{PA} mol_{Gly}^{-1}. In all the cases the volumetric productivity exceeded 1 g L^{-1} h^{-1} and reached a maximum of 1.63 g L^{-1} h^{-1} in glycerol: yeast extract combination of 90:30.

In the first two batches using 11.56 and 16.09 g_{CDW} L^{-1}, respectively, as initial cell density with glycerol: yeast extract combination of 60:20, the slopes of glycerol consumption and propionic acid production as a function of time were linear indicating the absence of substrate- or product inhibition. Propionic acid was produced at an average rate of 1.28 ± 0.12 g L^{-1} h^{-1}, which is 4.1 times higher than that for the similar medium composition and lower initial biomass concentration (0.09 g_{CDW} L^{-1}). Shifting from batch to batch the initial biomass concentration was increased. At 90 g L^{-1} glycerol the average production rate was 1.55 ± 0.11 g L^{-1} h^{-1}, which is the highest reported productivity from glycerol using batch mode of operation. Slight product inhibition was observed near the end of each batch and resulted in reduction of consumption rate of the residual 45 g L^{-1} glycerol to 2.19 g L^{-1} h^{-1} after a maximum of 4.19 g L^{-1} h^{-1}. As a consequence, propionic acid production rate reached 1.29 g L^{-1} h^{-1} after a maximum of 2.47 g L^{-1} h^{-1}. Increasing glycerol concentration to 120 g L^{-1} resulted in 29% reduction in production rate, which was still over 1 g L^{-1} h^{-1} and product inhibition was more significant.

With initial glycerol concentration of 150 g L^{-1}, product inhibition was significant and was accompanied by loss of metabolic activity at propionic acid concentration of 60 g L^{-1}. Product yield obtained was however in the same range as with lower glycerol concentrations.

In case of SBF system, comparison with the earlier reported results using heat-treated potato juice as a nitrogen/vitamin source, revealed increase in propionic acid productivity by 2 and 4-fold at glycerol: yeast extract concentrations (g L^{-1} each) of 90:30 and 120:40, respectively (Dishisha et al., 2013). This indicates the significance of the nitrogen source on propionic acid productivity, and also confirms that the lowered propionic acid productivity with heat-treated potato juice could be improved by increasing its concentration or supplementation with additional *N*-source/co-factors.

Production of propionic acid by Propionibacteria proceeds through the dicarboxylic acid pathway. The different steps beyond pyruvate to propionate through succinate require different co-factors and vitamins for enzymatic activity such as vitamin B12, biotin, and pantothenic acid (Hettinga and Reinbold, 1972a,b,c). The complex nitrogen source constitutes the main supply for these

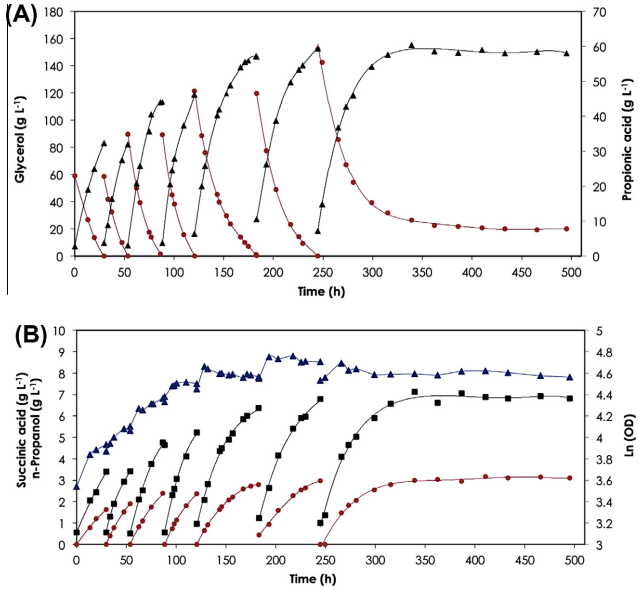

Fig. 4. Production of propionic acid by sequential batch fermentation (SBF) using *P. acidipropionici* DSM 4900 with cell recycle showing the concentrations of: (A) glycerol (●) and propionic acid (▲), (B) succinic acid (■), *n*-propanol (●), and biomass (▲). The initial concentrations of glycerol and yeast extract (g L^{-1}) were 60:20 for batches 1 and 2, 90:30 for batches 3 and 4, 120:40 for batches 5 and 6, and 150:50 for batch 7.

co-factors, and limited or unbalanced supply results in variation in by-products pattern and fermentation kinetics. For instance, 0.5 mg L^{-1} biotin was added to potato juice for enhancing the fermentation kinetics (Dishisha et al., 2013; unpublished data) and supplementation of whey with yeast extract was essential (Colomban et al., 1993). Increasing nitrogen/vitamin source concentration will ensure supply of these co-factors in excess, and hence higher propionic acid production rates and -yields. Also, the amino acids content of the nitrogen source, mainly arginine and aspartic acid act as a buffering system enhancing the propionic acid fermentation kinetics through lowering the inhibitory effect of the acid on the producing cells (Guan et al., 2013).

4. Conclusion

The present study clearly shows that the nitrogen/vitamin source plays an important role in propionic acid production. Modification of the conventional batch fermentation to CBF or SBF involving cell recycle, and operation using optimal nutrient composition and good pH-control, improved the process by shortening the fermentation time and maintaining high propionic acid productivity. It is possible that after optimization of the CBF and SBF processes with the cheap nitrogen source "e.g., heat-treated potato juice (Dishisha et al., 2013)", these strategies could be easy-to-apply for large scale propionic acid bioproduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.11.013.

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Electronic Supplementary Information (ESI)

Improved propionic acid production from glycerol: combining cyclic batch- and sequential batch fermentations with optimal nutrient composition

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Table S1. Effect of different concentrations of yeast extract (YE) and glycerol on cell growth and propionic acid production using *Propionibacterium acidipropionici* DSM 4900 in batch fermentations with uncontrolled culture pH.

Glycerol ($g\ L^{-1}$)	**YE ($g\ L^{-1}$)**	**μ_{max} (h^{-1})**	**Max OD**	**Max propionic acid ($g\ L^{-1}$)**	**Q_p ($g\ L^{-1}\ h^{-1}$)**
30	**10**	0.030	4	5.7	0.08
30	**20**	0.024	6	7.3	0.10
30	**30**	0.043	7	9.0	0.12
60	**10**	0.040	4	5.8	0.08
60	**20**	0.040	5	6.9	0.09
60	**30**	0.040	6	7.8	0.10
90	**10**	0.033	5	6.5	0.09
90	**20**	0.042	6	7.8	0.10
90	**30**	0.040	7	8.8	0.11

Fig. S1 Batch production of propionic acid from glycerol using *Propionibacterium acidipropionici* DSM 4900 with controlled culture pH at 6.5 at varying concentration of glycerol:yeast extract (g L^{-1} each). The symbols represent concentrations of glycerol (■), propionic acid [PA] (▲), succinic acid [SA] (x), acetic acid [AA] (∗), *n*-propanol [n-POH] (●), and cell density represented by OD at 620 nm (♦). Figures represents different combinations of glycerol and yeast extract.

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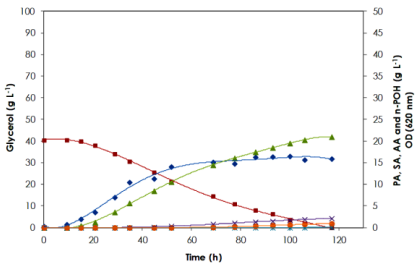

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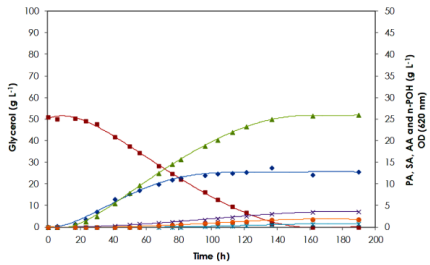

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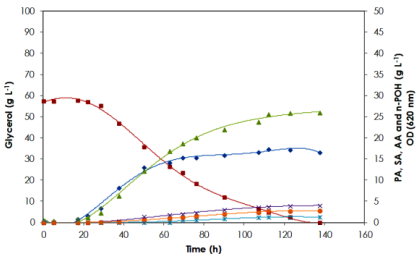

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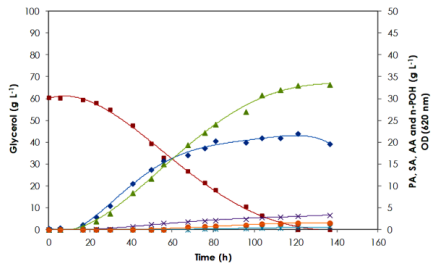

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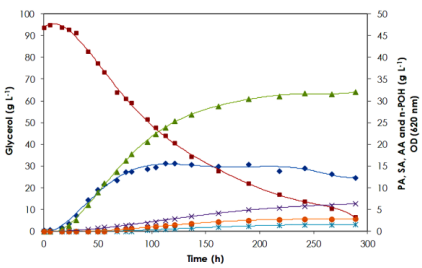

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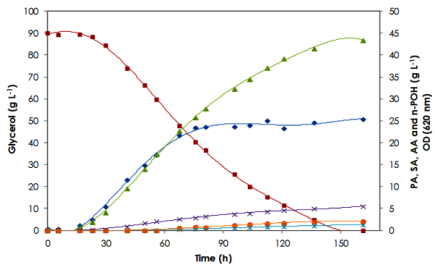
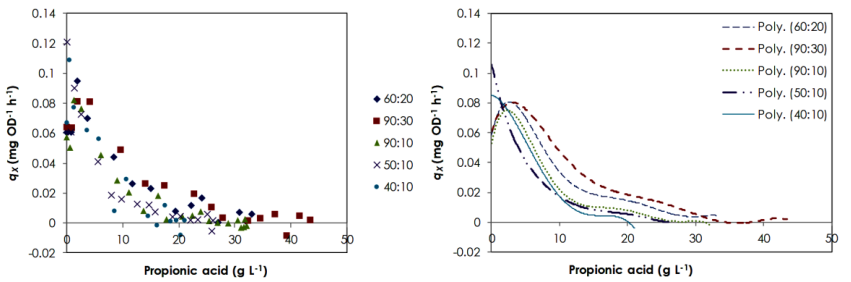


Fig. S2 (A) Specific growth rate (q_X) in h^{-1}, (B) specific propionic acid production rate (q_P) in g L^{-1} h^{-1}, and (C) specific glycerol consumption rate (q_S) in g L^{-1} h^{-1} as function of propionic acid concentration (g L^{-1}). The specific rates were determined for each sampling point of propionic acid fermentation using *Propionibacterium acidipropionici* DSM 4900 growing on different combinations of glycerol and yeast extract (g L^{-1} each).

A



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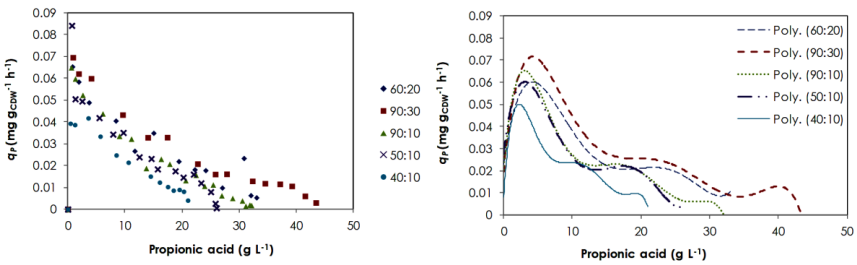

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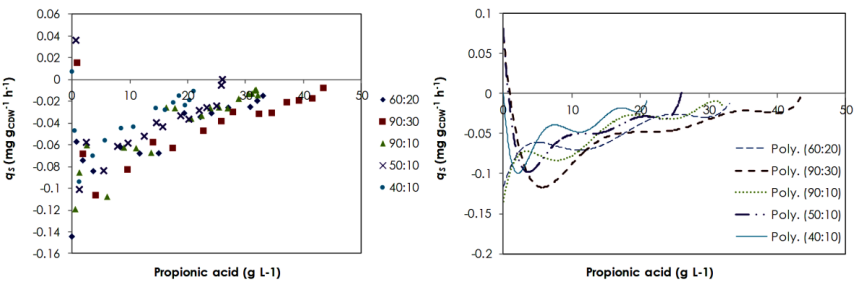

Paper II

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ORIGINAL PAPER

Stress induced biofilm formation in *Propionibacterium acidipropionici* and use in propionic acid production

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Abstract
Propionibacterium acidipropionici produces propionic acid from different sugars and glycerol; the production can be improved by high cell density fermentations using immobilized cells that help to overcome the limitations of the non-productive lag phase and product inhibition. In this study, the use of stress factors to induce *P. acidipropionici* to form biofilm and its use as an immobilization procedure in fermentations in bioreactors for producing propionic acid was investigated. Citric acid and sodium chloride increased exopolysaccharide production, biofilm forming capacity index and trehalose production. Analysis of the expression of trehalose synthesis-related genes *otsA* and *treY* by RT-qPCR showed significantly increased expression of only *treY* during log phase with citric acid, while FISH analysis showed expression of *treY* and *luxS* under the influence of both stress factors. The stress factors were then used for development of microbial biofilms as immobilization procedure on Poraver® and AnoxKaldnes® carriers in recycle batch reactors for propionic acid production from 20 g/L glycerol. Highest productivities of 0.7 and 0.78 g/L/h were obtained in Poraver® reactors, and 0.39 and 0.43 g/L/h in AnoxKaldnes® reactors with citric acid and NaCl, respectively.

Keywords Biofilm · Immobilization · Propionic acid · Stress factor · Trehalose

Introduction

Dairy Propionibacteria are an important group of microorganisms that have been traditionally used for the production of Swiss-type cheeses, and have also been used as animal and human probiotics (Langsrud et al. 1973; Cousin et al. 2011). They are even known to produce value added products such as Vitamin B12, trehalose, flavor compounds, antimicrobial compounds and propionic acid (Yongsmith et al. 1982; Woskow and Glatz 1991; Holo et al. 2002; Ruhal et al. 2012; Piwowarek et al. 2018). Considering the various ecological niches they inhabit, the Propionibacteria have the ability to adapt and survive under stressful conditions like high temperature, high salt concentration and low pH (Jan et al. 2000; Leverrier et al. 2004; Anastasiou et al. 2006). The adaptation to certain stress conditions is ascribed to induction of several stress response proteins (Leverrier et al. 2004; Guan et al. 2014) or trehalose synthesis (Cardoso et al. 2004; Ruhal et al. 2013).

Trehalose, a non-reducing disaccharide comprising two glucose molecules, is widely distributed in nature and is accumulated in organisms throughout all biological domains capable of surviving under complete dehydration conditions (Iturriaga et al. 2009). Several pathways for biosynthesis are known in different organisms including: (i) OtsA-OtsB pathway involving transfer of glucose from UDP-glucose to glucose-6-phosphate to yield trehalose 6-phosphate that is subsequently converted to trehalose (Brüggemann et al. 2004) (ii) TreS pathway involving the intramolecular arrangement of the α (1→4) glycosidic bond of maltose to α (1→1) bond to form trehalose (Nishimoto et al. 1996)

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(iii) TreY-TreZ pathway in which terminal α (1→4) bond of a glucan polymer is converted to α (1→1) bond followed by cleavage of the terminal disaccharide forming trehalose (Nakada et al. 1995) (iv) reversible conversion of glucose and ADP-glucose into trehalose catalyzed by trehalose glycosyltransferring synthase (TreT) (Qu et al. 2004), and (v) TreP (trehalose phosphorylase) catalyzed reversible reaction between glucose and glucose-1-phosphate (Ren et al. 2005). Trehalose is commonly found in propionibacteria; in particular *P. freudenreichii* subsp. *shermanii* has been found to accumulate very high levels of the sugar. OtsA-OtsB and TreS pathways have been identified in *P. freudenreichii*, the former used for trehalose synthesis and the latter for trehalose degradation (Cardoso et al. 2007).

Propionibacterium freudenreichii and *Propionibacterium acidipropionici* are the most commonly studied and industrially significant species. There are several reports on the use of these bacteria for production of propionic acid that is widely used as food and feed preservative, in cellulose plastics, pharmaceuticals, etc. (Liu et al. 2012). Propionibacteria utilize sugars and glycerol as carbon sources, the latter being the preferred substrate due to its highly reduced state that results in higher propionic acid yield with less byproducts as compared to sugar substrates (Coral et al. 2008). Propionic acid fermentation is characterized by slow cell growth, and is subject to inhibition by the product and low pH, resulting in low yield and productivity. Different approaches have been used to overcome these drawbacks and to boost the propionic acid production, e.g. by high cell density fermentations (Dishisha et al. 2012, 2013, 2015; Chen et al. 2013), extractive fermentation (Lewis and Yang 1992; Goswami and Srivastava 2001), and evolutionary- and metabolic engineering for enhancing tolerance of the bacteria to propionic acid and low pH (Woskow and Glatz 1991; Zhang and Yang 2009; Guan et al. 2016; Sapi et al. 2016).

Immobilization of Propionibacteria has been a common approach for achieving high cell density fermentations with improved fermentation kinetics (Rickert et al. 1998; Dishisha et al. 2012; Chen et al. 2013). Adsorption of cells to a solid matrix constitutes a simple form of immobilization. *P. freudenrichii* has been immobilized in a fibrous bed reactor in which the highest propionic acid concentration of about 136 g/L was reported (Chen et al. 2013). *P. acidipropionici* was adsorbed to recycled glass beads, Poraver® by way of electrostatic interactions with the cationic polymer, polyethyleneimine (PEI) used for coating the matrix (Dishisha et al. 2012). Although spontaneous biofilm formation on surfaces is widespread in bacterial consortia involving different mechanisms, several single bacterial species are able to develop biofilms for attachment to surfaces invariably through production of extracellular polymeric substances that are complex mixtures of mainly exopolysaccharides (EPS) and less amounts of proteins, DNA and lipids. Immobilization of *P. freudenrichii* was recently shown to be facilitated by the EPS produced by the microorganism (Belgrano et al. 2018). Biofilm formation can sometimes be triggered even in non-biofilm producers by cultivating them under sub-optimal growth conditions e.g. related to nutrition, temperature, osmolarilty (Fux et al. 2005; Rode et al. 2007). One strategy to obtain a biofilm is to stress the growth conditions making the microorganism survive by switching its metabolic resources for an adaptation to the new harsh environment where biofilm formation plays a protective role.

In the present work, biofilm formation by *P. acidipropionici* by exposure to stress conditions and propionic acid production were investigated, and the possible correlation to the synthesis of trehalose produced under the selected stress conditions was also studied.

Materials and methods

Strain and culture medium

Propionibacterium acidipropionici DSMZ 4900 was used in the present study. The basal culture medium used for cultivation of the organism consisted of 10 g yeast extract, 20 g glycerol, 0.25 g cysteine, 2.5 g K_2HPO_4 and 1.5 g KH_2PO_4 per liter of distilled water, with pH adjusted to 7 with 25% ammonia solution (Dishisha et al. 2013). The medium was flushed with oxygen-free nitrogen and autoclaved at 121 °C, 1.5 atm for 20 min prior to use. Inocula of *P. acidipropionici* were prepared from cryopreserved cultures, in 90 mL medium in serum bottles, grown at 30 °C for about a week prior to every new experimental procedure.

Screening of stress factors for inducing biofilm formation

Stress factors for developing biofilm of *P. acidipropionici* were chosen according to previous studies performed on *Salmonella enterica* (Gruzdev et al. 2011). The base culture medium was supplemented with the stress factors at different concentrations in serum bottles: 0.25, 0.5 and 0.75 M NaCl; 10, 20 and 30% (v/v) ethanol; 0.5, 1 and 1.5% hydrogen peroxide; 25, 50 and 75 ppm sodium hypochlorite; 25, 50 and 75 mM citric acid, and 40, 80 and 120 mM acetic acid, respectively. The concentrations of the chemicals (except for citric- and acetic acid) fall within the range described earlier (Gruzdev et al. 2011). Every stress factor was added in three different concentrations in order to find out possible triggering concentrations for biofilm formation. The bottles with 90 mL medium were inoculated with 5 mL of actively-growing inoculum and incubated at 30 °C for two

weeks prior to evaluation of EPS production and determination of Biofilm Forming Capacity (BFC) index, explained elsewhere.

Use of the stress factors in multi-stage fermentations

Propionibacterium acidipropionici was used for propionic acid fermentations in a reactor set up shown in Fig. 1, which consisted of a water-jacketed reactor vessel (500 mL) and a column (500 mL; 20 cm height and 5 cm internal diameter) packed with Poraver® (Dennert Poraver GmbH) or AnoxKaldnes® carriers as biofilm supports. The reactor vessel was equipped with ports for pH electrode, base addition, sampling, gas release and connection to the column for recycling of the culture medium. A total of 600 mL culture medium was used, half of which was in the reactor vessel and half in the column. Prior to use the entire reactor set up was sterilized by autoclaving at 121 °C, 1.5 atm for 20 min. During fermentations, temperature of the reactor set-up was maintained at 30 °C with a thermostat water-recirculating system, the medium in the vessel was magnetically stirred at 250 rpm and pH controlled at 6.5 using ammonia (5 M) through a pH control unit. The medium was constantly recycled at approximately 10 mL/min to acquire as much homogeneity as possible.

Four reactors were set up: two packed with Poraver® beads (Dennert Poraver GmbH) and two with AnoxKaldnes® carriers (Veolia Water Technologies). The two reactors with the same carrier material were prepared for experiments with citric acid- and NaCl-containing media, respectively, and were operated in parallel. The reactor operation comprised three stages: biofilm forming, rinsing in place, and propionic acid production. The biofilm forming stage was started by inoculating 15 mL of a fresh inoculum by means of a syringe through the base of the column and then followed immediately by pumping 300 mL of the cultivation medium containing either 30 mM citric acid or 1 M NaCl to fill up the column. The column was then left for a period of 4 days up to 3 weeks at 30 °C for biofilm formation without any recirculation of the medium or pH control. Subsequently, the column was emptied and rinsed by pumping 2 L of sterile 0.9% (w/v) NaCl solution prior to filling with 600 mL of fresh stress factor-free medium for propionic acid production in a recycle batch mode by recirculating the medium between the reactor vessel and the column and adjusting the pH in the vessel. Samples were withdrawn daily from the reactor vessel for determination of cell growth and product formation. Once all the substrate was consumed, the reactor was emptied and rinsed before starting the next batch of production.

In the case of Poraver® reactors, after a biofilm forming stage of three weeks, six batches of propionic acid production were carried out by including 50 mL inoculum in 600 mL of fresh medium during each run. After the sixth batch, three more fermentations were carried out without including inoculum in the medium. Operation of the AnoxKaldnes® reactors was initiated with only one initial inoculum which served for biofilm formation and propionic acid production. Three consecutive biofilm forming stages of 18, 6 and 4 days were carried out adding only fresh stress factor-containing media to each reactor, without any rinsing in between the stages. After the third biofilm-forming stage the column was rinsed and used for 3 cycles in batch of propionic acid production with rinsing in between each batch. Another experiment with AnoxKaldnes® reactors involved only one biofilm formation stage followed by rinsing and four production stages, with rinsing in between.

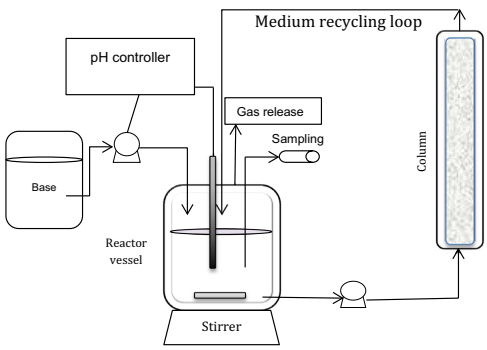

Fig. 1 The reactor set up used for stress-induced biofilm formation of *P. acidipropionici* and repeated recycle batch fermentations for propionic acid production. The arrows between the reactor vessel and the column show recycling of the medium through the reactor set up

Reverse transcription-quantitative PCR (RT-qPCR) and fluorescence in situ hybridization (FISH) experiments for detecting expression of genes involved in biofilm formation

RNA extraction was carried out following the Invitrogen protocol using TRIzol™ Reagent. Briefly, right after the samples were taken from the cultures, RNA protect™ Bacteria Reagent (QIAGEN, cat. no. 76506) was added in a reagent to sample volume ratio of 3:1 to protect the bacterial RNA, followed by centrifugation. The pelleted cells were homogenized by adding equal volume of the TRIzol™ Reagent and pipetting up and down several times in order to break the cells. Phase separation was carried out by adding 0.2 mL of chloroform per mL of TRIzol™ reagent used, shaking and centrifuging at 12,000×*g* for 15 min at 4 °C. The aqueous phase was harvested by pipetting and

transferred to a new tube, followed by RNA precipitation with 0.5 mL of 100% isopropanol per mL of TRIzol™ reagent used and then washing the precipitated RNA with absolute ethanol (vol/vol), shaking gently and centrifuging at 7500×*g*, 5 min at 4 °C. RNAse-free water was added for resuspension of the pellet followed by incubation at 55–65 °C to hydrate the RNA molecules. RT-qPCR was performed using a thermocycler (Applied Biosystems 7500) on the RNA samples in triplicates with the aim of analyzing *treY* and *otsA* expression and using 16S rRNA gene as control. The protocol used was from SuperScript™ III Platinum™ One-Step RT-qPCR Kit.

Primers and probes for RT-qPCR and FISH, respectively, were designed following the protocol described by Thornton and Basu (2011). The gene sequences of *P. acidipropionici* were obtained from NCBI gene data bank (NZ_KE386596), and BLAST bioinformatics tools were used for the design of primers. The cDNA and qPCR primers for *otsA* (trehalose 6-phosphate synthase), *treY* (maltooligosyl trehalose synthase) and 16S rRNA genes are listed in Table 1. The FISH probes for *treY* and *luxS* (gene coding for S-ribosylhomocysteine lyase) were 5′-GTCTCCCACCTGTTCTGCTC-3′ and 5′-TGGGAAGACGTACAAGGGAC-3′, respectively, both labeled at 3′ with cyanine Cy3™—Sp (Integrated DNA Technologies) while the probe for 16S rRNA gene was 5′-CACTCTTTGTACCGGCCATT-3′ labeled at 5′ with fluorescein 6-FAM™ (Integrated DNA Technologies).

Samples containing only biomass or biofilm were withdrawn from the growing cultures using a syringe. A sample (800 µL) was mixed with 500 µL of RNA protect TM Reagent (QIAGEN) and incubated for 15 min at room temperature. The mixture was centrifuged at 4800×*g* for 8 min, the pellet washed three times with phosphate buffer saline, and then 850 µL of absolute ethanol was added and incubated at 4 °C for 16 h (Inácio et al. 2003). Subsequently, 8 µL were fixed on to a slide and dehydrated with ethanol at 50, 80 and 96% (v/v), respectively. After that, 8 µL of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl, 0.01% SDS, pH 7.2) and 8 µL of the probe were added to the treated sample and the slide was incubated in a humid chamber in a hybridization oven at 45 °C for 2 h. Then the slide was treated using the washing buffer (5 M NaCl, 0.5 mM EDTA, 10% SDS, 1 M Tris–HCl pH 7.0) and incubated at 45 °C for 10 min. Finally, the slides were observed at × 10 and × 100 magnification using a fluorescence microscope (Olympus BX-40).

Analytical procedures

Cell growth

The cell growth was monitored by measuring the Optical Density (OD) as the absorbance of the cell suspension at 620 nm. Cell Dry Weight (CDW) was determined as a difference in the weight of a known volume of cell suspension before and after drying at 100 °C overnight. CDW was correlated with the OD_{620nm} for each sample.

Biofilm forming capacity index

The ability of *P. acidipropionici* to form biofilm was measured by determining Biofilm Forming Capacity index (BFC) as ratio of biofilm-related cells to planktonic cells, i.e. OD_{595nm}/OD_{620nm} (Gross et al. 2007). A culture sample of 7.5 mL was transferred to a sterile falcon tube containing the medium and a coverslip pre-sterilized by autoclaving. The tube was incubated at 30 °C for 24 h after which the medium was removed and the coverslip was rinsed with distilled water, dried and transferred to another falcon tube to be stained with 7.5 mL of 0.1% (w/v) crystal violet for 45 min, rinsed again with distilled water, dried and destained with 7.5 mL of 97% (v/v) ethanol for 10 min in yet another falcon tube. Absorbance of the solution was then measured at 595 nm. The absorbance of planktonic cells in the culture was directly measured at 620 nm as above.

Exopolysaccharides quantification

The EPS were quantified using a method described by Leyva et al. (2008) with some modification. Five milliliters of the

Table 1 Designed primers for studying the expression of *otsA* and *treY* genes, using 16S rRNA gene as control

	Primers			
	cDNA		qPCR	
	Left	Right	Left	Right
otsA	5′-TCGATGGAGATCGGGAAGG-3′	5′-CGTGTGGGTGCAGGACTACC-3′	5′-GATCGGAGGGAAGGGGATGT-3′	5′-CGTGTGGGTGCAGGACTACC-3′
treY	5′-GAGGACCGACAGGGAGTTCG-3′	5′-GCGGACTTCACCTACGACGA-3′	5′-CCATGTGATTGGGCACGAC-3′	5′-GCGGACTTCACCTACGACGA-3′
16S	5′-CGTGCTTTCGATACGGGTTG-3′	5′-CCAGGCGGGGTACTTAATGC-3′	5′-TGGACCTTTCCTGACGCTGA-3′	5′-CCAGGCGGGGTACTTAATGC-3′

culture medium was centrifuged at 16 000 × *g* for 10 min in order to separate the cells. The supernatant was dialyzed (Cellulose dialysis tube, Polylabo, Strasbourg, France; cut-off 2 kDa) against distilled water at 4 °C for 3 days, replacing the water three times a day. One milliliter of anthrone-sulfuric reagent (prepared using 0.2 g of anthrone, 10 mL of H_2O, 90 ml of 97% H_2SO_4) was added to 0.2 mL of the dialyzed sample, incubated at 90 °C for 15 min before measuring the absorbance at 620 nm. The EPS concentration was expressed in g/L. Glucose in a concentration range of 0–70 g/L was used as standard.

Capsular and released polysaccharides quantification

The quantification of capsular- and released polysaccharides was performed according to Di Pippo et al. (2013). Briefly, a volume of 10 mL culture broth was withdrawn after biofilm formation and centrifuged at 16,000×*g* for 10 min for separating the supernatant containing the released polysaccharide fraction. The pellet was incubated in 10 mL of H_2SO_4 at 95 °C for 1 h, and then centrifuged at 16,000×*g* for 5 min to release the capsular polysaccharides into the supernatant. The polysaccharide content in both fractions was determined using phenol–sulphuric acid method (Dubois et al. 1956) and standard glucose curve ranging from 0.05 to 1.0 g/L.

Trehalose quantification

Trehalose concentration was determined by the enzymatic procedure described earlier (Ruhal et al. 2011). Briefly, 10 ml of culture broth was centrifuged at 12,000–16,000×*g*, and the obtained pellet washed three times with 0.85% (w/v) NaCl, resuspended in 85% (v/v) ethanol, and boiled until the volume was reduced to 0.2–0.3 mL. Finally, 0.1 M citrate buffer pH 5.5 was added to make the volume to 1 mL, and after centrifugation the clear supernatant was separated for enzymatic analysis. One hundred microliter of 0.012 U Trehalase enzyme preparation (Sigma Aldrich) was added to 200 µL of the extract and incubated at 37 °C for about 12 h, and glucose released as a result of trehalose cleavage was determined by dinitrosalicylic acid (DNS) method (Miller 1959). Buffer was used as a blank for the experiment. The trehalose content was read from the calibration curve made using standard trehalose of 0–10 g/L treated under similar conditions.

Determination of substrate and products concentrations

Glycerol, propionic acid, acetic acid, succinic acid and other minor metabolites were analysed by a Jasco HPLC equipped with an Aminex HPX-87H organic acid analysis column (Bio-rad, Hercules, California, USA), CTO-6A oven (Shimadzu, Kyoto, Japan), Jasco AS 950-10 intelligent pump, PU 980 automatic intelligent injector (Jasco), ERC 7515A refractive index detector (ERC, Saitama, Japan), and a Chrompass Chromatography Data system (Jasco). Samples were diluted in ultrapure water, acidified with 20% (v/v) H_2SO_4 solution (20 µL H_2SO_4/mL sample), and then filtered through 0.45 µm membrane prior to analysis. Chromatography was performed using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, column temperature of 55 °C and refractometer temperature of 30 °C. The data obtained was used to calculate the product yield (g/g glycerol consumed) and productivity (g/L/h).

Scanning electronic microscopy

Scanning electron microscope (SEM) model JEOL JSM – T1000 was used for observing the effect of exposure to the stress conditions on the morphology of the cells. After preparing the samples onto the surface of the coverslip of the machine, they were subjected to a gold covering procedure for about 15 min and finally analyzed using the SEM.

Statistical analysis

Data statistical analyses were based on three replicates with the mean and standard deviations calculated. Data were subjected to analysis of variance (ANOVA) and Tukey HSD post-hoc tests at 5% significant level (p value < 0.05) using "car" (Fox and Weisberg 2011), package in R (R Core Team 2018).

Results

Effect of stress factors on biofilm formation and EPS production

The effect of including stress factors at different concentrations on growth and biofilm formation by *P. acidipropionici* grown in medium with glycerol as carbon source was evaluated. The NaCl containing cultures exhibited a three-day lag phase prior to cell growth just like the control cultures and acetic acid-containing medium, while citric acid induced a lag phase of one week. After two weeks of incubation, bacterial growth was measured by absorbance at 620 nm, and the OD values observed in cultivations with sodium chloride, citric acid and acetic acid were 7.8, 9.0 and 7.0, respectively in comparison to 8.4 for a control medium without addition of any stress factor. On the other hand, the presence of ethanol, sodium hypochlorite and hydrogen peroxide resulted in absorbance values of about 0, suggesting no growth and hence not considered for further experiments. EPS production and BFC index for the cultures were measured as indicators of biofilm formation (Table 2). The control culture

Table 2 Exopolysaccharide content (EPS) and biofilm forming capacity index (BFC) obtained in the media containing stress factors during different phases of cell growth in serum bottles

Stress factors	EPS (g/L)	BFC index in bacterial growth		
		Lag phase	Log phase	Stationary phase
Control[a]	0.169 ± 0.04	0.063	0.004	0.013
NaCl (0.8 M)	0.178 ± 0.06	0.644	0.005	0.004
Citric acid (25 mM)	0.187 ± 0.06	0.109	0.230	0.012
Acetic acid (80 mM)	0.126 ± 0.02	0.045	0.002	0.001

[a]Control included no stress factor in the medium

revealed the EPS production of 0.169 g/L, while there was a slight increase in the presence of NaCl to 0.178 g/L, and with citric acid to 0.187 g/L, while acetic acid containing cultures showed lower EPS values (0.126 g/L). ANOVA showed the EPS production between the different stress factors was significant ($p < 0.01$) and the post-hoc test showed that the addition of acetic acid decreases the EPS production.

Measurement of the BFC index showed the highest value to be 0.644 in the lag phase of the culture containing NaCl, followed by 0.230 in the log phase of citric acid-containing medium (Table 2). ANOVA showed statistical significance ($p > 0.05$) between the highest BFC indexes of NaCl, citric acid, acetic acid and control cultures There was no significant correlation between EPS production and BFC index in any of the growth phases. Acetic acid showed not only the lowest EPS value but also the lowest BFC indicators, even lower than the control (Table 2), and hence its use was abandoned at this point.

The concentrations of citric acid and NaCl were further varied between 15–40 mM and 0.8–1.8 M, respectively, in order to select an optimal concentration for further experiments. The most rigid cell aggregation was achieved at 25 mM citric acid and 0.8 M NaCl, respectively, during two weeks of cultivation. No inhibition of growth occurred at concentrations of citric acid up to 30 mM and of NaCl up to 1 M.

Citric acid and NaCl as stress factors differed in their effect on the production of the cell-bound capsular and released polysaccharides. In the base culture medium, *P. acidipropionici* produced 77.38 mg/L capsular polysaccharides and 55.87 mg/L of released polysaccharides. In cultures with citric acid as stress factor the produced polysaccharides were 162.85 mg/L ± 0.02 and 71.57 mg/L ± 0.01 capsular and released polysaccharides respectively. With NaCl, elevated levels of only capsular polysaccharides of 93.08 mg/L ± 0.01were observed and the released polysaccharide content was lowered to 50.64 mg/L ± 0.02. ANOVA showed the effect of both stress factors was of statistical significance on capsular polysaccharides production ($p < 0.01$) while none of the stress factors had statistical significance on released polysaccharide production ($p = 0.774$).

The SEM revealed the different shapes acquired by the cells during growth in the presence of a stress factor (Fig. 2). The cells from the citric acid containing cultures possessed a more spherical morphology and were shrunk and packed together, while the cells from cultures with NaCl retained a rod shape and showed a clear matrix around them, a typical structure of a biofilm.

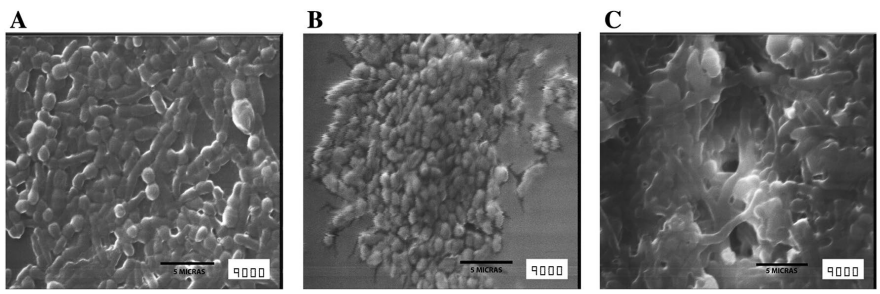

Fig. 2 Scanning electron micrographs of *P. acidipropionici cells* from different cultures at ×5000 magnification. **a** Cells in planktonic state when cultivated in basal medium, **b** cells aggregated in culture with citric acid as stress factor, **c** extracellular polymeric substance in culture with NaCl as stress factor, cells can be seen embedded into the matrix formed by the polymeric substance

Some experiments were performed using monosaccharides such as glucose, galactose and fructose, and disaccharides including sucrose, maltose and lactose as carbon sources instead of glycerol. Biofilms of varying rigidness, according to observation by naked eye, were formed with different sugars (data not shown). With NaCl as stress factor, more rigid biofilms were formed with the monosaccharides than with the disaccharides, while with citric acid, the rigidity was independent of the type of saccharides used, but was higher in all the cases than with NaCl.

Trehalose production and expression of treY, otsA and luxS genes in biofilm formation

The adaptation of *P. acidipropionici* to the presence of stress factors through possible accumulation of trehalose was studied. The amount of trehalose accumulated by the cells in cultures in serum bottles during two weeks was found to increase from 2.7 g/L ± 0.01 in the control culture to 6.8 g/L ± 0.02 and to 6.3 g/L ± 0.01 in citric acid- and NaCl-containing cultures, respectively. A significantly higher concentration of trehalose ($p < 0.01$) was obtained in the citric acid cultures with respect to the control and NaCl cultures..

The expression of *treY* and *otsA* was then monitored by qPCR in cultures containing 30 mM citric acid and 1 M NaCl, grown in serum bottles at 30 °C with no pH control. Samples were taken during lag-, exponential- and stationary phases. As shown in Fig. 3, a drastic increase in *treY* expression and minor increase in *otsA* was observed only in exponential phase of citric acid containing cultures, while the other phases showed no expression. In the case of NaCl containing culture, expression of neither *otsA* nor *treY* was observed.

Subsequently, FISH experiments were done using probes designed to hybridize with mRNA, on *P. acidipropionici* cells taken during exponential phase. Bioinformatics tools

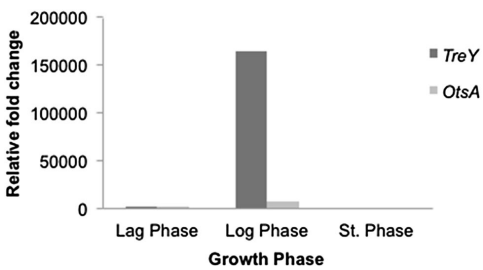

Fig. 3 Relative fold change of *otsA* and *treY* expression measured by RT-qPCR during lag-, exponential- and stationary phases in cultures containing citric acid. Standard deviation values in exponential phase were ± 1.12 and ± 1.0 for treY and for otsA, respectively. No detectable change was obtained in cultures with NaCl as stress factor

used to search for a control microorganism with high homology to the gene sequence with *P. acidipropionici* DSMZ 4900 to test the primers designed for *treY* and *otsA* did not result in identification of any closely related microorganism for use as control as their *treY* and *otsA* sequences were phylogenetically distant (supplementary data). Alignment tests using NCBI and BioEdit online resources showed Propionibacteria to be the microorganisms having high homology with the mentioned genes, while any possible control microorganism from another species has very different sequences. Therefore, the use of the protocol described by Thornton and Basu (2011) gave accurate and reliable results for both genes in terms of designed primers and designed probes. No expression of *treY* or *luxS* was observed in culttivations without the stress factors while *treY* and *luxS* were found to be expressed in all *P. acidipropionici* cultures with the stress factors (Fig. 4). The expression of *treY* was stronger than the one of *luxS* within both stress factors. Expression of 16S rRNA gene as a control was clear in every sample taken from the different media.

Repeated recycle batch fermentations in biofilm reactors for propionic acid production

Finally, the use of stress factors for promoting biofilm formation by *P. acidipropionici* on glass (Poraver®) and plastic (AnoxKaldnes®) matrices for use in propionic acid production was investigated. Table 3 provides a summary of the results of consecutive batch cultivations in Poraver® reactors with *P. acidipropionici* in citric acid and NaCl containing media using 20 g/L glycerol as carbon source. Cell growth was greatly affected in both reactors. At first, after switching the conditions from the three-week biofilm forming stage to the first production stage the growth rate of the planktonic cells improved from 0.04 to 1.85 g CDW/L/h and from 0.08 to 1.86 g CDW/L/h in citric acid- and NaCl-containing media, respectively. Even the product yield, -concentration and productivity were significantly increased. However, subsequently the growth was progressively reduced to 0.02 and 0.04 g CDW/L/h in citric acid- and NaCl-containing media, respectively, up to the sixth cycle. The volumetric productivity also showed an increase until the second fermentation cycle reaching a maximum productivity of 0.70 and 0.78 g/L/h in citric acid and NaCl, respectively, followed by a severe drop to 0.36 and 0.27 g/L/h, respectively, in the two reactors. Although the product yield was relatively more stable, higher average yields were obtained in NaCl containing cultures. The final product concentration obtained was also to some extent stable ranging from 9.3 to 11.79 g/L and from 9.2 to 14.5 g/L in citric acid- and NaCl-containing media respectively. Three more batches were run without any added inoculum. Product yield and -concentration showed

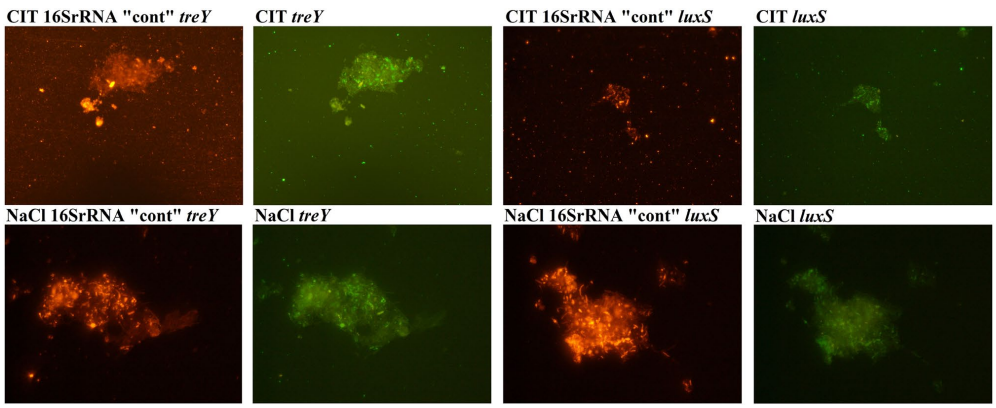

Fig. 4 Fluorescence in situ hybridization (FISH) images of gene expression for *treY* and *luxS* genes in cultures with citric acid and NaCl as stress factors. For each expression the images depict 16S rRNA expression as hybridization control and genes *treY* and *luxS* expression, fields in pairs respectively. Red-colored images show hybridization of control 16S rRNA; the expressions of *treY* and *luxS* for the respective stress factors are seen as light-orange cells contrasted in green-colored background fields. Cells are also seen green-colored in *treY* and *luxS* expression fields due to the hybridization of control 16S rRNA in the background. *CIT* citric acid-containing media, *NaCl* sodium chloride-containing media, *"cont"* control

Table 3 *Propionibacterium acidipropionici* growth and propionic acid production parameters in nine repeated batches after biofilm forming stage using Poraver® as the support

Batch	μ (g CDW/L/h)		q (g/L/h)		Y p/s (g/g)		P (g/L)	
	Cit[a]	NaCl	Cit[a]	NaCl	Cit[a]	NaCl	Cit[a]	NaCl
1	1.85	1.86	0.16	0.19	0.54	0.74	9.30	14.50
2	0.33	0.30	0.70	0.78	0.53	0.56	10.99	11.73
3	0.22	0.10	0.34	0.36	0.55	0.55	10.99	9.24
4	0.08	0.10	0.27	0.29	0.59	0.61	11.79	12.63
5	0.03	0.04	0.15	0.27	0.57	0.65	11.20	12.30
6	0.02	0.04	0.36	0.27	0.51	0.57	9.98	11.74
7	0.05	0.06	0.38	0.15	0.36	0.36	7.43	7.75
8	0.10	0.11	0.32	0.33	0.40	0.49	8.57	9.71
9	0.09	0.07	0.37	0.33	0.39	0.44	8.98	9.97

Batches 1–6 included the fresh inoculum of *P. acidipropionici*, while batches 7–8 were run without any added inoculum

μ growth rate, *q* volumetric productivity, *Y p/s* yield product/substrate, *P* final product concentration

[a]Citric acid

a decrease during the first run but increased again although not to the same extent as the previous batches (Table 3).

In the AnoxKaldnes® support reactors, three 1-week biofilm forming stages were conducted consecutively, followed by three sets of production stages. A second experiment consisted of having only one 9-day biofilm forming stage, followed by 4 production batches in a row (Table 4). The standard deviations for all calculations and measurements ranged between ± 0.05 and ± 0.43. The highest values for the growth rate in citric acid and NaCl containing media were 0.54 and 0.27 g CDW/L/h for the reactors that had three biofilm forming stages, productivities were 0.39 and 0.43 g/L/h, yields were 0.62 and 0.63 g/g, and the final product concentrations were 11.75 and 11.91 g/L, respectively. For the reactors that had only one biofilm forming stage the highest values for growth rate were 1.06 and 0.14 g CDW/L/h, product yields were 0.64 and 0.60 g/g, and highest product concentrations of 30.55 and 31.80 for citric acid and NaCl, respectively, while productivity was 0.35 g/L/h for both the stress factors. These similar values of the production parameters suggest that one stage of biofilm formation was enough for this multi-stage reactor operation, especially considering the higher values of the final product concentrations.

Table 4 *Propionibacterium acidipropionici* cell growth and propionic acid production parameters in multi-stage fermentation reactors with AnoxKaldnes® as support with two different modes of biofilm formation

Batch	μ (g CDW/L.h)		q (g /L. h)		$Y_{p/s}$ (g/g)		P (g/L)	
	Cit	NaCl	Cit	NaCl	Cit	NaCl	Cit	NaCl
1[a]	0.18	0.20	0.21	0.38	0.41	0.56	10.53	11.33
2[a]	0.54	0.27	0.39	0.43	0.56	0.59	11.08	11.91
3[a]	0.12	0.17	0.38	0.40	0.62	0.63	11.75	11.69
1[b]	1.06	0.03	0.20	0.21	0.64	0.60	16.65	17.34
2[b]	0.23	0.14	0.23	0.19	0.63	0.50	25.65	23.72
3[b]	0.15	0.14	0.35	0.35	0.56	0.52	30.55	31.80
4[b]	0.07	0.05	0.26	0.32	0.53	0.52	29.92	30.30

The symbols for the various parameters are the same as in Table 3

[a]Three consecutive batches run after three consecutive biofilm forming stages

[b]Four batches run after one only biofilm forming stage

Discussion

Propionic acid fermentation using immobilized propionibacteria has been shown to result in improved product yields and productivity (Dishisha et al. 2012; Zhu et al. 2012; Jiang et al. 2015). In case of *P. freudenreichii*, immobilization occurs spontaneously on the matrix by way of biofilm forming ability of the organism (Belgrano et al. 2018), while *P. acidipropionici* DSMZ 4900 cells required modification of the matrix to aid the immobilization e.g. by charge-charge interactions (Dishisha et al. 2012). Reports on biofilms produced by *P. acidipropionici* are few; weak biofilm formation was reported during cultivation in a medium based on glucose as the carbon source with addition of glycerol (Romero et al. 2016).

Following our earlier work on immobilization of *P. acidipropionici* DSMZ 4900 to Poraver beads coated with polyethyleneimine, in the present work we explored the possibility of biofilm formation by exposing *P. acidipropionici* cells to different stress factors that have earlier been used in a report to study the tolerance of *Salmonella enterica* after desiccation (Gruzdev et al. 2011). Biofilm formation was monitored through measurement of biofilm forming capacity as well as capsular and released exopolysaccharides. EPS production in propionibacteria is strain dependent, and influenced by medium composition and fermentation conditions. Increased EPS production has earlier been observed during growth of *P. acidipropionici* VM-25 in partially deproteinated whey (Racine et al. 1991) and of *P. acidipropionici* DSMZ 4900 in milk microfiltrate supplemented with yeast extract (Gorret et al. 2001).

Among all the stress factors used, we observed cell growth only in the presence of NaCl, citric acid and acetic acid. Citric acid induced a longer lag phase probably due to dissociation into its constituent ions that cause disturbance in the internal pH of the cells. A moderate increase in BFC was observed when NaCl or citric acid was included in the growth medium, the highest BFC values being obtained at 0.8 M NaCl and 25 mM citric acid. The two stress factors showed different effects on EPS production, although a distinct increase in capsular polysaccharides was observed with both. The effect of NaCl on triggering biofilm formation has been reported earlier in marine bacteria, *Piscirickettsia salmonis* (Marshall et al. 2012) and *Vibrio fischeri* (Marsden et al. 2017). Both cell growth and BFC of *P. acidipropionici* were reduced above 1.2 M NaCl or 35 mM citric acid. Tolerance to the high salt concentration seemed to be higher than that observed earlier for *P. freudenreichii* that showed total growth inhibition at around 1 M salt concentration (Boyaval et al. 1999).

Bacteria sense the changes in their environment and adjust their metabolic processes to utilize the available substrate as well as to protect them from harsh conditions (Jefferson 2004). Slow growth rate is a characteristic of bacterial biofilms when facing unfavorable environments and changes in gene expression result in metabolic heterogeneity in cell populations with specialized functions in order to preserve the integrity and to establish a more comfortable living state (Jefferson 2004).

The ability of the cells to resume planktonic growth after biofilm formation in media containing the stress factors by reseeding a piece of biofilm in fresh basal medium was confirmed for both the stress factors. The biofilms remained stable on shaking the serum bottles by hand, indicating no big dispersion as planktonic cells to the medium. Rigidity of the biofilms formed varied with the carbon source used, combination of sugars and citric acid giving the most rigid biofilms. Earlier studies with *Lactobacillus rhamnosus* showed that the nature of the carbon source used for cultivation influenced the molecular mass, morphology and consequently rheological properties of EPS formed (Polak-Berecka et al. 2015).

Several studies with *P. freudenreichii* subsp *shermanii* have reported elevated levels of trehalose produced at high concentrations of NaCl, ranging from 1,5 to 3% (w/v) (Boyaval et al. 1999; Cardoso et al. 2004; Ruhal and

Choudhury 2012). Suethao et al. (2015) have even reported trehalose concentration of 925 mg/L by *P. acidipropionici* DSM 20273 when grown in lactose at pH 7 without the addition of any stress factor. The concentrations of NaCl (0.8–1 M) and citric acid (30–35 mM) found in the present work for biofilm formation were higher than those reported earlier, and higher trehalose levels of 6.8 g/L and 6.3 g/L for citric acid and NaCl, respectively, were achieved.

Analysis of *P. acidipropionici* CGMCC 1.2232 genome sequence has revealed the presence of OtsA-OtsB and TreY-TreZ pathways for trehalose metabolism (Jiang et al. 2015). Using trehalose 6-phosphate synthase (*otsA*) and maltooligosyl trehalose synthase (*treY*) knockout mutants, the OtsA-OtsB pathway was suggested to be the major pathway for trehalose synthesis under acid stress. In contrast, higher activities of OtsA besides ADP-glucose pyrophosphorylase and GDP-pyrophosphorylase were noted in an osmotically sensitive mutant strain of *P. freudenreichii* compared to the parental strain (Ruhal et al. 2011). However, we observed elevated expression of *treY* and not *ostA* during cultivation in the presence of citric acid by RT-qPCR, and in both citric acid and NaCl containing cultures by FISH. Moreover, expression of *luxS* gene was clearly observed as also previously demonstrated in our laboratory (Romero et al. 2016). *LuxS* is involved in the synthesis of autoinducer AI-2 that plays a role in quorum sensing in some bacterial species. Involvement of *luxS* in biofilm formation has been studied in some pathogenic microorganisms like *Borrelia burgdorferi*, *Escherichia coli* and *Listeria monocytogenes* (Sapi et al. 2016; Zuberi et al. 2017; Bonsaglia et al. 2014). On the other hand, the role of *luxS* in biofilm formation in *Streptococcus mutans* is not totally clear, as some studies indicate this gene is not necessary for biofilm formation in this microorganism (Jefferson 2004), Also *luxS* reduces rather than induces biofilm formation in staphylococci by downregulating expression of biofilm EPS (Kong et al. 2006). Regardless of the differences in the various bacterial species, it is likely that auto-inducing signals influence the development of biofilms and it is also known that horizontal transfer of genetic information is easily supported in a matrix enclosed environment of a biofilm enhancing the possibilities for the cells to interchange useful genes and increase fitness of cell population (Jefferson 2004).

The results on propionic acid production obtained with stress adapted cells bound to Poraver® in recycle batch reactors are close to that obtained earlier in our laboratory (Dishisha et al. 2012), where PEI-treated Poraver® was used as support for immobilization of *P. acidipropionici* DSMZ 4900 in the same reactor set up, and the highest productivity obtained using 40 g/L of glycerol as substrate was 0.86 g/L/h compared to 0.70 g/L/h for citric acid-containing medium and 0.78 g/L/h for NaCl-containing medium reported here; and the product yield was 0.64 mol/mol (0.515 g/g) as compared to 0.59 g/g for citric acid-containing medium and 0.74 g/g for NaCl-containing medium. The productivity values were also similar to the productivities of 0.70 g/L/h or more obtained during cyclic and sequential batch fermentations of the organism in earlier studies (Dishisha et al. 2015). Higher productivity of 0.96 g/L/h has been reported for the acid-tolerant *P. acidipropionici* immobilized on a fibrous-bed reactor (Zhu et al. 2012).

The data obtained for the AnoxKaldnes® support reactors appeared to be similar to that in free-cell fermentations in batch giving productivity of 0.34 g/L/h and molar product yield of 0.64 mol/mol glycerol, and with propionic acid concentration of 19.5 g/L from 42 g/L glycerol (Dishisha et al. 2015), hence revealing no advantage of the stress treatment.

Conclusion

A bioreactor system in which the microbial cells immobilized on a matrix constantly serve as an inoculum source is an advantage especially for slow fermentation processes, avoiding the lag phase and directing the carbon source more for product formation. This study showed that exposure of *P. acidipropionici* to stress factors like citric acid and NaCl induced the microorganism to form biofilm as confirmed by the increase in the BFC values, production of EPS, electronic microscopy SEM and also visual observation. The biofilm cells were able to resume planktonic growth under normal conditions. Trehalose production was enhanced as a means of osmoprotection in the cells exposed to the stressed conditions; the genetic expression of trehalose metabolism pathways was however different in the two cases when tested by RT-qPCR. On the other hand, expression of *luxS* in biofilm formation was confirmed by FISH analysis for both stress factors. It was possible to use the *P. acidipropionici* biofilm for repeated fermentations although maintaining the stability of the system needs more in depth studies. Poraver® support was more efficient for biofilm formation and propionic acid production most likely due to its highly porous structure and perhaps better compatibility with the cell envelope as compared to the relatively hydrophobic AnoxKaldnes support.

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Compliance with ethical standards

Conflict of interest The authors declare there is no conflict of interest.

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SUPPLEMENTARY DATA

In order to confirm the sequences of the genes *otsA* and *treY* of *Acidipropionibacterium acidipropionici* DSM4900, BLAST search was carried out at first comparing with sequences of *Acidipropionibactera* ATCC and then with the general database of nucleotides.

Then after evolutive relations of *otsA* and *treY* genes of *Acidipropionibacterium acidipropionici* DSM4900 were analyzed with homologue sequences of strains which belong to genres *Acidipropionibacterium, Propionibacterium, Cutibacterium* and *Pseudopropionibacterium* of the family Propionibacteriaceae using as external groups *Corynebacterium humireducens* and *Corynebacterium maris* (Scholz & Kilian, 2016). A phylogenetical tree was constructed through Neighbor-joining method using BioNJ algorithm, considering a model of substitution T92 and bootstrap of 1000 replications. All the analyses were carried out in R (R Core Team, 2018).

Results

The sequence corresponding to otsA gene of *Acidipropionibacterium acidipropionici* DSM4900 shows an alignment highly significative (E value= $5x10^{-108}$) with the homologue reference sequences of *A. acidipropionici* ATCC 4875 (CP003493.1), WSH1105 (CP019400.1) and CGMCC 1.2230 (CP013126.1) with 100 % percentage of identity. Also, this sequence has 98.2 % identity with *P. acidipropionici* WGS7 (CP031057.1), F3E8 (CP015970.1) and ATCC 55737 (CP014352.1) (E value= $1x10^{-102}$).

The sequenced fragment of *treY* gene shows 100 % identity with the homologue sequences of *Acidipropionibacterium acidipropionici* ATCC 4875 (CP003493.1), WSH1105 (CP019400.1), CGMCC 1.2230 (CP013126.1), WGS7 (CP031057.1), F3E8 (CP015970.1) and ATCC 55737 (CP014352.1) (E value= $5x10^{-134}$).

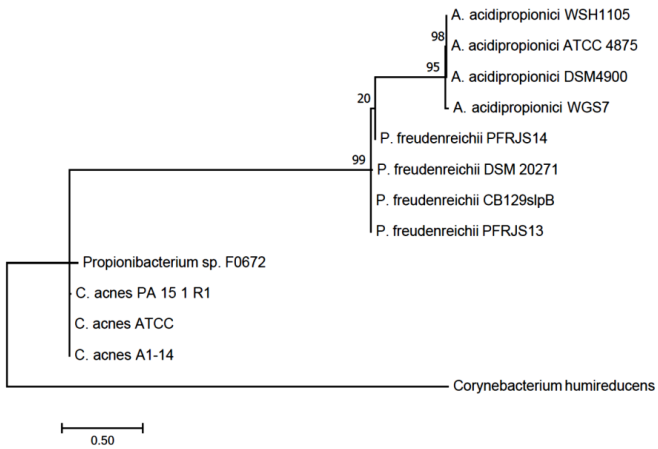

Fig.1. Neighbor-joining tree of gene *otsA* with strains from the family Propionibacteriaceae. The numbers at the branches come from a bootstrap of 1000 replicates.

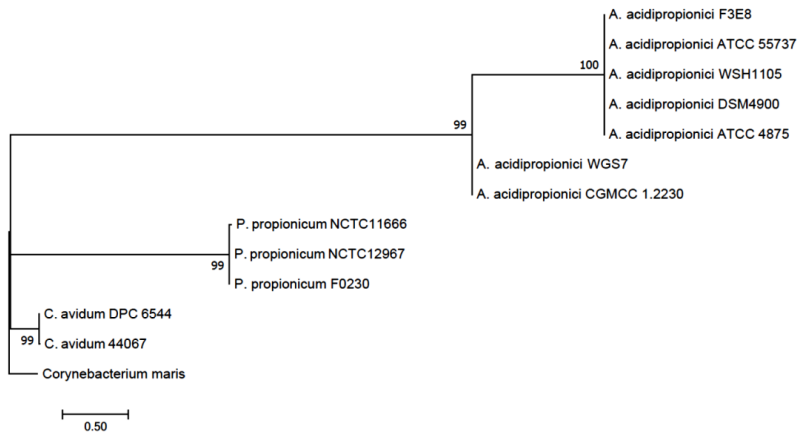

Figure 2. Neighbour-joining tree of gene *treY* with strains of the family. Propionibacteriaceae. The Numbers at the branches come from a bootstrap of 1000 replicates.

The phylogenetic trees of genes *otsA* and *treY* show clearly those sequences of *Acidipropionibacterium acidipropionici* DSM4900 obtained in this study are closely related with their homologues in the reference strains with a high statistical support in all their branches. This confirms the identity of both sequences.

GeneBank accession numbers for the nucleotides sequences are:

- BankIt2210632 otsA MK759674
- BankIt2210632 treY MK759675

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Paper III

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Propionic acid production from glycerol in immobilized cell bioreactor using an acid-tolerant strain of *Propionibacterium acidipropionici* obtained by adaptive evolution

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ABSTRACT

Propionic acid (PA) production from agro-industrial residues using propionibacteria has gained interest as an alternative to fossil-based process. Microbial production is however characterized by product inhibition, lowering the product titers and productivity. In this study, *Propionibacterium acidipropionici* DSMZ4900 was subjected to adaptive evolution to tolerate higher acid concentrations. The strain adapted to growth in medium spiked with 20 g/L PA exhibited improved product titer (16.8 vs 8.72 g/L) and productivity (0.52 vs 0.17 g/L·h) with glycerol as carbon source in batch fermentations. It was immobilized on polyethyleneimine coated recycled glass beads Poraver® and used for fermentations in recycle batch mode with increasing glycerol concentration and decreasing pH, respectively. Doubling yeast extract concentration raised PA yield and productivity by >1.5 fold. Glycerol at 100 g/L was completely consumed to give ~58 g/L PA at yield of 0.64 mol/mol and productivity of 0.28 g/L·h at pH 6.5. Decreasing fermentation pH to 5.0 increased PA productivity to 0.23 g/L·h from 0.14 g/L·h at pH 6.0 with 20 g/L glycerol, while immobilized cells exhibited no growth. The study shows combination of adaptive evolution and immobilization of cells to result in a robust system for PA fermentation at high glycerol concentration and lower pH.

1. Introduction

Dairy propionibacteria are anaerobic, gram-positive bacteria that besides being used for production of traditional Swiss-type cheeses and also as human and animal probiotics [1–3], are an important source of a variety of valuable products like Vitamin B12, trehalose, flavors, antimicrobial compounds including diketopiperazines, linear- and cyclic peptides, 3-phenyllactic acid and propionic acid [2,4–10]. Propionic acid is an important C-3 organic acid with applications in food and feed, pharmaceuticals and plastics [11,12]. Propionibacteria produce propionic acid via the Wood-Werkman cycle from different sugars or glycerol [13]. The commercially available propionic acid, however, is obtained by chemical synthesis from fossil based ethylene or propionaldehyde. Hence, developing an economically competitive production process from biobased feedstock using *Propionibacterium* spp. has received much attention during the past decades [11,12,14,15].

In order to lower the cost of the carbon source for propionic acid fermentation, a variety of inexpensive raw materials like whey lactose [16], cane- and soy molasses [17,18], sugarcane bagasse [19], Jerusalem artichoke hydrolysate [20], wheat flour [21], biodiesel derived glycerol [22–24], etc. have been used. Glycerol, being a more reduced carbon source than sugars, provides higher propionic acid yield, which is theoretically 1 mol/mol [13,25–27]. Under experimental conditions, however, the yield may vary between 0.6 and 0.9 mol/mol, with succinic acid, acetic acid and *n*-propanol being the major by-products [25]. Even cheaper alternatives for the complex nitrogen source required for propionibacteria have been tested [17,23]. The main bottleneck, however, is the product inhibition that is common with the microbial

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production of organic acids in general. In fact, propionic acid was reported to be the most inhibitory organic acid for the growth and survival of *Propionibacterium* strains [28]. Such inhibition results in low productivity and product titers, and challenging separation of the product from by-products including other organic acids as well as water, which limits cost-effective propionic acid production [29].

The main strategies used for improving the fermentation kinetics have been high cell density fermentations achieved by cell immobilization [15–17,22,30–32] or cell recycling [23,24,33,34], and developing acid tolerant strains by adaptive evolution [6,35,36] or metabolic engineering of the propionibacteria. Some examples of the latter are knockout of acetate kinase gene [37], overexpression of biotin-dependent carboxylases methylmalonyl-CoA carboxyltransferase and methylmalonyl-CoA decarboxylase [38] or engineering of phosphoenolpyruvate carboxylase [39] in *P. freudenreichii*, coexpression of heterologous glycerol dehydrogenase and malate dehydrogenase in *P. jensenii* [40], and genome shuffling via inactivated protoplast fusion [41,42]. Metabolic engineering has also been combined with controlling the fermentation by shift in pH or oxidoreduction potential in stages to promote cell growth and synthesis of propionic acid, respectively [43,44].

Earlier research in our laboratory has involved evaluating different modes of high cell density fermentations including immobilized cell fermentations, and free cell fermentations performed in sequential batch- and cyclic batch modes for improving propionic acid production from biodiesel derived glycerol using *Propionibacterium acidipropionici* [22–24]. The sequential batch fermentation resulted in highest productivity exceeding 1 g/L·h and yield of 0.71 g/g glycerol from 120 g/L glycerol at carbon:nitrogen ratio of 3:1 [24]. Immobilization of *P. acidipropionici* by adsorption to polyethyleneimine (PEI) treated foamed glass matrix, Poraver®, also enhanced propionic acid volumetric production rates considerably in sequential batch fermentations [22]. The usefulness of this matrix was subsequently confirmed by Jiang et al. [16], who reported propionic acid levels as high as 125 g/L from whey lactose during fed batch fermentation using immobilized *P. acidipropionici*. The cells with overexpressed trehalose-synthesis related gene *otsA* increased the titer further to 135 g/L at productivity of 0.67 g/L·h and yield of 0.67 g/g lactose [16].

Accumulation of trehalose, a non-reducing disaccharide, is known to have a protective effect on the bacterial cells including propionibacteria against physical and chemical stresses [45]. Three trehalose synthesis pathways have been identified, with synthesis from maltose via trehalose synthase (TreS) being the core functionality across all the propionibacteria species. The two-step synthesis pathway from ADP-glucose and glucose-6-phosphate via trehalose-6-phosphate synthase/phosphatase (OtsA-OtsB) is also common to most species except *P. propionicum*, while the third pathway linking glycogen metabolism to trehalose synthesis has been identified in only three species including *P. acidipropionici* [46]. In our previous studies, elevated *treY* gene expression accompanied increased trehalose accumulation in *P. acidipropionici* exposed to stress conditions in NaCl or citric acid containing medium [47].

The aim of the present study was to develop a *P. acidipropionici* strain that is tolerant to high propionic acid concentration by adaptive evolution. The adapted strain was tested for the expression of trehalose production related genes, and was evaluated in an immobilized cell bioreactor using PEI-treated Poraver® beads as the matrix, for production of propionic acid from increasing concentrations of glycerol and decreasing pH, respectively.

2. Materials and methods

2.1. Bacterial strains and culture medium

Propionibacterium acidipropionici DSMZ 4900 and its acid adapted strain were used in this study. The basal culture medium used for cultivation of the organism consisted of 10 g yeast extract, 20 g glycerol, 0.25 g cysteine, 2.5 g K_2HPO_4 and 1.5 g KH_2PO_4 per liter of distilled water, with pH adjusted to 7 using 28 % ammonia solution (Sigma–Aldrich, St. Louis, MO, USA) [23]. The medium was flushed with oxygen-free nitrogen and sterilized by autoclaving at 121 °C, 1.5 atm for 20 min prior to use. Glycerol and propionic acid (Sigma–Aldrich) were added to the culture medium at different concentrations. Inocula of *P. acidipropionici* DSMZ 4900 and the adapted strain were prepared from their respective cryopreserved cultures, in 90 mL basal medium in 100 mL serum bottles, grown at 30 °C for about a week prior to a new experimental procedure.

2.2. Adaptive evolution of P. acidipropionici DSMZ 4900 and propionic acid production in batch fermentations

P. acidipropionici DSMZ 4900 was first cultivated in the basal medium in serum bottles inoculated with 5% v/v of the culture suspension; the culture was harvested and the cells inoculated in the medium spiked with 10 g/L propionic acid while keeping the other conditions the same. After three sequential cultivations in this medium (each time using the previous culture as the inoculum), the cells were inoculated in the medium spiked with 20 g/L propionic acid, the procedure was repeated until the initial acid concentration in the medium was increased up to 40 g/L. The initial pH of the medium was adjusted to 6.7–7.0 at all propionic acid concentrations. The three sequential cultivations at each propionic acid concentration lasted totally 1–3 weeks (longer time needed at higher acid concentration) to achieve biomass concentration with OD_{620} higher than 7 (i.e. equivalent to cell dry weight of 2.56 g/L).

The *P. acidipropionici* culture, adapted in the medium supplemented with 20 g/L propionic acid, was then cultured in the serum bottles containing 90 mL medium without any propionic acid and that supplemented with 20 and 30 g/L of the acid, respectively. After two consecutive batches under each condition, the cells were used for inoculating pH-controlled cultivations in 300 mL media in water-jacketed reactors (500 mL total volume) equipped with ports for pH electrode, base addition, sampling and gas release through a 0.2 μm sterile filter. The inocula volumes were adjusted after determining the optical densities in order to have approximately similar cell concentration at the starting point. The OD_{620} values at the start of the pH-controlled fermentations were 0.092; 0.064 and 0.082 in the media spiked with 0, 20 and 30 g/L of propionic acid, respectively. As a control, the wild type *P. acidipropionici* DSMZ 4900 was also grown for two consecutive batches in the basal medium in serum bottles prior to being inoculated in the bioreactor to get the initial OD_{620} of about 0.098. The batch fermentations were then carried out at pH 6.5 and 30 °C until growth reached stationary phase. The pH was maintained using 5 M ammonia solution and a pH control unit. Samples were withdrawn at regular time intervals for monitoring bacterial growth and product formation in triplicates. From these experiments, the strain showing the best adaptation was selected for further use in packed bed bioreactors.

2.3. PEI treatment of Poraver®

Poraver®, foamed glass beads of 8 mm average diameter (Dennert Poraver GmbH, Germany), were treated with polyethyleneimine (PEI, Sigma-Aldrich, average MW – 25 000 by light scattering, average Mn: 10 000 by GPC, branched) for use as support for cell immobilization. A solution of 0.5 %–1 % (w/v) PEI, pH 3–4 was prepared by adding HCl (36 %) (Sigma–Aldrich), and the pH was then readjusted to 7 with 28 % ammonia solution. Poraver® carriers were soaked in the PEI solution and autoclaved at 121 °C for 20 min, rinsed with sterile distilled water and finally dried overnight at 60 °C prior to being packed in the reactor.

2.4. Packed-bed bioreactor set up

The setup shown in Fig. 1 consisted of a 500 mL water-jacketed

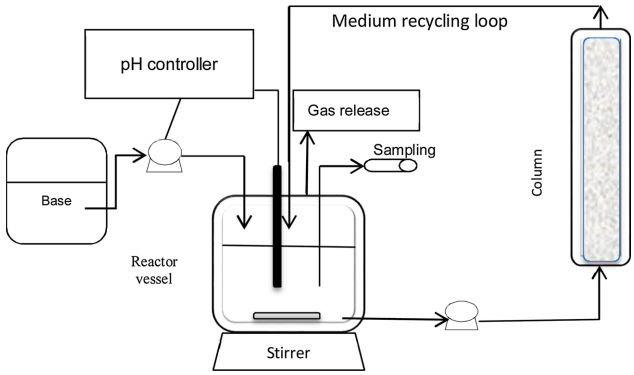

Fig. 1. Schematic view of the bioreactor set-up used for propionic acid production by immobilized *P. acidipropionici* cells.

reactor (similar to the one used for free cell fermentations) and a column (500 mL; 20 cm height and 5 cm internal diameter) packed with Poraver®. The reactor was connected to the column through a tubing for recycling the culture medium. The average volume of the medium in the whole setup was 600 mL, of which 300 mL each was in the reactor and the column, respectively. Once the PEI treated support was packed into the column, the whole system was sterilized by autoclaving at 121 °C and 1.5 atm for 20 min. The medium was autoclaved separately and pumped into the reactor in a sterile bench, and recycled at high rate through the system. Temperature of the reactor setup was controlled at 30 °C using a thermostatic water-recirculating system, and magnetic agitation in the reactor was set at 250 rpm. The reactor vessel alone was also used for pH-controlled batch cultivations of free *P. acidipropionici* cells.

2.5. Immobilization of the adapted cells of P. acidipropionici and fermentations in packed-bed bioreactors

Two packed-bed bioreactors were used for immobilized cell fermentations. Bioreactor 1 was operated at stepwise increasing glycerol concentrations, while bioreactor 2 was operated with stepwise decrease in pH values during consecutive batches in order to evaluate the effect of the changed parameters on propionic acid production. Both reactors were first filled with fresh sterile medium and any flow between the vessel and the column was blocked.

The reactor vessels were inoculated with actively growing cultures of the selected adapted strain of *P. acidipropionici*. The culture was first allowed to grow in batch mode with pH control in the respective reactor vessels at 30 °C until it reached an OD_{620} value higher than 10. Subsequently, the flow between the vessel and the column was opened and the culture broth was recirculated via the column with the PEI-treated Poraver® support for immobilization of the cells. The OD_{620} of the culture in the vessel was measured over time until it reached a constant value after a gradual decrease, implying that no more cells were being attached to the Poraver® beads. Both bioreactors were then emptied and then refilled with fresh medium, and the procedure was repeated two more times so as to immobilize as many cells as possible. No buffer salts were used in the medium to avoid any interaction with the charged bead surface that would compete with binding of the cells.

The bioreactor 1 was operated with increasing glycerol concentrations from 20 to 160 g/L during consecutive batches, with 10 or 20 g/L yeast extract (according to the experiment), 0.20 g/L cysteine, and pH adjusted to 6.5. In bioreactor 2, pH set for fermentation was lowered during consecutive batches from 6.0 to 5.5, 5.0 and 4.5, while the initial glycerol concentration was 20 g/L in every batch. Samples were withdrawn daily from the reactors for monitoring growth and product concentration.

Subsequent to cell immobilization- and between each fermentation cycle, the reactors were completely emptied and rinsed by pumping in 2 L of sterilized 0.9 % NaCl.

2.6. Analytical methods

All analyses were done on triplicate samples.

2.6.1. Cell density measurement

Cell growth was monitored by measuring the optical density of the culture at 620 nm. Cell dry weight (CDW) was determined as a difference in the weight of a known volume of cell suspension before and after drying overnight at 100 °C. CDW was correlated with the OD_{620nm} for each sample.

2.6.2. Determination of substrate and products concentrations

Glycerol, propionic acid, acetic acid, succinic acid and other minor metabolites were analyzed by HPLC (Jasco) equipped with an Aminex HPX-87H organic acid analysis column (Bio-rad, Hercules, California, USA), CTO-6A oven (Shimadzu, Kyoto, Japan), Jasco AS 950-10 intelligent pump, PU 980 automatic intelligent injector (Jasco), ERC 7515A refractive index detector (ERC, Saitama, Japan), and a Chrompass Chromatography Data system (Jasco). Samples were diluted in ultrapure water, acidified with 20 % (v/v) H_2SO_4 solution (20 µL H_2SO_4/mL sample), and then filtered through 0.45 µm membrane prior to analysis. Chromatography was performed using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, column temperature of 55 °C and refractometer temperature of 30 °C. The data obtained was used to calculate the product yield (mol/mol glycerol consumed) and productivity (g/L·h). The amount of propionic acid produced was calculated from the difference between the final concentration measured and the initial concentration included in the medium.

2.6.3. Reverse transcription-quantitative PCR (RT-qPCR) for treY and otsA expression

RNA extraction from the cells was performed following the Invitrogen protocol using TRIzol™ Reagent. Briefly, immediately after collecting samples from the cultures, RNA protect™ Bacteria Reagent (QIAGEN, cat. no. 76506) was added at a reagent to sample volume ratio of 3:1 to protect the bacterial RNA, followed by centrifugation. The pelleted cells were homogenized by adding equal volume of the TRIzol™ Reagent and pipetting up and down several times in order to break the cells. Chloroform (0.2 mL per mL of TRIzol™ Reagent used) was added,

and the tubes were shaken and centrifuged at 12 000×*g* for 15 min at 4 °C to obtain two phases. The aqueous phase was harvested by pipetting and transferred to a new tube, followed by RNA precipitation with 0.5 mL of 100 % isopropanol per mL of TRIzol™ reagent used and then washing the precipitated RNA by adding an equal volume of absolute ethanol, shaking gently and centrifuging at 7500×*g* for 5 min at 4 °C. RNAse-free water was added for re-suspension of the pellet followed by incubation at 55–65 °C to hydrate the RNA molecules.

RT-qPCR was performed on the RNA samples in triplicates using a thermocycler (Applied Biosystems 7500) with the aim of analyzing *treY* and *otsA* expression and using 16S rRNA gene as control as described earlier [47]. The protocol used was from SuperScript™ III Platinum™ One-Step RT-qPCR Kit. Primers for RT-qPCR were designed according to the protocol described by Thornton and Basu [48]. The gene sequences of *P. acidipropionici* were obtained from NCBI gene data bank (NZ_KE386596), and BLAST bioinformatics tools were used for the design of primers. The cDNA and qPCR primers for *otsA* (trehalose 6-phosphate synthase), *treY* (maltooligosyl trehalose synthase) and 16S rRNA genes are listed in Table S1.

3. Results

3.1. Adaptive evolution and propionic acid production by the adapted isolate of P. acidipropionici DSMZ 4900

The wild type *P. acidipropionici* DSMZ 4900 was subjected to stepwise increasing propionic acid concentration from 0 to 40 g/L in the medium with glycerol as carbon source. Cultivations with no propionic acid added to the medium lasted less than 7 days, reaching a maximum OD_{620} of 11.47 (SD ± 0.25), while those with 10, 20 and 30 g/L of propionic acid lasted 10, 13 and 21 days to reach the maximum OD_{620} of 9.7 (SD ± 0.28), 7.6 (SD ± 0.25) and 0.4 (SD ± 0.04), respectively. No cell growth was observed in the medium with 40 g/L of propionic acid even after 30 days of cultivation. The bacterial culture obtained from the medium with initial propionic acid concentration of 20 g/L was selected because of the good cell growth, inoculated in media with 0, 20 and 30 g/L in serum bottles, and allowed to grow for two rounds at each concentration prior to being used to initiate cultivations in batch mode in corresponding pH-controlled reactor vessels. Fig. 2 shows the growth profiles of the culture in the media spiked with varying concentrations of propionic acid in the pH-controlled reactors, and also the growth of the original *P. acidipropionici* DSMZ4900 only in the basal medium. The latter exhibited the shortest lag phase of about 2 days and growth rate of 0.16 g/L·h, reaching a final CDW of ca 4.43 g/L. On the other hand, the culture obtained from the medium supplemented with propionic acid and then grown at different acid concentrations of 0, 20 and 30 g/L (described above), had lag phases of about 4 days and longer, growth rates of 0.08, 0.03 and 0.001 g/L·h, and final CDW of ca 3.8, 3.7, and 0.2 g/L, respectively. The lower growth rate of *P. acidipropionici* mutant adapted in a fibrous-bed reactor compared to the wild type in the medium without propionic acid has been reported earlier [35]. The cells, obtained from the pH-controlled culture supplemented with 20 g/L propionic acid, were harvested and cryopreserved at −20 °C with 50 % (v/v) glycerol in test tubes for storage and for use as the adapted strain in all subsequent experiments after rejuvenating the cells for about a week in the basal medium at 30 °C.

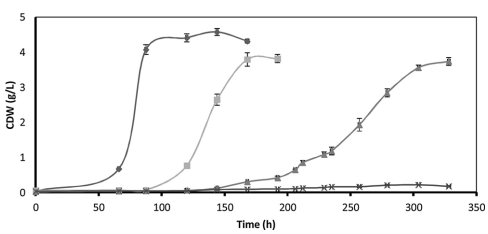

Fig. 2. Growth profiles of *P. acidipropionici* strain obtained after adaptation to growth in the culture medium supplemented with 20 g/L propionic acid in serum bottles and then grown in the medium supplemented with propionic acid at concentrations of 0 g/L (■), 20 g/L (▲), and 30 g/L (×), respectively, in the bioreactor at pH 6.5. Growth of wild type *P. acidipropionici* DSMZ4900 in basal medium (◆) is also shown.

The adapted strain was used for propionic acid production from glycerol in a batch mode at pH 6.5. As shown in Table 1 and also from the growth and production profiles in Fig. 3, the adapted strain when grown in the basal medium with 20 g/L glycerol and no added propionic acid in the medium, showed improved production of propionic acid (productivity of 0.52 vs 0.17 g/L·h, product concentration of 16.80 vs 8.72 g/L, and yield of 0.68 vs 0.58 mol/mol) compared to the wild type *P. acidipropionici* DSMZ 4900. Even the growth rate of the adapted strain was increased (0.23 vs 0.16 g CDW/L·h). During cultivation in the medium with initial propionic acid concentration of 20 g/L, the adapted strain showed about 2.3 and 2.7-fold decrease in the rates of cell growth and propionic acid production, respectively, and a slight decrease in product yield and concentration, but these parameters were still higher than that for the original DSMZ4900 strain grown without any supplementation of propionic acid (Table 1). With further increase in initial propionic acid concentration to 30 g/L for the adapted strain, the growth and product formation decreased more, nevertheless the product yield (0.65 mol/mol) and final net product concentration (11.5 g/L) remained still higher than in the wild type strain (Table 1). Succinic acid was also detected during all fermentations (Fig. 3). In contrast, cultivation of the wild type *P. acidipropionici* DSMZ 4900 in the medium containing 10 g/L propionic acid resulted in no visible cell growth.

3.2. Expression of trehalose-synthesis related genes treY and otsA

The adapted strain obtained above was cultivated in 20 g/L propionic acid-containing medium in serum bottles at 30 °C without pH control, and samples were taken during exponential growth on the 4th day and treated for RT-qPCR for analyzing the expression of genes *otsA* and *treY* involved in trehalose metabolism. Expression of only *treY* was found to be triggered, showing an increase of 82.32 (SD ± 0.43) fold with respect to the expression of the control 16S rRNA gene under the same conditions, while there was no detectable expression of *otsA* (data not shown).

3.3. Propionic acid fermentations using the immobilized adapted P. acidipropionici strain at increasing glycerol concentration

The adapted strain was immobilized by repeated recirculation of the culture suspension over the PEI coated Poraver® matrix in bioreactor 1. The three cell immobilization cycles lasted 2, 17 and 11 days, respectively. The absorbance of the culture after the last immobilization stage, i.e. a total period of more than 30 days, became stable at OD_{620} of 4.1 equivalent to CDW value of 1.5 g/L (SD ± 0.10).

Propionic acid fermentations in the bioreactor 1 were then conducted with increasing glycerol concentrations up to 160 g/L, and the results were compared with that obtained with batch fermentations using free adapted *P. acidipropionici* cells and free wild type cells from the earlier report from our laboratory [22]. As seen in Table 2, the fermentation kinetics of the immobilized cell reactor system in the present study showed slightly lower values for productivity and product yield as compared to the data reported by Dishisha et al. [22] at 40 and 60 g/L glycerol. Increasing the concentration of the nitrogen source was seen to be important for improving all the production parameters (Table 2). For example, increasing the yeast extract concentration from 10 to 20 g/L at 60 g/L glycerol in the medium increased the product yield from 0.48 to 0.73 mol/mol in a much shorter time (113.5 h vs 213 h) and hence an increase in productivity from 0.21 to 0.37 g/L·h.

Table 1
Propionic acid production by the wild type (WT) *P. acidipropionici* DSMZ4900 grown without added propionic acid and by the adapted strain (AS) in the medium with added 0, 20 and 30 g/L propionic acid at pH set to 6.5.

Initial propionic acid concentration in the medium (g/L)	μ (CDW g/L·h)	q (g/L·h)	Y (mol/mol)	Propionic acid (g/L)	Net propionic acid (g/L) *
WT, 0**	0.16 ± 0.01	0.17 ± 0.04	0.58 ± 0.06	8.72 ± 0.11	8.72 ± 0.11
AS, 0 ***	0.23 ± 0.01	0.52 ± 0.08	0.68 ± 0.30	16.80 ± 0.12	16.80 ± 0.12
AS, 20 ***	0.10 ± 0.02	0.19 ± 0.20	0.67 ± 0.41	31.60 ± 0.12	15.30 ± 0.12
AS, 30 ***	0.05 ± 0.02	0.12 ± 0.32	0.65 ± 0.34	38.10 ± 0.58	11.50 ± 0.58

* Net production in each case was the difference between the final and initial propionic acid concentration.
** Control cultivation in an identical reactor set up with the wild type.
*** For the adapted strain cultured at 0, 20 and 30 g/L of propionic acid in the medium, the actual concentrations obtained on analysis were 16.3 and 26.6 g/L for 20 and 30 g/L, respectively.

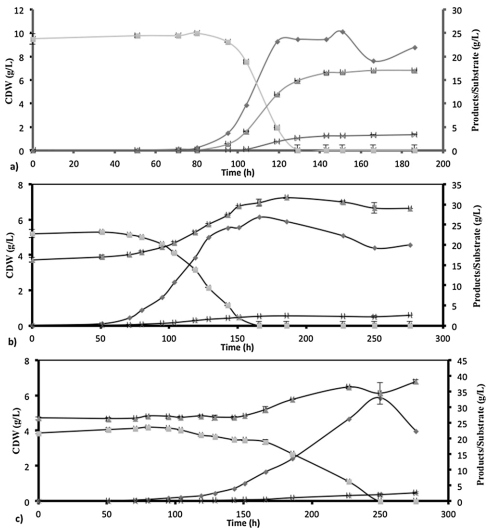

Fig. 3. Growth and propionic acid production profiles of the adapted *P. acidipropionici* strain cultivated in pH-controlled batch bioreactors in the medium supplemented with: a) no propionic acid, b) 20 g/L propionic acid, and c) 30 g/L propionic acid. Symbols indicate: (◆) growth expressed in terms of CDW, (▲) propionic acid, (■) glycerol, and (×) succinic acid.

Reducing the yeast extract concentration back to 10 g/L in the fermentation with 80 g/L glycerol decreased the productivity, yield and titer of propionic acid. These parameters were improved again when yeast extract concentration was raised to 20 g/L with glycerol at 100 g/L; the productivity, product yield and titer were 0.28, 0.64 and 57.73, respectively. Glycerol was completely consumed in every batch, except when used at 160 g/L, where after 25 days of fermentation the remaining amount of glycerol was 39.41 g/L, while propionic acid titer and yield were 61 g/L and 0.77 mol/mol, respectively, and productivity was 0.15 g/L·h. Hence, 120 g/L of glycerol could be completely consumed during propionic acid fermentation by the immobilized cells. Fig. 4 depicts the fermentation profiles in the 5 batches in bioreactor 1 run at glycerol concentration from 40 to 100 g/L.

Succinic acid and acetic acid were not detected in fermentations with low glycerol concentration of 40 g/L glycerol and 10 g/L yeast extract, but were detected as glycerol and yeast extract concentrations were increased in the medium; the highest concentrations obtained were 19.04 (±0) g/L succinic acid and 18.45 (±0.05) g/L acetic acid at 160 g/L of glycerol with 20 g/L of yeast extract. The suspended biomass concentration reached 2.45 and 10.26 g/L in the fermentation batches containing glycerol concentrations of 100 and 160 g/L, respectively.

Table 2
Production of propionic acid by adapted *P. acidipropionici* cells immobilized on PEI treated Poraver® beads during different batches in medium with increasing glycerol concentrations and comparison with batch reactors with free cells.

Batch	Glycerol: Yeast extract (g/L)	μ (g/L·h)	q (g/L·h)	Y (mol/mol)	Max propionic acid (g/L)
Free cells	20:10	0.33	0.52 ± 0.08	0.84 ± 0.03	17.00 ± 0.12
Free cells *	40:10	–	0.34	0.64	19.46
	60:10	–	0.26	0.64	26.31
1	40:10	0.09	0.27 ± 0.01 0.18 †	0.74 ± 0.03 0.68 †	18.20 ± 1.7 19.5 †
2	60:10	0.05	0.21 ± 0.01 0.22 †	0.48 ± 0.01 0.66 †	25.73 ± 0.02 26.0 †
3	60:20	0.07	0.37 ± 0.05 0.31 †	0.73 ± 0.02 0.78 †	32.92 ± 0.06 33.0 †
4	80:10	0.03	0.23 ± 0.01	0.41 ± 0.01	23.12 ± 0.35
5	100:20	0.01	0.28 ± 0.03	0.64 ± 0.05	57.73 ± 0.07
6	160:20	0.03	0.15 ± 0.24	0.77 ± 0.01	61.06 ± 0.04

* Data taken from Dishisha et al. (2012) on wild type *P. acidipropionici* cells.
† Data taken from Dishisha et al. (2015).

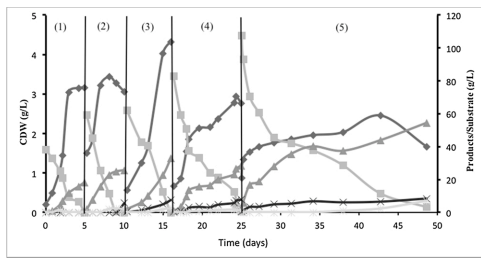

Fig. 4. Profiles of growth in terms of CDW (◆), glycerol consumption (■), and production of propionic acid (▲) and succinic acid (×) by the adapted strain of *P. acidipropionici* in the immobilized cell bioreactor during consecutive recycle batch cultivations in the medium with glycerol: yeast extract concentration ratios of (1) 40:10, (2) 60:10, (3) 60:20, (4) 80:10 and (5) 100:20, respectively separated by vertical bars. The high biomass at the beginning of the last batch was caused by a short previous batch of five days, which was aborted due to technical problems (not shown).

3.4. Propionic acid fermentations using the immobilized adapted *P. acidipropionici* strain at decreasing pH values

Immobilization of the adapted isolate in bioreactor 2 was performed in 2 stages of 17 days each, and the final OD_{620} of the culture was 4.54 equivalent to CDW of 1.7 g/L (SD ± 0.02). Propionic acid fermentations

were then performed sequentially at decreasing set pH values from pH 6.0–4.5, with an aim to reach the lowest possible pH at which the adapted strain would still have the ability to produce propionic acid. While the growth rate of the planktonic cells decreased from 0.03 to 0 g/L·h on pH reduction from 6.0 to 5.0, the propionic acid production was improved reaching the highest productivity of 0.23 g/L·h and product yield of 0.78 mol/mol at both pH 5.0 and 5.5 (Table 3). The fermentations lasted 8, 6 and 7 days at pH 6.0, 5.5 and 5.0, respectively. At pH 4.5, drastic inhibition of growth and production parameters were observed; no bacterial growth or product formation was noted during two weeks.

4. Discussion

Propionic acid is a strong growth inhibitor [28,49]; at the acid concentration of 1 % the specific growth rate of propionibacteria is reduced by more than 50 % [50]. Hence, improving the tolerance to both high propionic acid concentration as well as low pH is important for achieving a resource-efficient and economically competitive process. Adaptive evolution of *P. acidipropionici* DSMZ 4900 obtaining a tolerant strain in this study was done by gradual adaptation to successively increasing concentrations of propionic acid in the same way as reported earlier [6]; the difference being that the procedure was carried out over a shorter period of time (7 months) compared to one year used in the previous report [6], until reaching initial propionic acid concentration of 40 g/L where no cell growth was observed.

The tolerant *P. acidipropionici* strain, finally selected after adaptation in the medium supplemented with 20 g/L propionic acid, when cultivated without extra addition of propionic acid, exhibited 1.4-fold higher growth rate, 3.7-fold higher propionic acid production kinetics, 1.2 fold higher yield and almost 2-fold higher titer (16.8 g/L vs 8.72 g/L) in batch cultivations compared to the wild type cells in a normal cultivation medium with glycerol as carbon source (Table 1). Even at initial propionic acid concentration of 30 g/L, the fermentation data obtained for the adapted cells was comparable to that of the wild type *P. acidipropionici* grown in the medium without any supplemented propionic acid and which could not grow in the medium containing 10 g/L propionic acid. In contrast, in the report by Woskow and Glatz [6], only a slight increase in the propionic acid titer (13.6 versus 11.6 g/L) and volumetric productivity (0.39 vs 0.33 g/L·h) were reported for the tolerant *P. acidipropionici* strain compared to the wild type in batch fermentation on 30 g/L glucose as substrate.

In another report, batch cultivation of the developed tolerant *P. acidipropionici* strain on 20 g/L glycerol yielded 15.72 g/L propionic acid in 120 h with yield of 0.79 g/g and productivity of 0.13 g/L·h [36]. Fermentations in the semi-continuous or fed-batch modes have however resulted in higher propionic acid concentration (about 47 g/L) and less succinic acid and acetic acid from glucose [6,36]. But the fed-batch culture with glycerol gave no significant increase in productivity (0.2 g/L·h) and the highest product yield obtained (0.56 g/g) was lower than that in batch mode [36].

Increased tolerance to propionic acid has also been achieved by adaptation of *P. acidipropionici* for 3 months in a fibrous bed bioreactor [51]. The specific growth rate of the adapted culture was lower in the absence of propionic acid as compared to the wild type, but the extent of cell growth inhibition for the adapted culture was lower when grown in the presence of propionic acid, i.e. 50 % lower growth rate compared to 70 % for the wild type at 10 g/L propionic acid [51]. The acetate kinase deletion and adaptation in the fibrous bed bioreactor resulted in the mutant with improved tolerance, retaining 74 % of its specific growth rate at 20 g/L propionic acid but there was practically no improvement in the fermentation kinetics with free cells as compared to the non-adapted mutant in the normal cultivation medium [35]. In contrast, the adapted culture in this study had a better growth rate and fermentation parameters even in the presence of 20 g/L propionic acid (Table 1).

Table 3
Production of propionic acid by adapted *P. acidipropionici* cells immobilized on PEI treated Poraver® beads during fermentations controlled at different pH values.

pH	μ (CDW g/L·h)	q (g/L·h)	Y (g/g)	Propionic acid max (g/L)
6.0	0.03	0.14 ± 0.07	0.68 ± 0.01	10.04 ± 0.06
5.5	0.01	0.16 ± 0.11	0.78 ± 0.00	10.95 ± 0.31
5.0	0.00	0.23 ± 0.15	0.78 ± 0.00	10.88 ± 0.15

The strains with higher acid tolerance have been reported to exhibit different biochemical features such as increase in cellular straight chain saturated fatty acids and less unsaturated fatty acids [5,50], increased biosynthesis of H^+-ATPase and intracellular energy status for maintaining the intracellular pH in an acidic environment [35,52], increase in NAD^+/NADH ratio resulting from the decrease in H^+ concentration [52], and increased accumulation of arginine, aspartic acid and glutamic acid as their respective metabolisms involve consumption of H^+ and generation of ATP and NH_3 [52]. Several proteins/enzymes with roles in central metabolism, energy and redox balance, protein synthesis, molecular chaperone and transport of substances have been found to be up-regulated in the acid-tolerant strains obtained by genome shuffling [42]. The higher propionic acid production in the fibrous bed bioreactor was attributed to mutations in two key enzymes oxaloacetate transcarboxylase and propionyl CoA: succinyl CoA transferase involved in the production of propionic acid from pyruvate, and lower activity of phosphoenol pyruvate carboxylase giving lower succinic acid.

Increase in trehalose content has been an important response of Propionibacteria to oxidative, osmotic and acid stress [47,53,54]. The expanded trehalose metabolism in *P. acidipropionici* indicates its potential role in alleviating acid stress [46]. The adapted *P. acidipropionici* cells obtained in this study revealed a drastic increase in the expression of *treY* as in our earlier report when the bacteria were exposed to citric acid for inducing biofilm formation [47]. Other studies point to *otsA-otsB* pathway being primarily responsible for trehalose biosynthesis in *P. acidipropionici* [16], and higher expression of *otsA* than *treS* was also observed in *P. freudenreichii* under acid stress [55]. It is possible that the overexpression of *treY* observed in *P. acidipropionici* in the present work was related to the use of glycerol as carbon source in contrast to the other studies on propionibacteria grown on glucose [16,55]. Trehalose production was also favored in *Propionibacterium freundenreichii* ssp. *shermanii* cultures grown on pure glycerol [56].

Based on the earlier studies from our laboratory [22,57,58], the adapted *P. acidipropionici* cells were immobilized to the recycled glass beads Poraver® treated with PEI, the polycation that facilitates the adhesion of cells to the matrix by electrostatic interaction. PEI-Poraver bound *P. acidipropionici* have so far provided among the most efficient immobilized cell systems for propionic acid fermentation. We reported 35.2 g/L propionic acid from 85 g/L glycerol at a volumetric rate of 0.35 g/L·h by immobilized *P. acidipropionici* DSMZ4900 in a recycle batch fermentation [22]. Later on, Jiang et al. [16] reported the production of 32.4 g/L propionic acid from 60 g/L lactose with productivity of 0.56 g/L·h using PEI-Poraver immobilized *P. acidipropionici* CGMCC 1.2232 in batch mode, and up to 125 g/L propionic acid with a yield of 0.62 g/g and productivity of 0.56 g/L·h in a fed-batch mode. Fed-batch fermentations have been commonly adopted for increasing product titers [17,31,35,59].

As is clear from Table 2, the growth of the planktonic cells during recycle batch fermentations with varying glycerol concentrations was extremely low. This would lower the ATP demand for biomass formation and uncouple propionic acid production from cell growth. The productivity, although low but comparable to or better than many reports so far [34,51,60], was not significantly affected by increase in glycerol concentration up to 160 g/L. In accordance with our earlier report [24], propionic acid yield and titers were improved by increasing the yeast extract concentration (Table 2). The productivity is likely to further

increase with a higher concentration of the nitrogen source. As has also been reported earlier for other immobilized propionibacteria [51], the levels of succinic acid and acetic acid were maintained at low levels. An exception is the immobilized culture of the adapted acetate kinase deletion mutant of *P. acidipropionici* that started to produce significant amounts of acetic acid possibly for maintaining NADH/NAD^+ balance and providing additional ATP for cell growth [60].

P. acidipropionici and also other propionibacteria, including the ones evolved to adapt to high propionate concentration, are sensitive to increasing H^+ ion concentration rather than the anion concentration [28]. The bioreactor 2 with immobilized adapted *P. acidipropionici*, operated successively at decreasing pH values gave increased propionic acid yield and productivity while maintaining the product titer nearly constant at pH 5.0 (Table 3). No cell growth was observed since the cells utilize their energy to maintain intracellular pH rather than their growth at low pH [35,52,62]. On the other hand, an earlier study reported higher ratio of propionic acid to acetic acid (5:1) by free cells of *P. acidipropionici* at pH 5 but lower yield (0.387 g/g) and productivity (0.107 g/L·h) when shifting from pH 7.0 (0.426 g/g, 0.219 g/L·h) [61].

In conclusion, it can be stated that an adapted *P. acidipropionici* strain has been developed in this study that shows superior acid-tolerance than many of the earlier reports. Table S2 summarizes the propionic acid production data from this study and those reported in literature. Majority of the studies have been performed using sugar as carbon source and it is clear that higher product yields and concentrations were obtained in fed-batch fermentations. Our results are comparable with the studies reported with glycerol as carbon source using engineered *P. acidipropionici* by knock-out of the acetate kinase gene and adapted in a fibrous bed reactor [60], and we show the productivity and yield to be maintained at higher glycerol concentration. The immobilized cell system developed is robust; the bioreactor with increasing glycerol concentration was operated continuously for more than 2 months. The propionic acid production using this system can be improved further by fed-batch fermentations and even genetic engineering of the adapted strain e.g. by expression of glycerol dehydrogenase [63].

CRediT authorship contribution statement

Victor Hugo Cavero-Olguin: Methodology, Software, Validation, Investigation, Formal analysis, Data curation, Writing - original draft. **Farshad Rahimpour:** Methodology, Software, Validation, Investigation, Formal analysis, Data curation. **Tarek Dishisha:** Conceptualization, Methodology. **Maria Teresa Alvarez-Aliaga:** Supervision, Project administration. **Rajni Hatti-Kaul:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2021.08.005.

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SUPPLEMENTARY DATA

Propionic acid production from glycerol in immobilized cell bioreactor using an acid-tolerant strain of *Propionibacterium acidipropionici* obtained by adaptive evolution

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Table S1. Designed primers for studying the expression of *otsA* and *treY* genes, using 16S rRNA gene as control

	cDNA		qPCR	
	Left	Right	Left	Right
otsA	5'-TCGATGGAGATCGGGAAGG-3'	5'-CGTGTGGGTGCAGGACTACC-3'	5'-GATCGGAGGGAAGGGGATGT-3'	5'-CGTGTGGGTGCAGGACTACC-3'
treY	5'-GAGGACCGACAGGGAGTTCG-3'	5'-GCGGACTTCACCTACGACGA-3'	5'-CCATGTGATTGGGCACGAC-3'	5'-GCGGACTTCACCTACGACGA-3'
16S	5'-CGTGCTTTCGATACGGGTTG-3'	5'-CCAGGCGGGGTACTTAATGC-3'	5'-TGGACCTTTCCTGACGCTGA-3'	5'-CCAGGCGGGGTACTTAATGC-3'

Table S2. Comparison of the results from the bioreactors in this study with literature reports on propionic acid fermentation using free and immobilized cells in batch and fed batch bioreactors

Strain	Mode of operation	Carbon source	pH	Qp (g/L·h)	Y (mol/mol)	Titer (g/L)	Ref.
P. acidipropionici DSMZ 4900	Immobilized cell recycle batch (Bioreactor 1)	Glycerol (100 g/L) †	6.5	0.28	0.64	57.73	This work
	Immobilized cell recycle batch (Bioreactor 2)	Glycerol (20 g/L) †	5.0	0.23	0.78	10.88	
P. acidipropionici DSMZ 4900	Immobilized cell recycle batch	Glycerol (84.6 g/L)†	6.5	0.35	0.59	35.2	[1]
P. acidipropionici CGMCC 1.2232 (ATCC 4875)	Immobilized cell batch	Lactose (60 g/L)†	6.0	0.56	2.5 (0.54 g/g)*	32.4	[2]
	Immobilized cell fed batch	Lactose (200 g/L)††		0.56	2.87 (0.62 g/g)	125	
Propionibacterium freudenreichii CCTCC M207015 (cheese-isolated strain)	Immobilized cell fed batch	Hydrolysed cane molasses (200 g/L) †††	-	0.36	-	91.89	[3]
P. freudenreichii CCTCC M207015 (cheese-isolated strain)	Immobilized fed batch	Glucose (155 g/L) †††	6.9	0.14	-	67.05	[4]
	Immobilized batch	Glucose (80 g/L) †		0.19	-	32.65	
Adapted mutant ACK-tet *P. acidipropionici* ATCC 4875	Immobilized fed batch	Glucose (100 g/L) ††††	6.5	0.07	1.29 (0.53 g/g)	97	[5]
		Lactose ‡		0.12	2.13 (0.46 g/g)	104	
Adapted acid-tolerant *P. acidipropionici* CGMCC 1.2230	Immobilized repeated-batch	Pretreated sugar cane bagasse hydrolysate (25.4 g/L glucose and 32.1 g/L xylose) ‡ ‡	7.0	0.88	0.70 (0.29 g/g)	12.9	[6]
	Immobilized fed batch			0.38	0.9 (0.37 g/g)	58.8	
	Immobilized repeat- batch	Glucose (40 g/L) ‡ ‡ ‡		0.96	1.24 (0.51 g/g)	22.9	
	Immobilized fed batch			0.71	1.05 (0.43 g/g)	51.2	
P. acidipropionici ATCC 4875	High density culture + recycling cells batch	Glucose (40 g/L) ‡ ‡ ‡ ‡	6.0	0.32	0.97 (0.40 g/g)	75.9	[7]
Adapted mutant *P. acidipropionici* ATCC 4875	Immobilized fed batch	Glucose (100 g/L +)⁂	6.5	0.23 (5.23 g/L·day)*	0.97-1.58 (0.40-0.65 g/g)	71.8	[8]

Metabolically engineered (ACK-tet) *P. acidipropionici* ATCC 4875	Batch	Glycerol (40 g/L) †	7.0	0.25	0.73 (0.59 g/g)	23	[9]

* Data in parenthesis reflects the way they are presented in their respective references

† Carbon source completely consumed

†† Carbon source completely consumed in batches of 40 g/L

††† Carbon source completely consumed in batches of 10 g/L

†††† Carbon source not totally consumed in batches of 40 g/L

‡ Carbon source not totally consumed after different concentration batches ranging from 80 g/L to below

‡‡ Only glucose completely consumed

‡‡‡ Carbon source completely consumed in different batches of 40 g/L

‡‡‡‡ Carbon source consumed in different batches of 40 g/L and less

♣ Carbon source not totally consumed after three batches of c.a. 80 g/L

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Paper IV

Membrane-based continuous fermentation with cell recycling for propionic acid production from glycerol by *Propionibacterium acidipropionici*

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Abstract

Microbial production of propionic acid from renewable resources is characterized by slow growth of the producer bacteria and product inhibition. This report presents a study on high cell density propionic acid continuous fermentation from glycerol using *Propionibacterium acidipropionici* DSM 4900 in a membrane-based cell recycling system. A ceramic tubular membrane filter of 0.2 μm pore size was used as the filtering device for cell recycling while fermentations were run sequentially at dilution rates of 0.05 and 0.025 1/h using varying glycerol concentrations and two different yeast extract concentrations. Highest productivity of 1.23 g/L.h with product yield of 0.48 g/g substrate was obtained with 50 g/L glycerol at yeast extract concentration of 10 g/L. Increasing the glycerol and yeast extract concentration to 60 g/L and 20 g/L, respectively, led to increase in propionic acid productivity, product yield and concentration to 2.35 g/L.h, 0.78 g/g and 47.03 g/L, respectively. Lowering the dilution rate to 0.025 1/h reduced the production efficiency. The cell density increased from 6 to 90 g_{CDW}/L during the continuous operation of the system for a period of 5 months. A tolerant strain exhibiting growth at propionic acid concentration of 20 g/L was isolated at the end.

1. Introduction

Diary propionibacteria are anaerobic, Gram-positive bacteria that besides being used for production of traditional Swiss-type cheeses and as human and animal probiotics (Cousin et al. 2011; Thierry et al. 2011), are also an important source of valuable products like Vitamin B12, trehalose, flavors, antimicrobial compounds including diketopiperazines, linear- and cyclic peptides, 3-phenyllactic acid and propionic acid (Ruhal et al. 2011; Langsrud et al. 1973). Propionic acid, a C-3 organic acid, is a product of great interest with uses ranging from food industry, pharmaceutical, cosmetics and plastics (Liu et al. 2012; Ranaei et al. 2020).

The commercial production of propionic acid still depends on petrochemistry, although there have been ongoing research efforts to establish a bio-based route of production. Propionibacteria are the main producers of propionic acid from different carbon sources like glucose, lactose, lactic acid, xylose and glycerol (Carrondo et al. 1988; Coral et al. 2008; Jiang et al. 2015; Gonzales-Garcia et al. 2017; Dishisha et al. 2012, 2013, 2015; Wei et al. 2016; Cavero-Olguin et al. 2019, 2021;

Rogers et al. 2006, Barbirato et al. 1997, Yang et al. 1995). Glycerol, being a more reduced carbon source than sugars and lactic acid, gives a higher yield of propionic acid with much less acetic acid as the side product (Dishisha et al. 2012). Glycerol is a major co-product of biodiesel production (Clomburg and Gonzalez 2013), and finds several applications directly after refining, and also serves as an attractive feedstock for other chemical building blocks (Yang et al. 2012).

The propionic acid fermentation process is slow and is limited by product inhibition that results in low productivity and yield. Hence, use of high cell density reactors has been proposed to overcome the limitation. Several studies on enhancing propionic acid production using immobilized cell bioreactors have been reported (Huang et al. 2002; Suwannakham and Yang 2005; Feng et al. 2010; Chen et al. 2013; Dishisha et al. 2012; Cavero-Olguin et al. 2021, Yang et al. 1995, Lewis and Yang, 1992), majority of which utilize fibrous bed reactor. We have also reported sequential batch propionic acid fermentation with cell recycling using free cells of *Propionibacterium acidipropionici*, which were collected by centrifugation after each batch and then used to start a new batch of fermentation (Dishisha et al. 2013). Using a culture medium with 50 g/L glycerol, the cell biomass concentration increased 215 times during 9 batches that reached 21.5 g_{CDW}/L, while the productivity increased 6-fold to 1.35 g/L.h. By further optimizing the concentrations of carbon and nitrogen sources, the productivity reached 1.63 g/L.h with final propionic acid concentration of 60 g/L and a biomass of 31.28 g/L (Dishisha et al. 2015).

Membrane-based cell retention, applied primarily for cell separation during product recovery in biotechnology processes (Alexandri et al. 2018; Tomczak and Gryta 2021), offers a useful alternative for high cell density fermentation without any cell leakage (Chang et al. 1994). The latter technique has been used in lab scale for production of several metabolites like ethanol, acetone-butanol, citric acid, lactic acid and mannitol (Chang et al. 1994; Sawai et al. 2011; Sung et al. 2012; Mimitsuka et al. 2015; Fan et al. 2017). A number of studies have reported propionic acid production from sugars and lactic acid using continuous mode of operation with cell recycling (Goswami and Srivastava 2001; Crespo et al. 1990, 1991; Blanc and Goma 1989; Carrondo et al. 1988), while only one study employed glycerol as a carbon source achieving a productivity of 1 g/L.h with propionic acid concentration of 10 g/L (Boyaval et al. 1994).

The choice of a suitable membrane is important for the success of the application, the important criteria for selection being identified as mechanical strength, resistance to cleaning agents, pore

size and surface charge (Speed 2016). A major limitation with the membrane processes is the decrease in permeate flux due to membrane fouling, the extent of which depends on the process operating parameters and the interaction of the membrane with feed components. During the past two decades, there has been increasing interest in ceramic membranes that are based on alumina (Al_2O_3), zirconia (ZrO_2), titania (TiO_2) or a combination of these materials (Tomczak and Gryta 2021). The ceramic membranes have several advantages over the polymeric membranes including high resistance to aggressive physical and chemical cleaning, and corrosion, and inertness to biological components, allowing them to possess high flow capacity, high separation efficiency, and long shelf lives (Jabbari et al. 2019; Tomczak and Gryta 2020, 2021).

This report presents a study on continuous propionic acid fermentation involving cell recycle using a ceramic (TiO_2) membrane filter at varying concentrations of glycerol and yeast extract as carbon and nitrogen sources, respectively.

2. Materials and Methods

2.1. Chemicals

Glycerol (99%), ammonium hydroxide solution (28%) and L-cysteine HCl, anyhydrous (98%) were products of Sigma-Aldrich (St Louis, MO, USA). Bacto yeast extract (YE) was procured from Difco (BD laboratories, Detroit, MI, USA) and phosphate buffer salts from Merck (NJ, USA).

2.2. Microorganism and culture medium

Propionibacterium acidipropionici DSM 4900 was used in the present study. For preculture preparation, the basal culture medium consisted of 10 g/L yeast extract, 20 g/L glycerol, 0.25 g/L cysteine HCl, 2.5 g/L K_2HPO_4 and 1.5 g/L KH_2PO_4, pH 7 adjusted using ammonium hydroxide (Dishisha et al. 2013). The medium (90 mL) was prepared in 100-mL serum bottles, boiled, flushed with oxygen-free nitrogen, sealed and then autoclaved at 121 °C for 20 min. The bottles were then allowed to cool and used for pre-culture preparation. For bioreactor experiments, the same culture medium was used with varying concentrations of carbon- and nitrogen sources as follows: Glycerol (20, 40, 50, 60 and 70 g/L) and yeast extract (10 and 20 g/L).

2.3. Preculture preparation

For preculture preparation, 1 mL of stock culture in glycerol was aseptically transferred to 90 mL of sterile basal culture medium in 100-mL rubber-sealed serum bottle. The mixture was incubated at 30 °C for 7 days. The resulting culture was used as inoculum for bioreactor experiments.

2.4. Bioreactor set up

The bioreactor used for continuous production of propionic acid with cell recycling is shown in Figure 1. It consisted of a 500-mL water-jacketed vessel equipped with a headplate involving ports for pH electrode, base addition, sampling, gas release, liquid -outlet and -inlet for culture recycling to the filtration unit. The vessel was coupled to a tangential flow microfiltration module equipped with a tubular ceramic membrane (0.2 μm pore size, 25 cm length and 1 cm outer diameter) (Tami Industries, France). The liquid flow in the external recycling loop was controlled by a perstaltic pump (Watson Marlow 604S Ip55), which was manually adjusted to attain a constant volume in the bioreactor (chemostat). During the whole experiment, the temperature was maintained at 30 °C using a circulating water bath, the culture was mixed by stirring at 250 rpm on a magnetic stirrer, and the pH was controlled at 6.5 through addition of 5 M ammonia via a perstaltic pump controlled by an external pH control unit (Inventron AB, Mölndal, Sweden). Prior to use, the entire reactor set up including 300 mL of basal culture medium was sterilized by autoclaving at 121 °C for 20 min.

2.5. Continuous production of propionic acid in a bioreactor with cell retention

The continuous fermentation was preceded by batch cultivation. Initally, 30 mL of freshly-prepared preculture was aseptically transferred to 300 mL basal culture medium in the bioreactor vessel. After 3 days, the medium feeding was initiated at a rate of 18 mL/h until the empty spaces in the tubing and external filtration module were filled with the culture and keeping 300 mL inside the bioreactor. Hence, the total working volume reached ≈ 360 mL. At this point, the continuous mode of operation (chemostat) was initiated. Two dilution rates (D) were evaluated: 0.05 and 0.025 1/h corresponding to a medium feeding rate of 18 and 9 mL/h, respectively. Five different glycerol:yeast extract (Gly:YE) mixtures with concentration of 20:10, 40:10, 50:10, 60:10 and

60:20 g/L were evaluated at D = 0.05 1/h, while only three mixtures of 60:20, 60:10 and 70:10 were evaluated at D = 0.025 1/h. Each mixture was operated for at least 4 retention times (≈ 1400 mL at least). Samples were collected frequently from the bioreactor (5 mL) and the permeate (5 ml) for measuring the cell density and metabolite concentration, respectively.

2.6. Isolation of adapted P. acidipropionici variants

A dense suspension of *P. acidipropionici* cells was withdrawn from the 5-month continuous bioreactor, washed twice with sterile saline solution, and then inoculated in serum bottles used for isolation of adapted *P. acidipropionici* variants as described ahead. Two sets of media were prepared, the first was designed for the isolation of a low-pH resistant variant after three-week cultivation in 90 mL basal culture medium where the starting pH was adjusted to 7, 6 and 5, respectively. The second set was designed for the further isolation of propoinic acid-resistant variant; it consisted of liquid and solid culture media containing 10, 20, 30, 40 and 50 g/L propionic acid (pH 7), respectively, the former prepared for simultaneous cultivation in 90 mL and the latter prepared with agar at 2 % (w/v) for cultivation in petri dishes. Values of OD_{620} > 7 (i.e. equivalent to cell dry weight of 2.56 g/L), were adopted as *de facto* selective criterion as used earlier in our lab when obtaining an adapted strain by adaptive evolution (Cavero-Olguin et al., 2021). After sequential selection of adapted cultivations at low pH and high concentration of propionic acid, 100 μL culture samples were seeded on the surface of solid media to finally isolate adapted bacterial colonies after incubation at 30 °C for 7 days.

2.7. Quantitative analyses

The cell density was followed by measuring the optical density of the culture at 620 nm (OD_{620}). The cell dry weight (CDW) was determined by centrifugation of 5 mL fermentation broth at 4000 *g* for 20 min in a dried preweighed tube and drying the cell pellet for 12 h at 100 °C before weighing again and correlating with the volume.

Glycerol, propionic acid, acetic acid, succinic acid and other minor metabolites were analyzed by HPLC (Jasco) equipped with Aminex HPX-87H organic acid analysis column (Bio-rad, Hercules, California, USA), CTO-6A oven (Shimadzu, Kyoto, Japan), Jasco AS 950-10 intelligent pump, PU 980 automatic intelligent injector (Jasco), and ERC 7515A refractive index detector (ERC,

Saitama, Japan). Samples were diluted in MilliQ quality water, acidified with 20 % (v/v) H_2SO_4 (20 µL per 1 mL of sample), and then filtered through 0.45 µm syringe filter prior to analysis. Chromatography was performed using 5 mM H_2SO_4 as mobile phase flowing at a rate of 0.6 mL/min, column temperature was maintained at 55 °C and RI detector temperature at 30 °C.

2.8. Fermentation kinetics

For the batch mode of operation, the fermentation kinetics were calculated as follows:

- *Volumetric production rate:* Q_P *(g/L.h) = [∆P] / [∆t]*
- *Volumetric biomass productivity:* Q_X *(*g_X*/L.h) = [∆X] / [∆t]*
- *Product yield:* $Y_{P/S}$ (g_P/g_S) = | *[∆P] / [∆S]* |

While, for the continuous mode of operation with cell retention the fermentation kinetics were calculated as follows:

- *Dilution rate: D (1/h) = F/V*
- *Retention time:* t_r *(h) = 1/D*
- *Volumetric production rate:* Q_P *(*g_P*/L.h) = D. ∆P*
- *Product yield:* $Y_{P/S}$ *(*g_P/g_S*) =* |*∆P / ∆S*|

where P is the product concentration (g/L), *t* is the time (h), S is the substrate concentration (g/L), X is the biomass concentration (g_{CDW}/L), F is the continuous mode flow rate (mL/h) and V is the working volume (mL).

3. Results and Discussion

3.1. Batch cultivation

The continuous fermentation was preceeded by a batch fermentation in the bioreactor. Actively growing cells of *P. acidipropionici* were used to inoculate the basal medium containing 20 g/L glycerol and 10 g/L yeast extract. Glycerol was depleted in less than 70 h. The propionic acid volumetric productivity, yield and titer were 0.23 g/L.h, 0.55 g_P/g_S and 12.05 g/L, respectively. The biomass productivity was 0.09 g_{CDW}/L.h and the cell density reached ~ 6 g_{CDW}/L at the time of glycerol depletion (Fig. 2).

3.2. Continuous Chemostat with cell retention for propionic acid production

The continuous mode of operation of the bioreactor coupled to the membrane unit was started with the feed set at constant flow rate of 18 mL/h (D = 0.05 1/h) during 40 days followed by a feed rate of 9 mL/h (D = 0.025 1/h) for 18 days. Eight different ratios of glycerol (g/L) and yeast extract (g/L) were used along the whole fermentation period of 62 days. With the D = 0.05 1/h, Gly:YE ratios (w/w) of 20:10, 40:10, 50:10, 60:10 and 60:20 g/L were used sequentially. The operation was further continued for 90 more days (data not shown).

As seen in Table 1, the first condition used, i.e. with 20 g/L glycerol and 10 g/L yeast extract resulted in over 4.1-fold increase in productivity as compared to the batch fermentation. Raising the glycerol concentration from 20 g/L to 50 g/L while maintaining yeast extract concentration at 10 g/L, led to increase in productivity (1.23 g/L.h) and product concentration (24.5 g/L). The product yield was maintained in the range of 0.43-0.49 g/L with increase in the carbon source concentration (Table 1).

Glycerol was completely depleted when used up to 50 g/L, while only 83.5% glycerol was consumed when used at a concentration of 60 g/L (Figure 3). The volumetric biomass productivity was increased from 0.11 to 0.21 g_{CDW}/L.h with increase in glycerol concentration from 20 to 40 g/L, and then reduced with further increase in substrate concentration reaching a plateau at 50 g/L glycerol. As the lack or reduction of cell growth is possibly due to nutrient limitation, the yeast extract concentration was raised to 20 g/L with glycerol being maintained at 60 g/L. This led to enhanced fermentation kinetics, especially propionic acid productivity which reached 2.35 g/L.h and final propionic acid concentration of 47.03 g/L. This was concominant with increase in cell concentration up to 90.09 g_{CDW}/L with a low volumetric biomass productivity of 0.11 g_{CDW}/L.h (Table 1 and Figure 3). Inspite of this, only 74 % of the initial glycerol amount was consumed. Increasing the yeast extract concentration to 30 g/L was reported earlier to enhance fermentation kinetics (Dishisha et al., 2015), however, this was not considered in the present experiment as it would increase the cost of the raw material considerably.

The effect of lower feed rate (9 mL/h) equivalent to dilution rate (0.025 1/h) on the fermentation performance was then studied (Table 1 and Figure 3). Continuing fermentation with glycerol and yeast extract concentrations of 60 and 20 g/L, respectively, lowered the volumetric productivity and propionic acid titer as well as the cell growth rate. Subsequent increase in C:N ratio by

decreasing yeast extract concentration led to further decrease in the values, which were then partly regained by increasing the glycerol concentration to 70 g/L (Table 1).

From Table 1, it is evident that propionic acid production was more favorable at higher dilution rate (0.05 1/h), decrease in cell growth rate led to an increased carbon flux towards the product, and increase in nitrogen concentration had a dramatic effect on improving the productivity and product yield. Lowering the dilution rate lowered the productive parameters levels for propionic acid production but did not shift the carbon flux to biomass formation. Glycerol consumption at 60:20 and 70:10 g/L Gly:YE concentrations was 75.37 % and 86.42 %, respectively.

In principle, there are no limits to increase the dilution rates with cell-retention systems, since cell washout is prevented by the filtering device. The accumulated cells could stand high loads of substrate while possible inhibitory products are constantly removed (Naja et al. 2006). In the present work, the production of propionic acid was enhanced by adjusting the concentrations of carbon and nitrogen sources, and not by the retention time. Our observations seem to be in agreement with an earlier report showing no significant differences in the production of biomass, mannitol, lactic and acetic acid with *Leuconostoc citreum* in a ceramic membrane filter fermentation system at different dilution rates (Sung et al. 2012). During continuous fermentation for lactic acid production using *Bacillus coagulans* with a ceramic filter device, increase in glucose concentration in the feed from 50 to 70 g/L at 0.2 1/h resulted in the same concentration of lactic acid in the product stream along with 20 g/L of residual sugar (Fan et al. 2017).

The production parameters obtained in this work were higher than many of the reported data using immobilized cells or other forms of high-cell density systems for propionic acid production (Dishisha et al., 2012; 2013; 2015). With respect to cell retention using filtration system for propionic acid production, the first report made use of a spin filter in a continuous mode of operation for fermentation of lactose by *P. acidipropionici* at D = 0.05 1/h in a 7 L bioreactor, where 50 % of the cells were retained and resulted in almost four-fold enhancement of the productivity reaching 0.9 g/L.h compared to that in conventional batch fermentation (Goswami and Srivastava 2001). This was very similar to the 4.1-fold increase in productivity at the same dilution rate obtained in the first stage in the present work. Co-production of propionic acid and succinic acid by *P. acidipropionici* in a semi-continuous fermentation with a 0.22 μm membrane module for separation in a recycling loop coupled with a chromatography device, gave titer of

62.22 g/L and productivity of 0.43 g/L.h, an increase by 65 % (Li et al., 2021). In the present work the titer and productivity were increased by about 74 %, respectively. No contamination as well as no leakage of cells into the permeate was observed during the entire process.

While the accumulated cell concentration reached ~ 6 g_{CDW}/L during the batch fermentation, the cell density during the continuous fermentations with the membrane filter device ranged from 32 to 90 g_{CDW}/L (Table 1 and Figure 3). According to Chang et al. (2011) a tenfold density of a batch culture is accepted as high-cell density, which implies that the high-cell density state with ~ 59 g_{CDW}/L was reached already at Gly:YE concentrations of 40:10 g/L. Nonetheless, uneven rates between the inflow and outflow occurred during the entire fermentation.

The system was highly affected by the retained cells on the membrane filter and possible cake formation, leading to decrease in the filtration rate as also reported earlier for lactic acid fermentation using *B. coagulans* and *Lactococcus lactis* ssp. *lactis*, respectively (Fan et al. 2017; Persson et al. 2001). An earlier study on lactic acid fermentation lasting 3-5 days with cell concentration of only 1.1-3.1 g/L reported that the cake formation was prevented when the recycling pump was operated at a lower velocity than that needed for the critical flux through the membrane (Persson et al. 2001), both factors being far lower than that in the present work. Membrane fouling and the need for high pressures for operation could be the main bottlenecks when using the membrane filters with high cell-densities and even with high molecular weight products. For example, performance of the system involving cultivation of *Saccharomyces cerevisiae* with integrated tangential filtration device was affected by the accumulation of polymeric substances onto the surface of the filter (Naja et al. 2006). Other studies showed that materials like polyvinylidene difluoride had antifouling properties when used for pyruvic acid production by *Torulopsis glabrata* (Sawai et al. 2011).

The membrane filter retained both live and dead cells, and it would be useful to determine the proportions of the two cell states during long term operation of the reactor in order to understand the physiological state of the cells.

3.3. Isolation of acid tolerant strain

Continuous fermentation over a long period in the membrane integrated bioreactor exerts extensive stress on the cells that were subjected to mechanical stress due to the constant shearing through pumping and high pressures. The suspension of washed cells from the bioreactor were batch cultivated in serum bottles for three weeks at pH values of 7, 6 and 5, respectively. The final cell density of the three cultivations gave values of OD_{620} > 7; cells from pH 5 serum bottle were harvested and transferred to another liquid media at initial propionic acid concentrations of 10, 20, 30, 40 and 50 g/L. Bacterial growth with OD_{620} > 7 were obtained only in media with 10 and 20 g/L of propionic acid. Agar plates with 10 and 20 g/L propionic acid were then used and both showed isolated colonies, while no growth was observed in cultures with higher propionic acid concentrations.

The isolated colony from the solid media with 20 g/L propionic acid was cultured in liquid medium supplemented with the same propionic acid, grown for 3 weeks and the culture was stored in 50 % glycerol medium at -20 °C for further use. The resulting variant was able to grow at pH 5 with 20 g/L glycerol in presence of 20 g/L propionic acid.

4. Conclusion

Efficient performance of the ceramic filter was demonstrated during a long fermentation period. Significantly higher production efficiency was obtained in comparison with previous reports. Nonetheless, such a system would benefit by the use of a sensor for automated control of fouling on the filter surface and the volume in the reactor vessel to enhance the stability and performance for long-term fermentations. The concomitant adaptation of the strain exposed to mechanical stress and also exposure to high concentrations of glycerol and propionic acid, made possible an easy isolation of a tolerant strain for further investigation.

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Legends to figures

Figure 1. Schematic presentation of the cell recycling fermentation with membrane filter bioreactor set-up used for propionic acid production by *P. acidipropionici*

Figure 2. Batch cultivation of *P. acidipropionici* with glycerol:yeast extract ratio of 20:10 g/L. The different symbols represent biomass (□), glycerol (□), propionic acid (□), succinic acid (□) and acetic acid (□) concentrations

Figure 3. Continuous propionic acid production from glycerol using *P. acidipropionici* with cell recycling via a tangential flow filtration module with a ceramic membrane filter. The concentrations of biomass (▲), propionic acid (□), glycerol (□), acetic acid (□), succinic acid (□), and n-propanol (+) are presented during two different dilution rates. The first five stages correspond to D = 0.05 and the last three stages correspond to D = 0.025, with different medium composition (Gly:YE ratio of 20:10, 40:10, 50:10, 60:10, 60:20, 60:20, 60:10, 70:10 g/L, respectively).

Table 1. Propionic acid production parameters under continuous operation with cell recycling at different concentrations of glycerol and yeast extract and at varying dilution rates

	D = 0.05					**D = 0.025**		
	20:10	**40:10**	**50:10**	**60:10**	**60:20**	**60:20**	**60:10**	**70:10**
X **(g_{CDW}/L)**	32.16 ±4.34	58.90 ±0.84	61.57 ±8.97	49.12 ±7.99	90.09 ±1.44	63.56 ±0.35	77.49 ±3.79	74.81 ±3.84
Q_P **(g/L/h)**	0.96 ±0.09	0.92 ±0.07	1.23 ±0.02	1.11 ± 0.01	2.35 ±0.09	0.90 ±0.07	0.61 ±0.04	0.79 ±0.05
$Y_{P/S}$ **(g_P/g_S)**	0.47 ±0.04	0.43 ±0.03	0.48 ±0.03	0.49 ± 0.01	0.78 ±0.05	0.74 ±0.08	0.39 ±0.03	0.53 ±0.06
P **(g/L)**	19.17 ±0.09	18.39 ±1.12	24.57 ±0.31	22.15 ± 0.14	47.03 ±0.09	36.02 ±1.02	24.50 ±0.36	31.59 ±0.99

9. Figures:

Figure 1

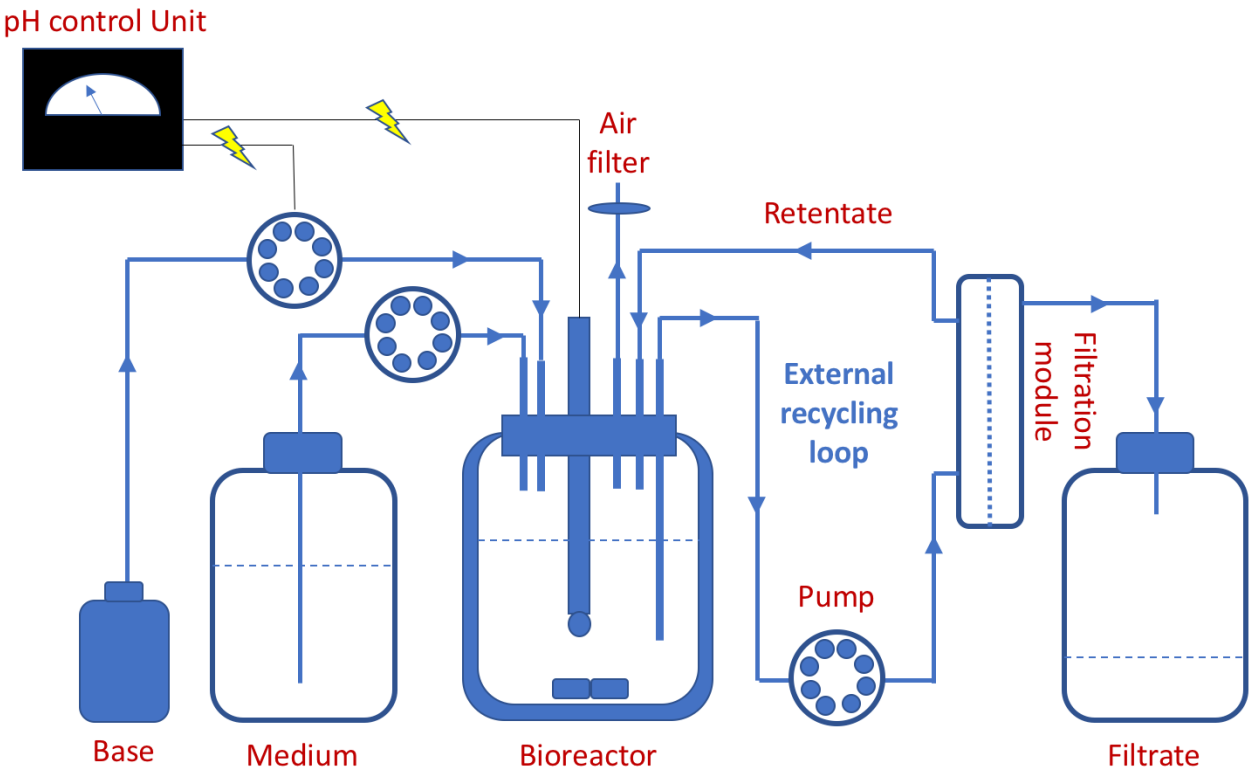


Figure 2.

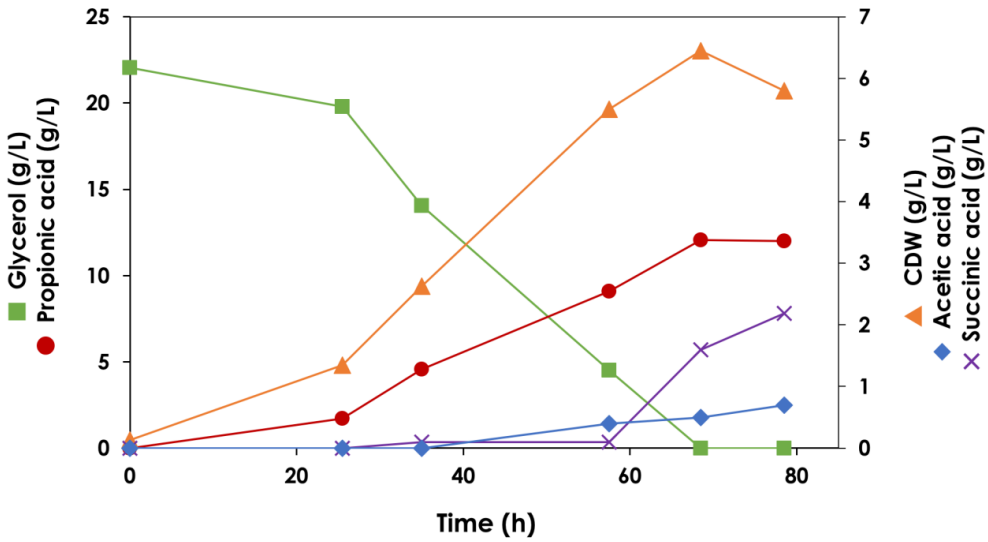
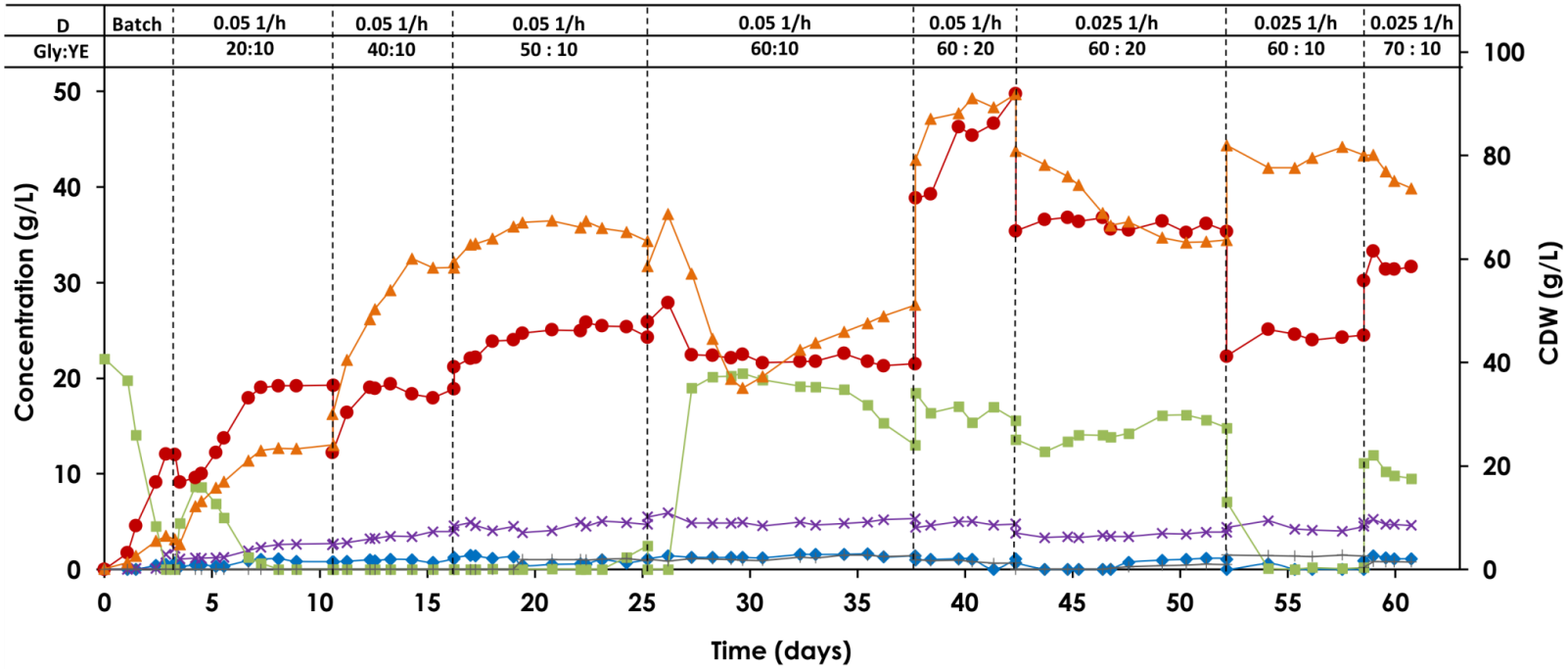

Glycerol (g/L)
Propionic acid (g/L)
CDW (g/L)
Acetic acid (g/L)
Succinic acid (g/L)
Time (h)
0
5
10
15
20
25
0
1
2
3
4
5
6
7
0
20
40
60
80

Figure 3.


D
Gly:YE
Batch
0.05 1/h
20:10
0.05 1/h
40:10
0.05 1/h
50 : 10
0.05 1/h
60:10
0.05 1/h
60 : 20
0.025 1/h
60 : 20
0.025 1/h
60 : 10
0.025 1/h
70 : 10
Concentration (g/L)
CDW (g/L)
Time (days)
0
5
10
15
20
25
30
35
40
45
50
55
60
0
10
20
30
40
50
0
20
40
60
80
100

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