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Genetic Engineering of *Clostridium acetobutylicum* to enhance isopropanol-butanol-ethanol production with an integrated DNA-technology approach

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Abstract

Acetone being a non-fuel solvent produced during the traditional acetone-butanol-ethanol (ABE) fermentation by Clostridium acetobutylicum; reduces the overall fuel alcohol yield. However, the conversion of acetone into isopropanol has been recommended to improve the process economy. The present study aims to develop an engineered C. acetobutylicum DSM 792 strain to convert acetone into isopropanol by introducing the secondary alcohol dehydrogenase gene from C. beijerinckii NRRL B593 using the allele-coupled exchange approach. Batch and continuous fermentation experiments were carried out with a modified strain C. acetobutylicum DSM 792-ADH to improve the isopropanol yield and titer. The growth and production behavior of the modified strain in stationary flask culture and controlled batch culture was studied. Almost 50% of acetone was converted into isopropanol with highest total solvent yield to be 0.39 g.g⁻¹ glucose. The modified strain also utilized sugar mixture and SO₂–ethanol–water spent liquor as a substrate to produce the solvents.

Keywords: Clostridia, IBE, immobilization, isopropanol, fermentation, column reactor
1. Introduction

The demand for a clean and renewable biofuel has dramatically increased as a new benchmark on the petroleum industry from last few decades. Butanol being a superior biofuel to ethanol has attracted an increased attention because of its chemical properties including higher energy density and less hygroscopicity [1]. However, the productivity and yields during biofuel production are limited by chaotropicity, which is reported to be extreme for butanol [2-5]. Butanol has traditionally been produced by acetone-butanol-ethanol (ABE) fermentation using clostridia [6]. However, ABE fermentation is currently considered as less economical process for biofuel production with respect to carbon recovery. The acetone produced during this process cannot be used as a fuel due to several reasons including its corrosiveness [7-11]. The co-production of acetone with butanol (and ethanol) is considered undesirable because of the reduction in butanol yield per unit mass of substrate utilized during the process [9]. Thus, a reduction in the acetone production has been an important objective of clostridial metabolic engineering. Hence, it is desirable to suppress the formation of acetone or to convert it into another fuel additive such as isopropanol. Isopropanol is a simple secondary alcohol that has been used as a cleaning agent and plasticizer in the plastics industry. Recently, it has been reported that isopropanol can also be used as a fuel additive for the preparation of high-octane gasoline [12,13].

Many bacterial metabolites released from bacterial cells, including alcohols, act as antimicrobials and they frequently presents an obstacle to biotechnologists [2,4,14]. Furthermore, the microorganisms used to carry out fermentations are already stressed by other factors including high sugar concentrations and temperature fluctuations [4,15,16]. However, isopropanol is considerably more polar and less chaotropic than butanol and, hence is typically less inhibitory to the bacterial cell [4,5,17]. The attempts to reduce the acetone production by metabolic engineering have so far resulted in decreased butanol production and large accumulation of acetic and butyric acids in the broth [7,10,11,18]. Acetone formation is essential in the cell culture for cytosolic
detoxification from carboxylic acids and protons to increase the culture pH in response to acetic acid and butyric acid to produce butanol and ethanol [19]. Hence the conversion of acetone into isopropanol is desirable instead of suppressing the acetone production in metabolic pathway. The conversion of acetone into isopropanol is a single reduction step biochemical reaction, which improves the total yield of fuel alcohols [9,13]. The conversion of acetone into isopropanol in ABE fermentation can produce mixed alcohols as IBE (isopropanol-butanol-ethanol) without the disruption of pathway that can directly be used as a biofuel mixture. Isopropanol is produced in nature by several solventogenic clostridia which simultaneously produce butanol and ethanol [20,21]. However, the IBE production in these natural isopropanol producers is still low (total solvent concentration of 5.87 g.l\(^{-1}\)) and slow (productivity of 0.12 g.l\(^{-1}\).h) in batch cultures [22,23].

The future of fuels produced by fermentation largely depends on the availability of inexpensive and abundant raw materials as well as efficient conversion process. For sustainable industrial scale IBE production, a number of obstacles such as choice of feedstock, low product yield and product toxicity need to be overcome. Lignocellulosic biomass being abundantly available in the nature offers great potential for the production of biofuels with clostridial strains. SEW (SO2–ethanol–water) pulping as a fractionation process for lignocellulosic fibrous material such as spruce wood chips, fiber crops or waste paper has been used as a promising pretreatment method [24-26]. The use of SEW spent liquor for the production of solvents can promote the biorefinery industry which harvests and processes the trees for their subsequent use. The SEW process which has a potential to use hardwood, softwood and recycled fibers can minimize the feedstock cost and also help in improving the current waste water treatment.

The present work was aimed to develop an improved IBE producing strain of \textit{C. acetobutylicum} and to optimize its bioprocess. \textit{Clostridium acetobutylicum} is a commonly used microorganism for ABE fermentation. It does not possess a gene for secondary alcohol dehydrogenase (\textit{adh}) which is required to produce 2-propanol (isopropanol) from acetone [27]. In
order to modify the organism to produce isopropanol the \textit{adh} gene for isopropanol dehydrogenase from other species belonging to clostridium genus \cite{28,29} can be inserted into \textit{C. acetobutylicum}. For example, \textit{C. beijerinckii} NRRL B593 and \textit{C. aurantibutyricum} NCIB 10659 are capable to produce a mixture of isopropanol and \textit{n}-butanol with standard production media \cite{29}. These strains contain a NADPH dependent primary/secondary alcohol (isopropanol) dehydrogenase (EC 1.1.1.1), which convert both acetone and butyraldehyde to the corresponding alcohols isopropanol and \textit{n}-butanol, respectively. The \textit{adh} gene for primary/secondary alcohol dehydrogenase has been characterized, cloned and sequenced from \textit{Clostridium beijerinckii} (NRRL B593 and NESTE 255) by various researchers \cite{30,31}.

The modified strain \textit{C. acetobutylicum} DSM 792-ADH was developed by incorporating the gene encoding the secondary alcohol dehydrogenase (\textit{adh}) from \textit{C. beijerinckii} NRRL B593 into the \textit{C. acetobutylicum} DSM 792 chromosome using allele-coupled exchange approach \cite{32}. The engineered strain was further used in the optimization of fermentation parameters to evaluate its performance and stability in industrially pertinent conditions. It was also attempted to further intensify the total alcohol production in batch and continuous fermentations to increase the titer and productivity of the solvents. The use of SEW spent liquor from spruce chips for the production of IBE as described here will help to promote the biorefinery concept. Genetically modified clostridium strains capable of producing isopropanol could be used in industrial production of “green” chemicals from renewable and sustainable resources, such as lignocellulosic biomass.

2. Materials and methods

2.1. Materials

All the nutritional components required for the fermentation were purchased from the local vendors from Finland \cite{33}. PCR amplification was done with Phusion Flash High-Fidelity
PCR Master Mix (Thermo Fisher Scientific Inc., USA). Restriction (FastDigest) and ligation enzymes for DNA cloning were obtained from Thermo Fisher Scientific Inc., USA. Vector pUC18 was from Thermo Fisher Scientific Inc., (USA). Oligonucleotides for the cloning were synthesized by Oligomer Oy (Finland). QIAquick PCR Purification Kit (QIAGEN, USA) was used for purification of the products after PCR reaction and Wizard® Genomic DNA Purification Kit (Promega, USA) for the isolation of genomic DNA. One Shot® TOP10 Electrocomp™ *Escherichia coli* competent cells were from Invitrogen (USA). Plasmids pMTL-JH16 and pAN2 were obtained from Prof. Nigel Minton, The University of Nottingham, University Park, Nottingham, NG7 2RD, UK. The SO$_2$–water–ethanol (SEW) spent liquor was obtained from the Department of Forest Products Technology, School of Chemical Technology, Aalto University, Finland.

### 2.2. Microorganism and medium

*C. beijerinckii* NRRL B593 (synonym: DSM6423) and *C. acetobutylicum* DSM792 (synonym: ATCC 824) strains were procured from DSMZ (Germany). The cultures were maintained on a reinforced clostridia medium (RCM) as explained earlier [34]. The production medium reported by Tripathi et al. [35] was modified to use a sugar mixture as a carbon source containing glucose, mannose, arabinose, galactose, and xylose as a replacement to sole glucose (60 g L$^{-1}$). Other medium components were (in g L$^{-1}$), magnesium sulfate 0.2, sodium chloride 0.01, manganese sulfate 0.01, iron sulfate 0.01, potassium dihydrogen phosphate 0.5, potassium hydrogen phosphate 0.5, ammonium acetate 2.2, biotin 0.01, thiamin 0.1, and p-aminobenzoic acid 0.1. Sugar mixture contained (g L$^{-1}$) glucose 32.73, mannose 14.78, arabinose 2.14, galactose 3.95, and xylose 6.41 to get total sugars to be 60 g L$^{-1}$. The medium was adjusted to pH 6.5 with HCl, if necessary and purged with nitrogen and autoclaved at 203.4 kPa (121 °C) for 20 min.
2.3. SEW spent liquor preparation and conditioning

The SEW spent liquor was produced and conditioned as reported by Sklavounos et al. [25]. The fractionation of spruce wood chips was carried out by using SEW method and the spent liquor obtained from it was processed further. The spent SEW liquor was further conditioned with evaporation, steam stripping, liming and catalytic oxidation to be directly used for the fermentation. The concentrations of fermentation inhibitors including acetic acid, formic acid, furfural and hydroxy methyl furfural (HMF) were below the lethal level for clostridia [25]. The pH of spent liquor was finally adjusted to 6.5 with Ca(OH)\(_2\) before the addition of medium components. The total sugar concentration in final liquor was approximately 43.65 g.l\(^{-1}\). The individual sugar concentrations were (in g.l\(^{-1}\)) glucose 12.4, mannose 16.88, galactose 4.18, arabinose 2.95 and xylose 7.24. This liquor was diluted to 4 times to further reduce the inhibitory components and was supplemented with other nutritional components as detailed in previous section 2.2. to make final sugar concentration to be 60 g.l\(^{-1}\).

2.4. Bacterial plasmids and maintenance

Actively growing cells of *C. beijerinckii* after overnight incubation were transferred (5% v/v) into larger volume of RCM medium to produce higher biomass for DNA isolation. Clostridial cells were harvested after 24 h by centrifugation (4500×g, 10 min) and further used for DNA extraction by Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). *C. acetobutylicum* DSM792 aliquots were activated and inoculated (2% v/v) under anaerobic conditions into MSS media [36].

Transformants of *E.coli* were grown aerobically in Luria–Bertani (LB) medium at 37°C. The media were supplemented with antibiotics as required in the following concentrations: for *C. acetobutylicum*, erythromycin (40 µg.ml\(^{-1}\)) and thiamphenicol (15 µg.ml\(^{-1}\)); for *E.coli*, ampicillin (100 µg.ml\(^{-1}\)), tetracycline (10 µg.ml\(^{-1}\)) and chloramphenicol (25 µg.ml\(^{-1}\)). Plasmid DNA
was isolated from *E. coli* and *C. acetobutylicum* transformants using QIAprep Spin Miniprep Kit (QIAGEN, USA).

### 2.4.1. Plasmid construction

The plasmids and primers used in this study are shown in Table 1 and Table 2. For the pMTL-JH16-ADH construction first the *adc* terminator and promoter of the *C. acetobutylicum* were cloned into plasmid pUC18 creating pUC-adcP-adcT plasmid. For this the fragment containing an EagI site and the *adc* terminator region was created by annealing primers P01 and P02. To obtain plasmid pUC-adcT the fragment was BamHI-EcoRI digested, ligated with BamHI-EcoRI digested pUC18 and transformed into *E.coli* TOP10. Further, the *adc* promoter region was amplified from the *C. acetobutylicum* genomic DNA using primers P03 and P04 and purified with Qiagen PCR purification kit. The PCR product and pUC-adcT were digested with PstI-BamHI, ligated and transformed into *E.coli* TOP10 to obtain plasmid pUC-adcP-adcT. Then, the *adh* gene was amplified from *C. beijerinckii* NRRL B-593 genomic DNA using P05 and P06 as primers. The PCR product was digested with BamHI-EagI, ligated with BamHI-EagI digested plasmid pUC-adcP-adcT and transformed into *E.coli* TOP10 to obtain pADH. On further step, using the primers P07 and P08, the ‘*adc* promoter-*adh*-*adc* terminator’ fragment was amplified from pADH, digested with NotI-NheI, ligated with digested with NotI-NheI plasmid pMTL-JH16 [32] and transformed into *E.coli* TOP10 to obtain pMTL-JH16-ADH.

### 2.4.2. Transformation and integration procedures

Plasmid pMTL-JH16-ADH was methylated by the methyltransferase from pAN2 [37] in *E. coli* TOP10 prior to transformation of *C. acetobutylicum* DSM 792 as described before [38]. *C. acetobutylicum* DSM792 was grown in MSS media until the exponential growth stage (OD 1.2 for about 4 hours). All manipulations were carried out in a specially constructed anaerobic chamber equipped with a mini-centrifuge, heating block and electroporation device. The cells were
centrifuged at 4.5×g for 10 min at 4°C and washed in cold electroporation buffer (EPB) (270mM sucrose 15 ml; of 686mM NaH₂PO₄ 110µL (pH 7.4)). The cells were centrifuged further and re-suspended in 1.5 ml of cold EPB. The cells (500 µl) were mixed subsequently with DNA (0.8 µg) in 50µl of water and transferred into cold 0.4 cm gap electroporation cuvette which was chilled for 1 min. After the pulse was applied (600 Ω, 1.8 kV, 50µF) [39] the cells were diluted with 1 ml of warm MSS medium and incubated for 4h at 37°C for outgrowth. Aliquots of 100 µl were spread on MSS plates supplemented with 15 µg.ml⁻¹ thiamphenicol to select the plasmid-borne resistance marker catP. Plates were incubated anaerobically for 2-3 days at 37 °C. The clones were transferred to MSS plates supplemented with 40 µg.ml⁻¹ erythromycin in order to specifically select double-crossover clones [32].

2.5. Batch experiments

The preliminary experiments to check the growth and production profile with the modified strain were carried out as batch experiments in 125 ml screw cap bottles with 50 ml standard production medium. The medium was inoculated (5% v/v) with 20 h old actively growing seed culture and incubated at 37 °C for 210 h. The samples were taken after every 12 h and analyzed for the solvent production and sugar utilization.

The batch experiments in the bioreactor were also carried out under controlled pH and zero oxygen conditions. These experiments with both pure glucose and sugar mixture as a carbon source were carried out with otherwise standard production medium to study the behavior of the modified strain and its solvent production abilities. The samples were analyzed after every 12 h for the solvent production and sugar utilization. All the experiments were carried out at least in triplicates and the results reported are the average of them.
2.6. Continuous fermentation

In order to improve the solvent productivity and reduce the operational cost of the process, the continuous production of IBE solvents are desirable. Chemostat cultivations were carried out in 1 L jacketed glass bioreactor with a 500 mL fermentation volume. The bioreactor was inoculated with 10 % inoculum of highly motile cells of C. acetobutylicum DSM792-ADH. After cultivating the cells in batch mode for 24 h, the fermentation feed medium was continuously pumped into the bioreactor at different dilution rates. Dilution rates were varied from 0.01 h$^{-1}$ to 0.14 h$^{-1}$ with reactor temperature of 37 °C. During the course of fermentation, samples were collected at regular intervals and analyzed for biomass, acetone, isopropanol, butanol, ethanol, residual sugar and acids. Unless otherwise stated, all the continuous fermentations were carried out in duplicates and results reported are the average of two fermentations. Samples for biomass and product analysis were taken after five reactor volumes for three consecutive days at steady state condition. The steady state was confirmed by stable biomass and product concentrations at a specific dilution rate. The continuous fermentations were carried out in three sets of experiments viz. glucose as a carbon source, sugar mixture as a carbon source and SEW spent liquor as a carbon source in addition to the standard production medium [33,34].

2.7. Determination of substrates and products

The solvents (n-butanol, isopropanol, acetone and ethanol) and acids (acetic acid and butyric acid) were quantified by gas chromatography (Hewlett Packard series 6890) [26]. Glucose, mannose, arabinose, galactose, and xylose were determined by high-performance liquid chromatography (Bio-Rad Laboratories, Richmond, CA), equipped with an Inores S 259-H column (Inovex, Vienna, Austria) packed with Inores cation exchanger (particle size, 9 µm) [33]. The bioprocess parameters including the solvent productivity and yield were calculated as explained earlier [33,34].
3. Results and discussion

Metabolic engineering of the *C. acetobutylicum* DSM792 strain was carried out with an aim to convert acetone to isopropanol, and consequently produce mixed fuel alcohols. The intracellular conversion of acetone into isopropanol is an attractive alternative to avoid acetone excretion to produce a valuable alcohol. As genetic tools are available for *C. acetobutylicum* ATCC824 [40,41], there is a strong interest to transform this native and industrially recognized ABE producer, into an efficient IBE producer. Although some *C. beijerinckii* strains reduce acetone naturally with an endogenous *adh* gene, the final titers of the solvents have been lower than those of the best ABE producers [23,29,42].

3.1. Introduction of *adh* gene into *C. acetobutylicum*

*C. acetobutylicum* being an industrial organism producing commercial biofuels such as butanol, demands robust fermentation conditions and strains for a feasible and economic bioprocess. The use of genetic engineering tools to enhance the yield, specificity and feedstock utilization in the fermentation process is an attractive way to improve its feasibility.

Traditional introduction of *adh* gene into *C. acetobutylicum* using replicative plasmids [9,43,44] was tried (unpublished data) and found not promising from economics and industrial point of view because of major additional cost of antibiotics required in the growth media and inherently unstable nature of the plasmid carrying the *adh* gene. Hence, there was a need to opt for an economic process with a more effective genetically engineered microorganism. Allele-coupled exchange approach as a novel DNA integration method into a chromosome was tried with *C. acetobutylicum* to enhance the solvent titer and productivity. As a result, the modified *C. acetobutylicum* DSM792-ADH was constructed and tested on new SEW spent liquor and sugar mixture during batch and continuous experiments.
3.2. IBE production in batch fermentation

The preliminary batch experiments containing pure glucose as a carbon source in stationary flask culture were performed to observe the microbial behavior during the fermentation. The time course of batch fermentation (Fig. 1) showed that the solvent production started at the end of the exponential phase. The flask culture resulted in maximum level of total solvents 15 g.l⁻¹ after 144 h. At this time interval the amount of isopropanol observed was 2.51 g.l⁻¹. The production profile of acetone, isopropanol, ethanol and butanol can be seen in Fig. 1. The flask culture utilized 70.0 % of the sugars and yielded 0.34 g.g⁻¹ of total solvents (supplementary Fig. 1; Table 1). Although, the acid production and solvent ratio of IBE with remaining acetone formation during the flask culture were close to standard behavior (supplementary Table 2), the lower total solvent titers recommended to use controlled batch fermentation in a bioreactor and continuous fermentation as optional approaches. The time course of batch fermentation in a controlled bioreactor can also be observed in Fig. 1. When using glucose and the sugar mixture as the substrate for the fermentation with *C. acetobutylicum* DSM792-ADH the growth and production profiles were similar to those with *Clostridium* wild type strains [34,45]. The batch fermentation in the controlled bioreactor with medium containing pure glucose resulted in the maximum total solvent titer to be 18 g.l⁻¹ with 2.51 g.l⁻¹ and 10.78 g.l⁻¹ isopropanol and butanol respectively after 72 h fermentation with no further significant increase thereafter (Fig. 1). Almost 81 % of the glucose was utilized after 72 h fermentation with the total solvent yield of 0.37 g.g⁻¹. The highest total solvent titer with the sugar mixture as a substrate in the controlled bioreactor was 15.18 g.l⁻¹ after 72 h fermentation and resulted in total solvent yield of 0.30 g.g⁻¹. *C. acetobutylicum* DSM792-ADH could utilize both pentose as well as hexose sugars as detailed in supplementary Table 2. The order of utilization of sugars was found to be – glucose>arabinose>mannose>xylose>galactose (supplementary Table 2). The conversion efficiency (with respect to acetone) during controlled batch fermentation, with glucose and the sugar mixture as a substrate was found to be almost similar (~50%). Moreover, the
fermentation profile and performance of the *C. acetobutylicum* DSM792-ADH were similar to wild
type strains of *C. acetobutylicum* DSM792 and *C. beijerinckii* DSM 6423 [23,26,34,45]. The results
indicated that *C. acetobutylicum* DSM792-ADH could successfully utilize hexose and pentose
sugars during the fermentation and can be best suited for the use in biorefinery processes. A
behavioral pattern of the modified strain encouraged us to perform the continuous fermentation
studies with SEW spent liquor as a substrate.

3.3. IBE production in continuous fermentation

The use of SEW spent liquor for the production of ABE solvents have been studied
previously [26]. SEW pulping is a fractionation process for lignocellulosics to fractionate them into
pulp, lignin and monomeric hemicellulose sugar mixtures for further production of value-added
chemicals [25]. The limitations of batch fermentation such as low productivity and high solvent
toxicity were expected to be partly overcome with continuous fermentation of *C. acetobutylicum*
DSM792-ADH. Three independent continuous experiments were carried out with pure glucose,
sugar mixture and SEW liquor as a substrate to produce AIBE (acetone:isopropanol:butanol:ethanol). The effect of different dilution rates on IBE production is
shown in Fig. 2. The concentrations of solvents and acids were decreased with increase in dilution
rate in all the three experiments as expected because of increasing share of unconsumed
monosaccharides. The isopropanol concentration at a dilution rate of 0.01 h⁻¹ was found to be 1.5
g.l⁻¹ and 1.3 g.l⁻¹ with pure glucose and the sugar mixture as a substrate. Since the modified strain
have already proved its capability to use sugar mixture for the production of solvents, the studies
with SEW liquor were also carried out to confirm its suitability of to the continuous fermentation
process. *C. acetobutylicum* DSM792-ADH could utilize the sugars (64.05 %) from SEW liquor and
produced total solvents to the concentration of 6.7 g.l⁻¹ with 0.9 g.l⁻¹ isopropanol at the dilution rate
of 0.01 h⁻¹ (Fig. 2). The results obtained with *C. acetobutylicum* DSM792-ADH are in accordance
with the results obtained with the wild type isopropanol producing strain *C. beijerinckii* DSM 6423
[23]. The other solvent titers as well as solvent yield and productivities with SEW liquor were
found to be lower than those with pure glucose and the sugar mixture as a substrate (Fig. 2, Fig. 3
and Fig. 4). A general trend of increase in solvent productivity with increase in dilution rate up to
0.075 h\(^{-1}\) followed by decrease in productivity with higher dilution rates, was observed with all the
fermentation experiment. The highest solvent yield with pure glucose, sugar mixture and SEW
liquor as a substrate, with continuous fermentation were found to be 0.39 g.g\(^{-1}\), 0.29 g.g\(^{-1}\) and 0.17
g.g\(^{-1}\) respectively (Fig. 4). The highest solvent yield, when glucose was used as a substrate in flask
cultivation, controlled batch fermentation and continuous fermentation were found to be 0.34 g.g\(^{-1}\),
0.36 g.g\(^{-1}\), 0.39 g.g\(^{-1}\) respectively. This confirms that the solvent yield based on substrate
consumption did not depend on the genetic modifications, but rather on the physiological conditions
of the culture. Interestingly the solvent ratio (acetone:isopropanol:butanol:ethanol) remained
consistent with all the three continuous experiments as with the wild type strain except the decrease
in acetone concentration (Table 3). Table 3 also depicts the percent conversion of acetone into
isopropanol during the continuous fermentation of *C. acetobutylicum* DSM792-ADH. Acid
assimilation was also comparable in the modified culture, to improve the C3 compound (acetone or
isopropanol) in the solvents. The conversion of acetone with pure glucose as a substrate was
observed to be in the range between 45 – 50 %. When the sugar mixture was used as substrate the
percentage conversion decreased slightly with increase in the dilution rate and varied in between 40
– 50 %. Besides, the percentage acetone conversion with SEW liquor as a substrate was further
reduced, and observed in the range between 28 – 43 %. Table 3 clearly depicts that, the solvent
conversion ratio remains stable at around 40 – 50 % when all the favorable conditions produced
higher solvent amounts. As the dilution rate increased, the concentration of total solvents was found
to be decreased, which disturbs the standard ratio of solvents (acetone:isopropanol:butanol:ethanol)
as well as the percentage conversion of acetone into isopropanol. The disturbance in conversion
efficiency of the modified strain from acetone into isopropanol was probably due to the imbalance in cofactor regeneration for the secondary alcohol dehydrogenase [30].

A study to develop an engineered strain suitable for IBE production by introducing a primary/secondary alcohol dehydrogenase from *C. beijerinckii* NRRL B-593 has been tried recently by several researchers [9,32,44]. The performance of the modified strain *C. acetobutylicum* DSM792-ADH was better than that of a natural IBE producer (*C. beijerinckii*) as detailed in a previous study [23,45]. Although the complete conversion of acetone into isopropanol was not achieved in the present study, the conversion up to 50% was in accordance with the other reports [46,47]. The incomplete conversion of acetone into isopropanol with the modified strain is probably due to the cofactor imbalance, as the secondary alcohol dehydrogenase is NADPH dependent [30]. Further increase in IBE yield and productivities were observed with advanced bioprocessing such as use of immobilized column reactors [48].

4. Conclusions

The fuel alcohol yield with clostridia can be improved by converting acetone to isopropanol, and producing mixed IBE instead of ABE. The exclusive production of fuel alcohols in fermentation broth can significantly reduce the downstream processing cost. Hence, a modified *C. acetobutylicum* strain was developed to produce IBE with improved titer and yield. The *adh* gene from *C. beijerinckii* NRRL B593 was constitutively expressed in *C. acetobutylicum* DSM 792 to convert acetone into isopropanol. The efficient consumption of glucose in batch and continuous fermentation without strain degeneration suggests that the engineered strain can be used for long-term fermentation. Moreover, the effective use of sugar mixture and SEW liquor by *C. acetobutylicum* DSM792-ADH made it suitable for a cost effective industrial application. The modified strain converted almost 50% of the acetone into isopropanol with higher solvent yields. However, the complete conversion of acetone into isopropanol was not achieved in this study. Since
the secondary alcohol dehydrogenase is NADPH dependent enzyme, increased availability of the cofactor may improve the conversion efficiency. Moreover, the use of advanced bioprocesses for IBE solvent production such as the use of immobilized column technique and *in-situ* solvent recovery module can further improve the yield as well as productivity of the process.

### 5. Acknowledgments

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### 6. References


**Figure legends**

**Figure 1** The solvents produced in the flask cultivation and controlled batch fermentation with *C. acetobutylicum* DSM 792-ADH

**Figure 2** The solvent production with respect to dilution rate in continuous fermentations of *C. acetobutylicum* DSM 792-ADH with glucose as a substrate (a); sugar mixture as a substrate (b); and SEW spent liquor as a substrate (c)

**Figure 3** Total sugars consumed and total solvents produced in a continuous culture of *C. acetobutylicum* DSM 792-ADH

**Figure 4** Overall solvent productivity and solvent yield in a continuous culture of *C. acetobutylicum* DSM 792-ADH
Table 1 List of strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<td>DSMZ⁷</td>
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<tr>
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<tr>
<td>pUC-ADC-P-ADC18</td>
<td>pUC-ADC-P-ADC18 derivative with adh gene under adc promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pMQL-JH16-ADH</td>
<td>pMQL-JH16 derivative containing adc promoter, adh gene and adc terminator</td>
<td>This study</td>
</tr>
</tbody>
</table>

⁴ Abbreviations: Ap⁴, ampicillin resistant; Cm⁴, chloramphenicol resistant; adh, a primary/secondary alcohol dehydrogenase gene; ermB, macrolide–lincosamide–streptogramin (MLS) antibiotic-resistance marker; MCS, multiple cloning site; adc, acetoacetate decarboxylase gene from *C. acetobutylicum*.

⁵ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of microorganisms and Cell Cultures), Germany.

⁶ Life Technologies Corporation, Invitrogen, USA.

⁷ The University of Nottingham, University Park, Nottingham, NG7 2RD, UK.
Table 2 List of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence from 5’to 3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P01</td>
<td>GATCCACTACGGCCGTAAAAATAAGAGTTACCTTAAAT GGTAACTCTTTATTTTTTAATGC</td>
<td>[9]</td>
</tr>
<tr>
<td>P02</td>
<td>AATTGCATTTAAAAAAATAAGAGTTACCATTAAAGGTAA CTCTTATTTTTACGGCCGTAGTG</td>
<td>[9]</td>
</tr>
<tr>
<td>P03</td>
<td>ATATCTGCAGAAGTGTACTTTTATTTTCGAAAGC</td>
<td>[9]</td>
</tr>
<tr>
<td>P04</td>
<td>ATATGGATCCTAATAATGTTTAGCTTTTCTAACAT</td>
<td>[9]</td>
</tr>
<tr>
<td>P05</td>
<td>ATATGGATCCTAAGGAGGAACATATTTTATGGAAG</td>
<td>[9]</td>
</tr>
<tr>
<td>P06</td>
<td>ATATCGGCCGTTATAATATAACTACTGCTTTAAATTA</td>
<td>[9]</td>
</tr>
<tr>
<td>P07</td>
<td>ATGGCGGCCGCTTCGCTATTACG</td>
<td>This study</td>
</tr>
<tr>
<td>P08</td>
<td>ATATGCTAGCGGAATTGTAGCGGATAAC</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3 Solvent ratio and percent acetone conversion into isopropanol during the continuous fermentation of *C. acetobutylicum* DSM 792-ADH

<table>
<thead>
<tr>
<th>DR (h⁻¹)</th>
<th>Pure glucose</th>
<th>Sugar mixture</th>
<th>SEW liquor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.010</td>
<td>1.5:1.4:6.1:1.0 (47.803)</td>
<td>1.6:1.4:6.0:1.0 (46.737)</td>
<td>1.8:1.3:5.9:0.9 (43.209)</td>
</tr>
<tr>
<td>0.025</td>
<td>1.4:1.4:6.2:1.0 (49.958)</td>
<td>1.5:1.5:6.2:0.9 (49.877)</td>
<td>1.9:1.3:6.0:0.9 (40.991)</td>
</tr>
<tr>
<td>0.050</td>
<td>1.4:1.3:6.3:1.0 (48.014)</td>
<td>1.4:1.3:6.3:0.9 (48.181)</td>
<td>1.8:1.3:6.0:1.0 (40.707)</td>
</tr>
<tr>
<td>0.075</td>
<td>1.5:1.3:6.1:1.0 (46.595)</td>
<td>1.7:1.4:6.0:0.9 (45.900)</td>
<td>1.8:1.1:6.2:1.0 (37.035)</td>
</tr>
<tr>
<td>0.100</td>
<td>1.6:1.3:6.1:1.0 (44.709)</td>
<td>2.0:1.3:5.9:0.9 (39.562)</td>
<td>1.8:0.7:6.2:1.2 (28.555)</td>
</tr>
<tr>
<td>0.120</td>
<td>1.7:1.4:6.1:0.8 (45.219)</td>
<td>2.1:1.4:5.8:0.6 (40.307)</td>
<td>-</td>
</tr>
<tr>
<td>0.140</td>
<td>1.8:1.5:5.8:0.9 (45.303)</td>
<td>2.3:1.4:6.1:2.0 (37.282)</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1 The solvents produced in the flask cultivation and controlled batch fermentation with C. acetobutylicum DSM 792-ADH.
Figure 2 The solvent production with respect to dilution rate in continuous fermentations of C. acetobutylicum DSM 792-ADH with glucose as a substrate (a); sugar mixture as a substrate (b); and SEW spent liquor as a substrate (c).
Figure 3 Total sugars consumed and total solvents produced in a continuous culture of *C. acetobutylicum* DSM 792-ADH
Figure 4 Overall solvent productivity and solvent yield in a continuous culture of *C. acetobutylicum* DSM 792-ADH