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Differential Solvent Production by *Clostridium beijerinckii* NRRL-B593 Grown on Carbohydrates and Peptides

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Abstract

Several *Clostridium* species are well known for their Acetone-Butanol-Ethanol (ABE) fermentation. *Clostridium beijerinckii* NRRL-B593 is an obligately anaerobic and spore-forming bacterium possessing the ability to produce isopropanol due to the presence of a primary/secondary alcohol dehydrogenase (ADH) catalyzing the reduction of acetone. The production of butanol, isopropanol, and ethanol by *C. beijercinkii* NRRL-B593 using sugars is well studied, however, it is not clear whether peptides can support growth and be utilized for the production of these solvents. *C. beijerinckii* NRRL-B593 was grown on four different types of media: tryptone, sucrose, dextrose, and mixed (tryptone, dextrose, yeast extract) to determine the effects. Growth and end-product production were highest for *C. beijerinckii* NRRL-B593 grown on mixed media. Ethanol was produced on all types of media, while only mixed and sucrose (or dextrose) media supported solvent production for butanol, acetone, and isopropanol. ADH activities corroborated this data as mixed media resulted in the highest enzyme activity. These results suggest that *C. beijerinckii* NRRL-B593 can only utilize carbohydrates to produce the highest amount of solvents, and peptide substrates do not support the production of butanol, acetone, and isopropanol.

Introduction

Solvents, which are mainly petroleum based [1], are commonly used substances for a plethora of applications ranging from house-hold uses (ie. detergents) to more industrial purposes (ie. chemical synthesis). With the growing concerns of utilizing petrochemicals and the consequences they put onto the environment, more research efforts have been focused on using sustainable renewable sources, namely, microbial based sources to combat such impacts [1]. The use of microorganisms is quite versatile in that the type of end-products that are produced, including solvents and biofuel, can vary depending on the microorganism and type of substrate used [2]. Most microbes can readily convert sugars, starches, and fats to solvents. *Escherichia coli*, for example, is used for the production of solvents for biofuel from biomaterials, with reports showing 30 and 25 g/L of butanol and ethanol produced, respectively [2]. Substrates can range from a variety of sources including plant- and animal-based biomass. The metabolic pathway(s) involved are also important in determining which end-product(s) will be produced. *Saccharomyces cerevisiae* is a commonly used ethanol-producing eukaryotic microorganism, whose pathway involves the decarboxylation of pyruvate to produce ethanol [3]. Clostridia are a group of anaerobic bacteria capable of producing solvents in large quantities consisting of a few representative species including *Clostridium acetobutylicum*, *Clostridium pasteuranium,* and *Clostridium beijerinckii*, among others [4]. This group is known for their solvent producing capabilities particularly in acetone, butanol, and ethanol production using the butyrate and butanol-acetone fermentation pathway (a pathway which is well-studied in *C. acetobutylicum* [5]). These solventogenic clostridia are able to utilize carbohydrates as their main carbon and energy sources to grow and produce solvents, however, it is unclear whether proteinaceous materials can be used as substrates for solvent production or not.

C. beijerinckii NRRL-B593 is a particular solvent producing bacterium that is able to produce isopropanol in addition to butanol and ethanol (IBE) - where most clostridia are unable to produce isopropanol but rather acetone [6]. For industrial biofuel production, isopropanol is more desired as acetone is corrosive to parts of engines made up of rubber or plastic [7]. *C. beijerinckii* NRRL-B593 can produce solvents from various carbohydrate sources, however, it is unclear whether it is able to utilize peptides to produce solvent using the same butyrate and butanol-acetone fermentation pathway.

In this study, *C. beijerinckii* NRRL-B593 was grown on both carbohydrates and peptides. *C. beijerinckii* NRRL-B593's growth, production of solvents, and production of hydrogen were compared to determine whether it is able to produce solvents using peptides or not.

Materials and Methods

Organism and Chemicals

C. beijerinckii NRRL-B593 from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany was used for this study. Spore suspensions of *C. beijerinckii* NRRL-B593 were also prepared and stored at -80 ˚C until use [17]. All chemicals were commercially available.

Growth Conditions

C. beijerinckii NRRL-B593 was cultured in media adjusted to pH 7.0 and grown in an anaerobic serum bottle at 37 ˚C. All media contained a salt solution, which was prepared according to DSMZ (Germany). The salt solution contained the following (in 1 L unless otherwise specified): 0.01 g calcium chloride, 0.02 g magnesium sulfate, 0.04 g dipotassium phosphate, 0.16 g sodium bicarbonate, 0.08 g sodium chloride. The basal media contained (in 1 L unless otherwise specified): 5.0 g yeast extract, 40 mL salt solution (as described above), 0.001 g resazurin, 4.0 g ammonium acetate, 0.5 g L-cysteine-HCl, 0.01 g biotin, and 0.01 g p-aminobenzoic acid. Peptide sources added to the basal media included: beef extract, beef peptone, wheat peptone, casein, tryptone powder (to a final concentration of 5 g/L).

Four different media compositions were used for this experiment (in 1 L unless otherwise specified): 1) mixed media: 5 g tryptone, 10 g yeast extract, 5 g dextrose (or sucrose), 40 ml salt solution, 1 mg resazurin, 0.5 g L-cysteine-HCl; 2) tryptone media: 20 g tryptone, 40 ml salt solution, 1 mg resazurin, 0.5 g L-cysteine-HCl, 4 g ammonium acetate, 0.268 g L-alanine, 0.162 g L-isoleucine, 0.189 g L-valine, 45 mg vitamin B_1, 45 mg vitamin B_6, 0.2 mg folic acid; 3) sucrose media: 60 g sucrose, 40 ml salt solution, 1 mg resazurin, 0.5 g L-cysteine-HCl, 4 g ammonium acetate, 0.268 g L-alanine, 0.162 g L-isoleucine, 0.189 g L-valine, 45 mg vitamin B_1, 45 mg vitamin B_6, 0.2 mg folic acid; and 4) dextrose media: 60 g dextrose, 40 ml salt solution, 1 mg resazurin, 0.5 g L-cysteine-HCl, 4 g ammonium acetate, 0.268 g L-alanine, 0.162 g L-isoleucine, 0.189 g L-valine, 45 mg vitamin B_1, 45 mg vitamin B_6, 0.2 mg folic acid.

Batch Fermentation

After autoclaving, serum bottles containing media were sealed with a sterile gray butyl stopper (20 mm, Fisher Scientific, Ontario) and crimped with an aluminum seal (20 mm CS/M, Fisher Scientific, Ontario). Bottles were then made anaerobic with a manifold system. Batch fermentations were carried out in 160 mL serum bottles with an overpressure of 3 psi nitrogen gas without agitation. Culture bottles were inoculated using either a *C. beijerinckii* NRRL-B593 seed culture (grown to late-log phase) or a *C. beijerinckii* NRRL-B593 spore suspension.

Analyses of Fermentation Products

Fermentation products including butanol, ethanol, acetone, and isopropanol were measured using a Shimadzu GC-14A Gas Chromatograph (GC) equipped with two connected capillary columns (DB-624 and DB-5) and a Flame Ionization Detector (FID). GC analyses were performed under the following conditions: helium, 50 psi; hydrogen, 30 psi; air, 10 psi; injector temperature, 200 ˚C; detector temperature: 250 ˚C. The temperature program was the following: initial temperature of 35 ˚C, held for 10 minutes; program rate: 2 ˚C/min to 45 ˚C and 40 ˚C/min to 150 ˚C (hold for 1 min). The sample (1 µl) was directly applied to the injector for GC analyses. Peaks were identified using external standards and peak areas were calculated by integration using Peak Simple software (SRI Instruments, California, USA).

Hydrogen Detection

The GC-Buck Model 910 GC equipped with a Supelco 60/80 molecular sieve 5A column and a Thermal Conductivity Detector (TCD) was used for hydrogen detection. The carrier gas was nitrogen, and pressure was 11 psi (~76 kPa), and flow rate was 16 ml min^{-1}. The injector, TCD and column temperatures were set to 110, 100, and 60 °C, respectively. A standard curve was prepared for the determination of hydrogen produced. Peak Simple was used as the integrating software (SRI Instruments, USA).

Preparation of Cell-Free Extract

Frozen cells of *C. beijerinckii* NRRL-B593 (1 g) were re-suspended anaerobically in 5 ml of 6 mM Tris/HCl buffer (pH 7.5) containing 2 mM sodium dithionite (SDT), 2 mM dithiothreitol (DTT), and 5% (v/v) glycerol. French press was used to lyse cells in lysing buffer then, the supernatant was collected as cell-free extract after 10 min of centrifugation (10,000 x g).

Protein and Enzyme Assays

Protein content was determined using the Bradford method [8] and bovine serum albumin was used as the standard. Alcohol dehydrogenase (ADH) activity was measured by monitoring the acetone-dependent absorbance change of NADPH at 340 nm at 45 ˚C (ε_{340} = 6.3 $mM^{-1}cm^{-1}$, acetone + NADPH + $H^+ \rightarrow NADP^+$ + H_2O + 2-propanol). The assay mixture (2 ml) contained 100 mM EPPS (pH 8.5) and 30 mM acetone, and the reaction started by the addition of the enzyme. One unit of ADH activity is defined as 1 µmol of NADPH formed or oxidized per min.

Results and Discussion

C. beijerinckii NRRL-B593 was able to grow on all types of media prepared (Figure 1). Either dextrose or sucrose (data not shown) supported the highest growth, followed by mixed media, and lastly tryptone. Similar growth curve patterns were shown on mixed and tryptone media. Both mixed and tryptone media supported a much faster growth compared to that on dextrose (or sucrose) media (Figure 1). The slowest growth on either dextrose or sucrose may be a result from the supplement with vitamins only. There are likely other factors present in yeast extract or tryptone that are required for its fast growth (Figure 1). The result also showed that peptides alone could only support its growth to a lower cell density. This observation is similar to what was noted by Mead, which was that saccharolytic clostridia showed little to no growth on 3% casein hydrolysate [9]. It was also observed in those studies that there was an increase in growth proportional to the concentration of glucose added [9].

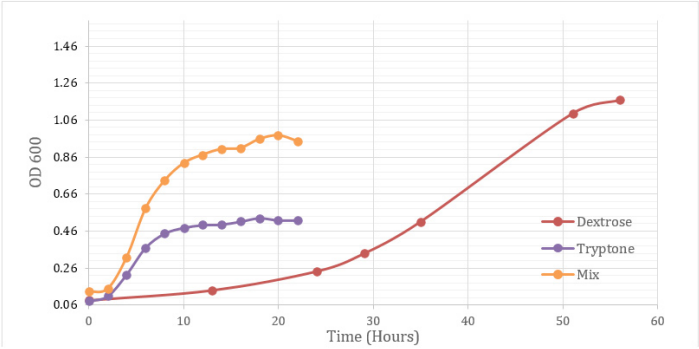

Figure 1: Growth of *C. beijerinckii* NRRL-B593 on three different media types (dextrose, tryptone, and mix, respectively). Growth was monitored and measured using a Genesys 10UV Spectrophotometer at 600 nm. Similar results were obtained for both non-spore culture and spore cultures of *C. beijerinckii* NRRL-B593 however, only non-spore culture data is presented here.

Growth of *C. beijerinckii* NRRL-B593 on tryptone and mixed media, respectively, was further extended to determine whether varying substrate concentrations influenced growth. In samples that varied dextrose concentrations in mix media (30 g/L - Mix30 media, 60 g/L - Mix60 media), no significant variation in growth was visible (Figure 2). It is possible that *C. beijerinckii* NRRL-B593 cells did not utilize all the glucose provided, suggesting possible repressive effects by a component in the media causing cells to reach stationary phase and eventually sporulating. A similar trend was observed for cells grown on tryptone. In samples with 15, 20, and 25 g/L tryptone concentrations, respectively, *C. beijerinckii* NRRL-B593 displayed similar growth patterns (Figure 2). The results indicate growth is not proportional to the amount of substrate added under the growth conditions specified.

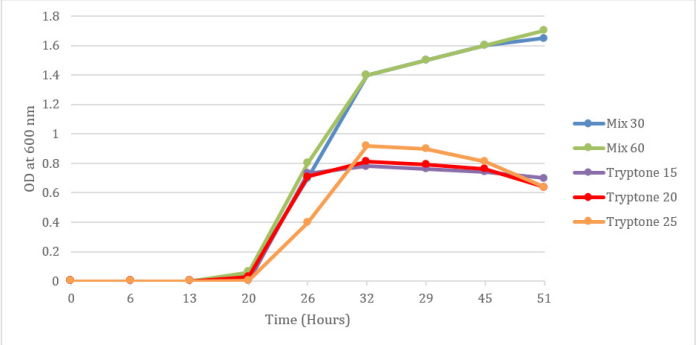

Figure 2: Growth of *C. beijerinckii* NRRL-B593 on two different media (mix and tryptone, respectively) at various concentrations. Growth was monitored and measured using a Genesys 10UV Spectrophotometer at 600 nm. Both dextrose and tryptone media sources supported growth of *C. beijerinckii* NRRL-B593, with an increase in concentration resulting in an increase in growth. Both concentrations of mixed media (tryptone, sucrose, yeast extract) (30, 60 g/L) tested resulted in higher growth as compared to all concentrations of tryptone (15, 20, 25 g/L).

Solvent production by *C. beijerinckii* NRRL-B593 was determined using gas chromatography, namely, butanol, ethanol, acetone, and isopropanol production. In nearly all the solvents tested (with the exception of ethanol), *C. beijerinckii* NRRL-B593 grown in mixed media resulted in the highest solvent production overall (Figure 3). Ethanol was the only solvent produced in all three media conditions (Figure 3b), while butanol (Figure 3a), acetone (Figure 3c), and isopropanol (Figure 3d) were only produced in mixed and sucrose media, indicating that tryptone could not be used to support the production of butanol, acetone, and isopropanol. Other peptides substrates including beef extract, beef peptone, yeast extract, wheat peptone, and casein were tested, however, results show that they also could not support the production of butanol, acetone, and isopropanol. Solvent production in *C. beijerinckii* NRRL-B593 grown in a "peptone-yeast extract - glucose" medium has been previously reported to be 8 mM and 61.7 mM of isopropanol and butanol, respectively [4]. A more recent study reported that butanol and isopropanol production in *C. beijerinckii* NRRL-B593 batch culture grown on glucose was approximately 30 mM and 60 mM, respectively [10].

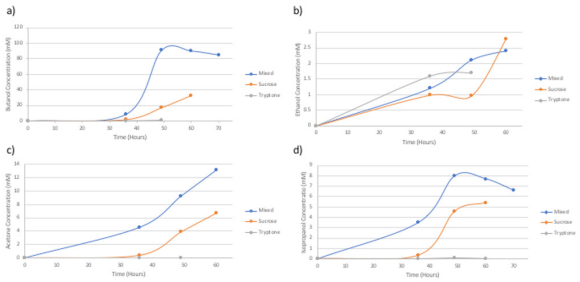

Figure 3: Production of butanol, acetone, isopropanol and ethanol by *C. beijerinckii* NRRL-B593 on three different media types (dextrose, tryptone, and mix, respectively). Production of (a) 1-butanol, (b) ethanol, (c) acetone, (d) isopropanol (in mM) detected using a Shimadzu GC 14-a equipped with an FID. Similar results were obtained for both non-spore culture and spore cultures of *C. beijerinckii* NRRL-B593, however, only non-spore culture data is presented.

Hydrogen production by *C. beijerinckii* NRRL-B593 grown on various substrates were measured (Figure 4). Its growth on dextrose media resulted in the highest hydrogen production while growth on all other peptide sources tested resulted in similar hydrogen concentrations (Figure 4). Hydrogen production in cultures grown on the basal medium and peptide supplemented provided media were similar. Out of the different peptides tested, wheat peptone supplemented media had cultures that produced the most hydrogen (2.4 ± 0.14 mM; Figure 4). This data correlates to the solvent production data obtained from GC analyses (Figure 3) where little to no solvent production was observed for culture grown on "peptide only" media. For culture grown on peptides, hydrogen production was lower compared to culture grown solely on carbohydrates (Figure 4), which is expected as it has been previously reported that the major source of hydrogen produced by fermentation is from carbohydrates [11]. Butanol production detected on samples grown on dextrose had similar concentrations as reported in literature (74 mM by [12]; 93 mM by [13]). Isopropanol on dextrose, however, had lower concentrations than reported in literature (50 mM by [12]; 53 mM by [13]).

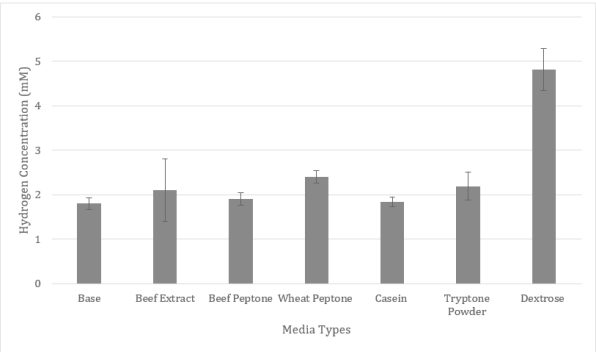

Figure 4: Hydrogen production by *C. beijerinckii* NRRL-B593 grown on various substrates. Error bars are in ± SD.

ADH activities in *C. beijerinckii* NRRL-B593 grown on three different media were determined (Table 1). Cells grown on mixed media had the highest specific activity (0.209 ± 0.052 U/mg), followed by tryptone then glucose media (0.150 ± 0.018 U/mg and 0.109 ± 0.003 U/mg, respectively). The specific activity measurements taken from *C. beijerinckii* NRRL-B593 cells grown in carbohydrate media and peptide media suggests that metabolic processes may be dependent on substrate availability. Specific activity is higher for cells grown in peptide media compared to glucose media, further suggesting that the expression of solvent-producing genes may be partially dependent on the carbon source available for *C. beijerinckii* NRRL-B593 [14].

It has been speculated that only one enzyme is present that catalyzes the production of butanol and isopropanol in *C. beijerinckii* NRRL-B593 [14]. *C. acetobutylicum* ATCC 824, contrastingly, is known to produce the same solvents with the exception of isopropanol (it instead produces acetone) [4]. ADH activity of *C. beijerinckii* NRRL-B593 grown on mixed media resulted in the highest specific activity, followed by tryptone, then glucose with the lowest activity (Table 1). Similar results were obtained for both non-spore culture and spore cultures of *C. beijerinckii* NRRL-B593, however, only non-spore culture data is presented. With supporting data from the solvent production (Figure 3) and enzymatic assay data (Table 1), it is evident that *C. beijerinckii* NRRL-B593 is unable to utilize peptide sources to produce butanol, isopropanol, and acetone. Although this organism can utilize peptides to support its growth (Figute 1), peptides that are metabolized into keto-acids (such as pyruvate) cannot be utilized to support the production of solvents (other than ethanol). Ethanol can still be produced by *C. beijerinckii* NRRL-B593 as pyruvate (a product of peptide metabolism) is a precursor to its production, which is catalyzed by a three-enzyme pathway [15].

Cells grown on media	Specific Activity (U/mg)*
Mixed	0.209 ± 0.052
Tryptone	0.150 ± 0.018
Sucrose	0.109 ± 0.003
*(value ± SD)	

Table 1: ADH activity of *C. beijerinckii* NRRL-B593 grown on three different media types (mixed, tryptone, and sucrose, respectively).

Although the pathway for butanol production from the fermentation of peptides has not yet been elucidated in *C. beijerinckii* NRRL-B593, there are pathways that are known to produce butanol in other microorganisms. In clostridia, the butanol production pathway and key enzymes involved have been extensively studied in *C. acetobutylicum* ATCC 824, an Acetone-Butanol-Ethanol (ABE) producing bacterium that grows on sugars [16]. It is thought that *C. beijerinckii* NRRL-B593 may share such a similar pathway [17]. In *C. acetobutylicum* ATCC-824, fermentation of sugars is biphasic – with the first phase being

acidogenesis and the second phase being solventogenesis [18]. Its solvent production is pH-dependent where a lower pH resulted from an accumulation of organic acids (ie. butyric and acetic acid) signals the pathway to initiate the solventogenesis phase and begin converting these acids to their corresponding alcohols (ie. butanol and acetone) [18]. Contrastingly, reports have shown solvent production by *C. beijerinckii* NRRL-B593 to be independent of pH changes [19,20], suggesting a possible rerouting of carbon and electron flow in the conversion of acid to solvent. Acetoacetyl-CoA:butyrate:CoA transferase, a key enzyme in *C. acetobutylicum* ATCC 824, allows for the production of acetone and the conversion of acetate and butyrate to ethanol and butanol, respectively [7]. The complete genome of *C. beijerinckii* NRRL-B593 has yet to be sequenced, however, its pH-independent solvent production suggests that its butyrate and butanol-acetone fermentation pathway may be regulated differently.

In non-clostridial species, alternative butanol production pathways also exist, such as the amino-acid pathway naturally present in *S. cerevisiae* which involves the catabolism of amino acids [21]. In this pathway, amino acids are converted into a keto-acid (ie. 2-ketovalerate), which is then further decarboxylated into its corresponding aldehyde (ie. butanal) and ultimately its corresponding alcohol (ie. butanol) [21]. A similar keto-acid pathway was successfully engineered into *E. coli* by Atsumi et al. [22] for the production of long-chained alcohols (ie. butanol). It is unlikely, however, that any of such butanol production pathways of non-clostridial species would be present in *C. beijerinckii* NRRL-B593.

Conclusion

The lack of solvent production by *C. beijerinckii* NRRL-B593 grown on peptide sources indicates the inability for the bacterium to utilize these sources for significant growth and, furthermore, solvent and hydrogen production solvent and hydrogen production. It appears that it is only favourable for *C. beijerinckii* NRRL-B593 to utilize peptide sources for the production of ethanol explaining the inability of butanol, acetone, and isopropanol production. This study provides clear data in helping to understand the metabolism and growth of *C. beijerinckii* NRRL-B593 on peptides. Further investigation is required to elucidate its regulatory metabolic mechanism when grown on different substrates.

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References

1. Tobiszewski M, Namieśnik J (2012) PAH diagnostic ratios for the identification of pollution emission sources. Environmental Pollution 162: 110-119.
2. Kumar R, Kumar P (2017) Future microbial applications for bioenergy production: a perspective. Frontiers in Microbiology 8: 450.
3. Liao JC, Mi L, Pontrelli S, Luo S (2016) Fuelling the future: microbial engineering for the production of sustainable biofuels. Nature Reviews Microbiology 14: 288.
4. George HA, Johnson JL, Moore WEC, Holdeman LV, Chen JS (1982) Acetone, Isopropanol, and Butanol Production by *Clostridium beijerinckii* (syn. *Clostridium butylicum)* and *Clostridium aurantibutyricum*. Applied and Environmental Microbiology 45: 1160-1163.
5. Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50: 484-524.
6. De Gérando HM, Fayolle-Guichard F, Rudant L, Millah S, Monot F, Lopez-Contreras AM (2016) Improving isopropanol tolerance and production of *Clostridium beijerinckii* DSM 6423 by random mutagenesis and genome shuffling. Appl. Microbiol. Biotechnol 100: 5427-5436.
7. Lee J, Jang YS, Choi SJ, Im JA, Song HH, Cho JH, Seung DY, Papoutsakis ET, Bennet GN, Lee SY (2012) Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. Appl. Environ. Microbiol 78: 1416-1423.
8. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72: 248-254.
9. Mead GC (1971) The amino acid fermenting Clostridia. Journal of General Microbiology 67: 47-56.
10. Survase S, Jurgens G, Heiningen A, Granstrom T (2011) Continuous production of isopropanol and butanol using *Clostridium beijerinckii* DSM 6423. Applied Microbiology and Biotechnology 91: 1305-1313.
11. Gomez-Flores M, Nakhla G, Hafez H (2017) Hydrogen production and microbial kinetics of *Clostridium termitidis* in mono-culture and co-culture with *Clostridium beijerinckii* on cellulose. AMB Express 7: 84.
12. De Gérando HM, Wasels F, Bisson A, Clement B, Bidard F, Jourdier E, Lopez-Contreras AM, Lopes Ferreira N (2018) Genome and transcriptome of the natural isopropanol producer *Clostridium beijerinckii* DSM-6423. BMC genomics 19: 242.
13. De Vrije T, Budde M, van der Wal H, Claassen PA, López-Contreras AM (2013) In situ removal of isopropanol, butanol and ethanol from fermentation broth by gas stripping. Bioresour Technol 137: 153-159.
14. Hui SF, Zhu C, Yan R, Chen J (1987) Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenases in two strains of *Clostridium beijerinckii* (*Clostridium butylicum*). Applied and Environmental Microbiology 53: 697-703.
15. Eram M, Ma K (2013) Decarboxylation of pyruvate to acetaldehyde for ethanol production by hyperthermophiles. Biomolecules 3: 578-596.
16. Qi F, Thakker C, Zhu F, Pena M, San K, Bennett GN (2018) Improvement of butanol production in *Clostridium acetobutylicum* through enhancement of NAD(P)H availability. J. Ind. Microbiol. Biotechnol 45: 993-1002.
17. Kasap M (2002) Nitrogen metabolism and solvent production in *Clostridium beijerinckii* NRRL B593 (Doctoral dissertation, Virginia Tech).
18. Johnson MJ, Peterson WH, Fred EB (1931) Oxidation and reduction relations between substrate and products in the acetone butyl alcohol fermentation. Journal of Biological Chemistry 91: 569-591.

19. George HA, Chen JS (1983) Acidic conditions are not obligatory for onset of butanol formation by *Clostridium beijerinckii* (synonym, *C. butylicum*). Appl. Environ. Microbiol 46: 321-327.

20. Yan RT, Zhu CX, Golemboski C, Chen JS (1988) Expression of solvent-forming enzymes and onset of solvent production in batch cultures of *Clostridium beijerinckii* ("*Clostridium butylicum*"). Appl. Environ. Microbiol 54: 642-648.

21. Branduardi P, Longo V, Berterame NM, Rossi G, Porro D (2013) A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae*. Biotechnology for biofuels 6: 68.

22. Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJ, Hanai T, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metabolic engineering 10: 305-311.