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Effects of Temperature, pH, and Agitation on Growth and Butanol Production of *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *Clostridium saccharoperbutylacetonicum*

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***Abstract* – Butanol is a promising alternative to fossil-derived fuels. *Clostridium* genus bacteria are known for their ability to produce butanol as one of the metabolites, however, at the moment this solution is not economically viable. To solve it, the process of butanol production should be optimized. While ABE fermentation has been extensively studied, information about the optimal growth conditions for specific microorganisms often differs from one study to another. Therefore, this study aims to search for optimal growth conditions in sealed serum bottle tests for three widely used strains in ABE fermentation. In this study effects of temperature, pH, and agitation were tested on *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *Clostridium saccharoperbutylacetonicum*. The optimal temperature for *C. beijerinckii* growth and butanol production was 32 °C, the optimal agitation speed for growth was 0 rpm, but for butanol production, it was 200 rpm. For *C. saccharoperbutylacetonicum* growth and butanol production pH 7.5, 30 °C temperature and an agitation rate of 100 rpm were optimal, however, this effect was slight. For *C. acetobutylicum* cultivation optimal temperature, pH, and agitation rate were respectively 37 °C, 6.5, and 200 rpm.**

***Keywords* – ABE fermentation; anaerobic fermentation; biobutanol; butanol; *Clostridium*; external fermentation factors; IBE fermentation.**

Nomenclature	
GHG	Greenhouse gases
ABE	Acetone-butanol-ethanol
OD	Optical density
rpm	Rounds per minute

1. INTRODUCTION

Currently, the demand for fossil fuels is increasing while the supply is slowly decreasing. Moreover, due to continuous efforts to decrease greenhouse gas (GHG) emissions, an

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alternative to fossil fuels is needed. Fuels that are based on alcohol, including butanol, are a potential solution since they have many similar properties to gasoline. Butanol has such advantages as high energy content, low vapour pressure, and high octane number and it can be blended with conventional fuels even to high concentrations. It is also less volatile, hazardous, and hygroscopic than ethanol [1]. Butanol can be produced either via fermentation or using fossil fuels, however, currently the latter route is used mostly [2].

Butanol that is produced by fermentation is called biobutanol. The fermentation is done using bacteria from the genus *Clostridium*. During this glucose is used by bacteria to produce three parts acetone, six parts butanol, and one part ethanol. This process is named acetone-butanol-ethanol (ABE) fermentation [3]. ABE fermentation can be divided into two stages – acetogenesis and solventogenesis. During acetogenesis, sugars are converted into acetate and butyrate, but during solventogenesis, these metabolites are converted into acetone, butanol, and ethanol [4]. Afterwards, butanol can be extracted using various methods [3].

At the moment, bioethanol production using ABE fermentation is not economically viable. This is due to high substrate costs and product toxic effects on the bacteria, which causes low final product yields. Expensive product extraction also must be considered [5]. To increase final product yield, generally, there are two main approaches: to develop superior microbial strains or to optimize the bioprocess [6]. To optimize bioprocess, many different parameters must be tested, including temperature, pH, and agitation [3]. While some of these parameter optimal values are provided by the culture collection from which strains were obtained, they are usually based on cell growth rather than the product amount. Because of this, it is necessary to optimize all fermentation parameters to achieve the desired outcome.

Based on the previously conducted literature review [35], in this study, three *Clostridium* genus strains were selected – *C. acetobutylicum*, *C. beijerinckii*, and *C. saccharoperbutylacetonicum*, for which we will search for the optimal cultivation conditions.

2. MATERIALS AND METHODS

2.1 Microorganism strains

In the research bacteria strains *Clostridium acetobutylicum* DSM 792, *Clostridium beijerinckii* DSM 6423, and *Clostridium saccharoperbutylacetonicum* DSM 14923 were used. Microorganisms were purchased from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). Stock samples were stored at −40 °C.

2.2 Media and culture preparation

For pre-culture and inoculum preparation PY+X medium was used. It consisted of trypticase peptone 5 g/L, meat peptone (pepsin digested) 5 g/L, yeast extract 10 g/L, salt solution 40 mL/L, sodium resazurin (0.1% w/v) 0.5 mL/L, L-Cysteine HCl×H_2O 0.5 g/L, D-Glucose 5 g/L. Salt solution consisted of $CaCl_2$×$2H_2O$ 0.25 g/L, $MgSO_4$×$7H_2O$ 0.5 g/L, K_2HPO_4 1 g/L, KH_2PO_4 1 g/L, $NaHCO_3$, 10 g/L, NaCl 2 g/L.

For experimental cultures, standard fermentation media was used. It consisted of glucose 50 g/L, yeast extract 5 g/L, NH_4Cl 2 g/L, L-cysteinum chloride 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4×$3H_2O$ 0.655 g/L, $MgSO_4$×$7H_4O$ 0.2 g/L, $MnSO_4$×$6H_2O$ 0.01 g/L, $FeSO_4$×$7H_2O$ 0.01 g/L, $CaCO_3$ 5 g/L. In the case of *C. beijerinckii* DSM 6423 in all experiments and for all bacteria strains in the pH tests, NaCl was added to a concentration of 2 g/L.

To prepare L-cysteine chloride solution, distilled water was autoclaved. After autoclaving water was flushed with nitrogen gas to remove oxygen, L-cysteine chloride was added in a concentration of 12.6 g/100 mL and the solution was immediately flushed again and then autoclaved again. L-cysteine chloride solution was added to the media just before use by pouring it into the 100 mL serum bottle. Then the bottles were flushed with nitrogen gas.

L-cysteine chloride was prepared the same way for inoculum media as for standard fermentation media. First, all components of the media except L-cysteine chloride were diluted and pH was adjusted to 6.5 with 4M HCl and 4M NaOH. The media was sterilized by autoclaving and while it was still hot, flushed with nitrogen gas.

Pre-culture was made by adding bacteria culture from an agar plate with an inoculating loop to a 100 mL serum bottle containing 45 ml PY+X media and incubated overnight. As indicated by the DSMZ, *C. acetobutylicum* was incubated at 37 °C, *C. beijerinckii* was incubated at 35 °C, and *C. saccharoperbutylacetonicum* was incubated at 30 °C. Inoculum was prepared by transferring 5 mL of preculture to a 100 mL serum bottle containing 45 mL PY+X media and incubated for three days at the same temperature as pre-culture. The experimental culture was prepared the same way as inoculum, only it was incubated at previously described temperatures for four days.

All tests were done in triplicates.

2.3 Ensuring anaerobic conditions

Flushing media and cell suspensions with nitrogen gas was necessary to ensure anaerobic conditions during fermentation. To avoid contamination, a sterile 0.22 μm filter was inserted in the tube that is connected to the nitrogen gas tank and provides nitrogen flow to the media and cell suspensions.

When the experiment was prepared, already flushed media was poured into the bottles, 45 mL of each bottle. 100 mL bottles were flushed by inserting two sterile needles in the rubber part of the cup. One was used to connect the bottle to the in-flowing nitrogen gas, the other was needed as an outlet for outgoing gasses from the bottle. Each bottle must be rinsed for at least 10 minutes. Then 5 mL inoculum was added and bottles were flushed again.

Each day samples for optical density (OD) measurements and gas chromatography analysis were taken. First, a sterile needle was put into the rubber part of the bottle cap. When the pressure was normalized (no sound of outflowing gasses from the bottle), a syringe was added to the needle and 2 mL of cell suspension was collected. One mL was used for spectrophotometry analyses and 1 mL was used for gas chromatography. After sample collection, the bottles were rinsed with nitrogen again as described before. Samples for gas chromatography were stored at −20 °C until used.

2.4 OD measurements using spectrophotometry

To determine culture growth, OD was measured daily using the pre-programmed OD600 method where the sample's absorption of 600 nm wavelength light was measured. On the day of inoculation, the OD of the medium with added inoculum was measured and this value was subtracted from further measurements. If the OD value was higher than 1, samples were diluted and measured again. The final results are calculated by multiplying dilution times with the measured OD. To perform the measurements, a spectrophotometer BioMate 160 (Thermo Scientific) with the software Genesis was used.

2.5 Butanol measurements using gas chromatography

Butanol content was analyzed by GC chromatograph (Shimadzu GC-2030) equipped with a flame ionization detector and a fused silica column (DB-FATWAX UI, 30 m, 0.25 mm ID and film thickness 0.25 µm; Agilent) with an external calibration by the standard solutions of individual GC or HPLC grade compounds – *n*-butanol, isopropanol, methanol, ethanol, acetone (Sigma-Aldrich). Before injection into the GC, the samples were filtered using a 0.20 µm filter and diluted 2-fold with distilled water. A volume of 1 µL was injected into the column with a split ratio of 100:1, injector temperature 245 °C, and detector temperature 250 °C. The initial oven temperature was 40 °C, maintained for 4 min, before increasing by 15 °C/min to 235 C and holding at 235 °C for 4 min; helium was used as the carrier gas, with a constant column flow rate of 2 mL/min.

3. RESULTS AND DISCUSSION

3.1 Temperature effect of cell growth and butanol production

To test the temperature effect on cell growth, each strain was incubated not only at the temperature recommended by the supplier (DSMZ) but also at a few degrees above and below the recommended temperature. This is to ensure that the used temperature is optimal not only for cell growth but also for butanol production. Results are shown in Figs. 1–3.

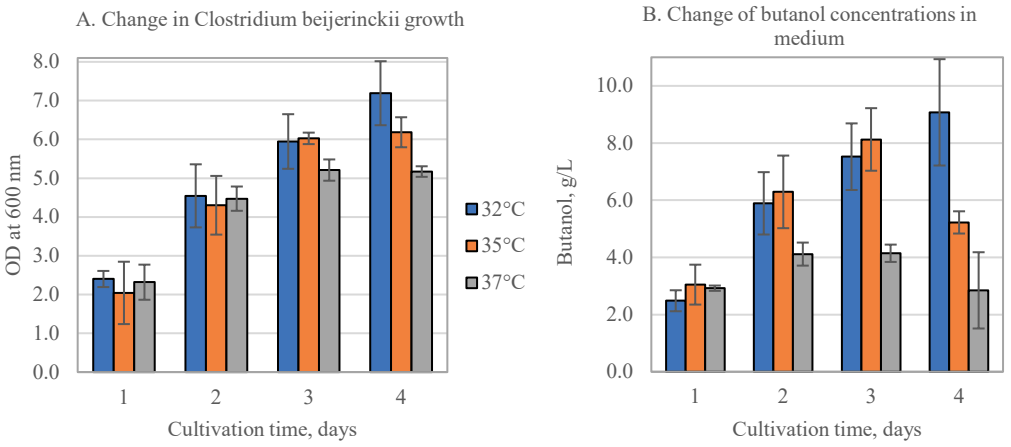

Fig. 1. Temperature effect over time on *C. beijerinckii* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

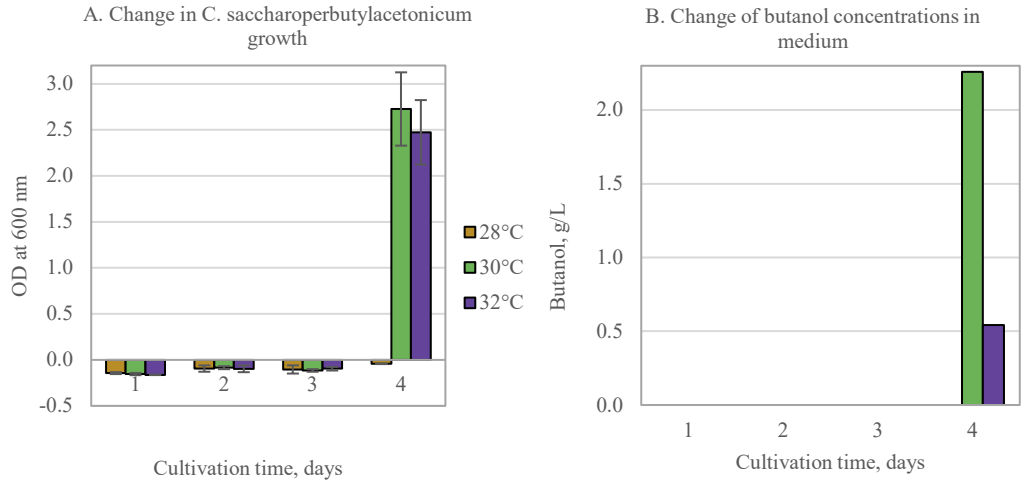

Fig. 2. Temperature effect over time on *C. saccharoperbutylacetonicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation (SD). SD was not calculated for some of the mean values as due to poor growth samples were excluded from triplets. In this test the strain had poor growth and negative OD values in some of the samples were measured due to $CaCO_3$ slowly dissolving in the medium during the experiment, thus reducing the absorption. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

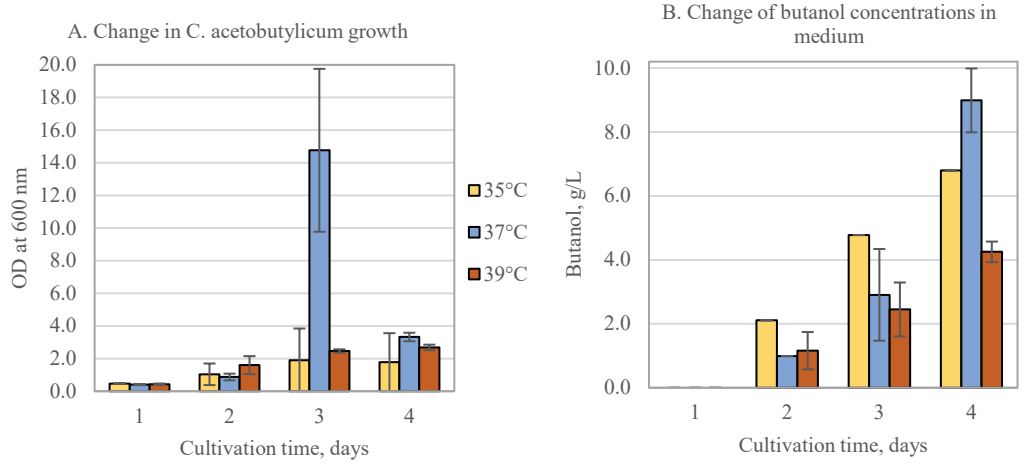

Fig. 3. Temperature effect over time on *C. acetobutylicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. SD was not calculated for some of the mean values as due to poor growth samples were excluded from triplets. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

The recommended fermentation temperature by DSMZ for *C. beijerinckii* was 35 °C [7], however, the results indicate that the optimal fermentation temperature is 32 °C (Fig. 1). When the strain was cultivated at a lower temperature (in this case, 32 °C), it took a longer time to reach the maximum butanol concentration. However, the final butanol concentration is higher at 32 °C compared to when the strain was cultivated at 35 °C, where the maximum

biobutanol concentration is achieved more quickly, but with a lower final concentration. The highest butanol concentration when cultivated at 32 °C was 9.1 g/L, but, when cultivated at 35 °C it was 8.1 g/L. It should be also emphasized that the standard deviations are quite broad for both (Fig. 1). In addition, temperatures lower than 32 °C should also be assessed in future studies, when using *C. beijerinckii*.

In the experiment done by Carrie *et. al.* 34 °C temperature was used to cultivate *C. beijerinckii*, however, it was done using immobilized cells in a fixed bed bioreactor. In that study maximum obtained butanol concentration was 7.5 g/L [8]. There are also a few studies where *C. beijerinckii* is cultivated at 37 °C. During continuous cultivation at 37 °C Survase *et. al.* obtained 7.51 g/L butanol results [9], but Cebreiros *et. al.* were able to reach 4.2 g/L butanol concentration using hydrolyzed eucalyptus sawdust [10]. However, most of the experiments have been done by cultivating *C. beijerinckii* at 35 °C. In this experiment, it was observed that, for *C. beijerinckii*, a cultivation temperature of 32 °C not only appeared to be optimal but also led to the attainment of a relatively high butanol concentration of 9.1 g/L, surpassing the results from previously mentioned studies.

During this experiment, *C. saccharoperbutylacetonicum* started to grow only on the third day, and butanol was detected only on day four when incubated at 30 °C and 32 °C (Fig. 2). At that time butanol concentration was only 2.3 g/L and 0.5 g/L, respectively. The recommended fermentation temperature for *C. saccharoperbutylacetonicum* is 30 °C [11]. Since *Clostridium* genus bacteria are very sensitive to oxygen, it is possible that during the preparation of these cultures flushing with nitrogen was not effective enough. The negative results were obtained because $CaCO_3$ solubility in water-based media is very low and, when the initial OD measurement with media and inoculum was made, it was higher than in later days, when $CaCO_3$ had dissolved more. When *Clostridium* growth is occurring $CaCO_3$ solubility is not a significant problem in these tests, but in this case, its effect could be observed in samples where growth did not occur.

Very rapid growth was observed in strain *C. acetobutylicum* from day 2 to day 3 at 37 °C and consequently, butanol production was also increased more than in samples that were incubated at other temperatures (Fig. 3). The maximum butanol concentration was reached on day four and it was 9.0 g/L. DSMZ recommended temperature for *C. acetobutylicum* is 37 °C [12], and in this case, this temperature also ensures the highest butanol concentration. While there is a report of cultivating *C. acetobutylicum* at 35 °C [13], in most cases 37 °C is used. For this strain, the maximum butanol concentration is reported to be 13 g/L without product removal during fermentation [14].

3.2 Initial pH effect of cell growth and butanol production

The recommended pH for the media recipe provided by DSMZ (media 104b) is 6.8–7. However, this media is used only for pre-culture and inoculum. To learn which initial pH is optimal for cell growth and butanol production, a pH test was performed. Each bacteria strain was cultivated at four different pH for four days. Each day OD and butanol content were measured. Results of this test are displayed in Figs. 4–6.

As can be seen in Fig. 4, pH has almost no effect on *C. beijerinckii* growth and butanol production, but nonetheless, high butanol concentrations of up to 13.66 g/L were achieved in this test. Similarly, pH had very little effect on *C. saccharoperbutylacetonicum* growth (Fig. 5), however small effect can be observed on butanol production. When pH was 7.5, *C. saccharoperbutylacetonicum* butanol concentration was 8.15 g/L.

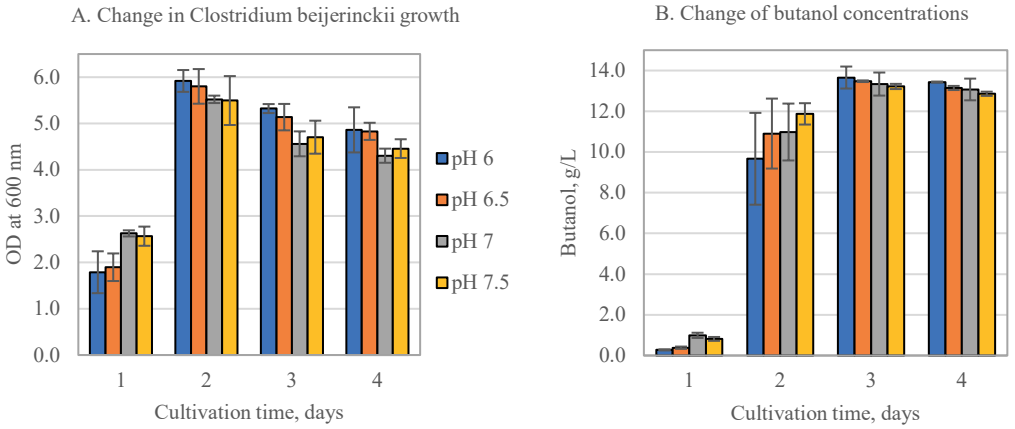

Fig. 4. pH effect over time on *C. beijerinckii* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 35°C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

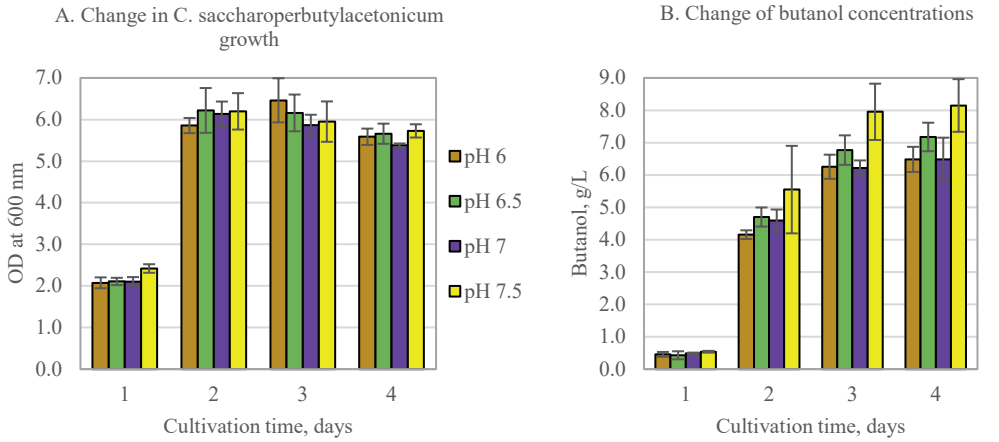

Fig. 5. pH effect over time on *C. saccharoperbutylacetonicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 30°C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

However, pH had a greater effect on *C. acetobutylicum* (Fig. 6). While during this experiment all OD values and butanol concentrations were lower than in *C. beijerinckii* and *C. saccharoperbutylacetonicum*, pH 6.5 was slightly better than pH 7 and 7.5. When cultivated at pH 6.5, the OD value was 4.12, and the butanol concentration was 4.01 g/L. The lowest results were observed when *C. acetobutylicum* was cultivated at pH 6. OD value and butanol concentration were respectively 2.5 and 0.94 g/L.

In the literature, various pH values are used for the cultivation of *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. acetobutylicum*. When multi-staged systems are used for fermentation in bioreactors, the pH of the first-stage fermentation for *C. acetobutylicum* can be 4.5 to 6.1, pH values are more consistent with first-stage fermentation for *C. beijerinckii*, which varies from 4.6 to 4.8 [15].

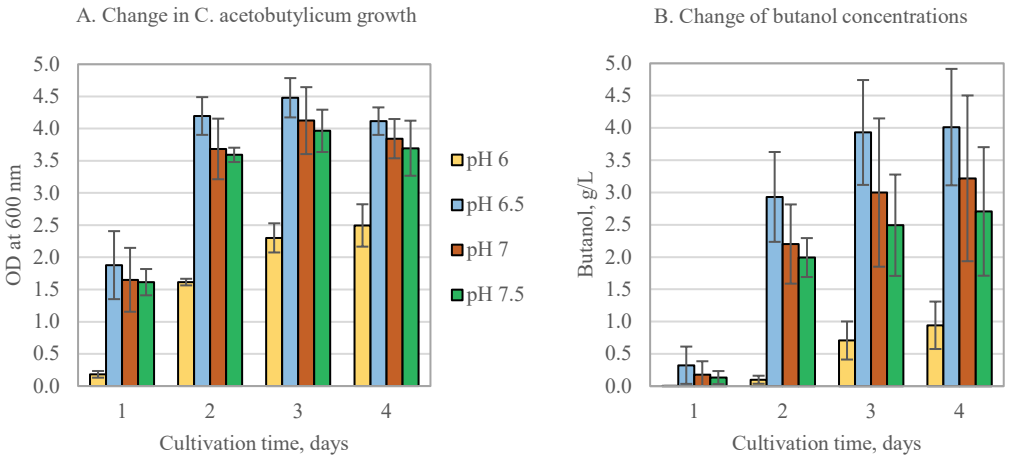

Fig. 6. pH effect over time on *C. acetobutylicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 37 °C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

Regulation of pH during cultivation has also been studied. Using cultivation with active pH regulation, pH 5.5 is reported to be optimal for *C. beijerincki* [16], [17], although one study suggests that when pH is kept constant at 7, the highest butanol concentration was achieved [18]. It is worth mentioning that the concentration achieved in that study was still quite low [18].

Initial pH has been studied in a few papers with different strains. According to Al-Shorgani *et. al.* [19] optimal pH value for *C. saccharoperbutylacetonicum* N1-4 is 5.8. In that study, the optimal temperature was 30 °C [19]. On the other hand, the best pH value for butanol and hydrogen production for *C. saccharoperbutylacetonicum* DSM 14923 in a study done by Singh *et. al.* [20] optimal pH value was 6.5. In that particular study, the optimal temperature was 37 °C [20]. The difference in results between studies is most likely explained by the interaction and mutual influence these factors have on each other.

More information about the initial effect of initial pH is available for *C. acetobutylicum*. Generally, when the initial pH is lower, solvent yields are higher, but, when the initial pH is lower, acid yields are higher [21]. For various *C. acetobutylicum* strains optimal pH can be 6, 6.05, and 6.5 [22], [23], [24]. This correlates well with this study's results as well.

3.3 Agitation effect of cell growth and butanol production

Another important environmental factor is agitation. Usually, this factor provides aeration during cultivation, but in anaerobic fermentation, it ensures homogeneous dispersion of ingredients. However different microorganisms can respond differently to agitation.

During the experiment, different responses to agitation were observed. A more pronounced effect was observed on butanol concentration than on OD (Figs. 7–9).

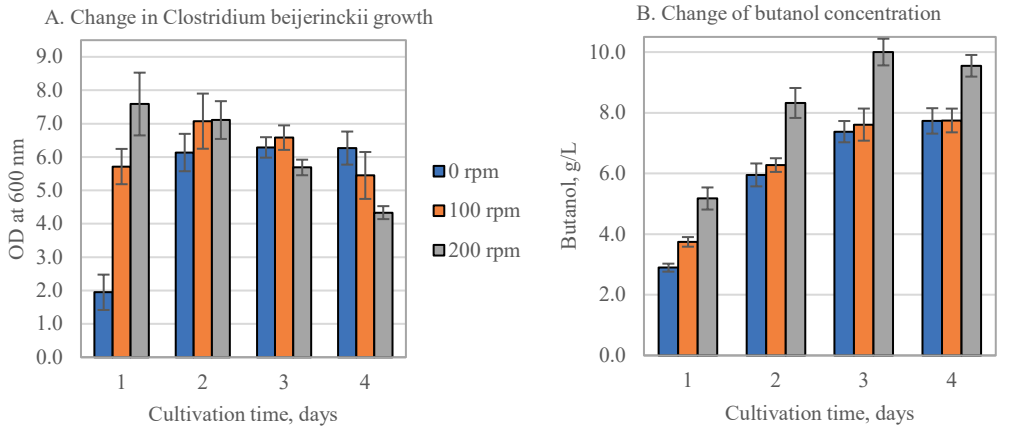

Fig. 7. Agitation effect over time on *C. beijerinckii* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 35 °C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

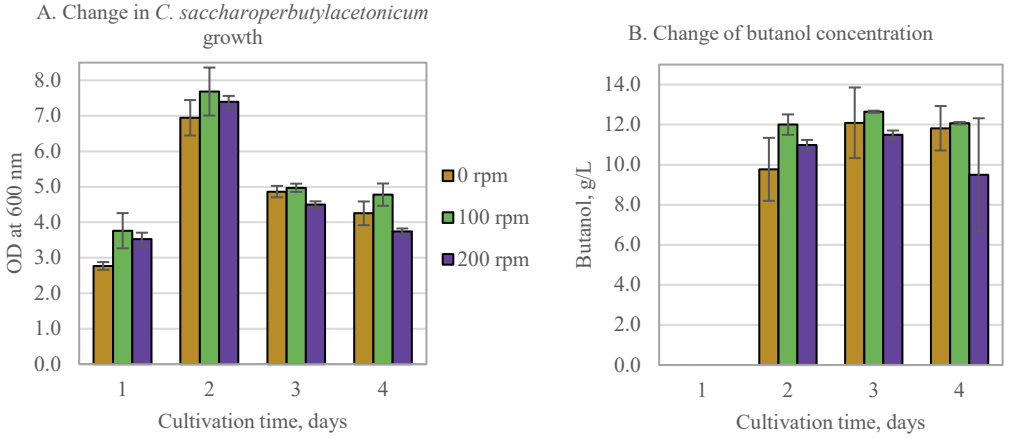

Fig. 8. Agitation effect over time on *C. saccharoperbutylacetonicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 30 °C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

Interestingly, for *C. beijerinckii* no agitation was preferable to obtain the highest OD, but 200 rpm was optimal for butanol production, and the highest concentration was 10 g/L on the third cultivation day (Fig. 7). Other researchers have found that slight agitation has a beneficial effect on butanol production. Wang and Blaschek [25] used response surface methodology to find the optimal agitation rate, which was determined to be 48 rpm. When tested in the laboratory, these results were consistent, as the maximum butanol yield was obtained at 50 rpm [25]. In one of the experiments done by Quershi *et al.,* [26] continuous agitation of 130 rpm was used to cultivate *C. beijerinckii*, but a very low acetone-butanol yield was achieved. However, when the same experiment was repeated but continuous agitation was replaced with gentle agitation once per day for 3 to 5 minutes, the yield increased from 1.06 g/L to 14.2 g/L [26]. These studies suggest that slight agitation is necessary for butanol production, but this is not consistent with the results acquired in this experiment.

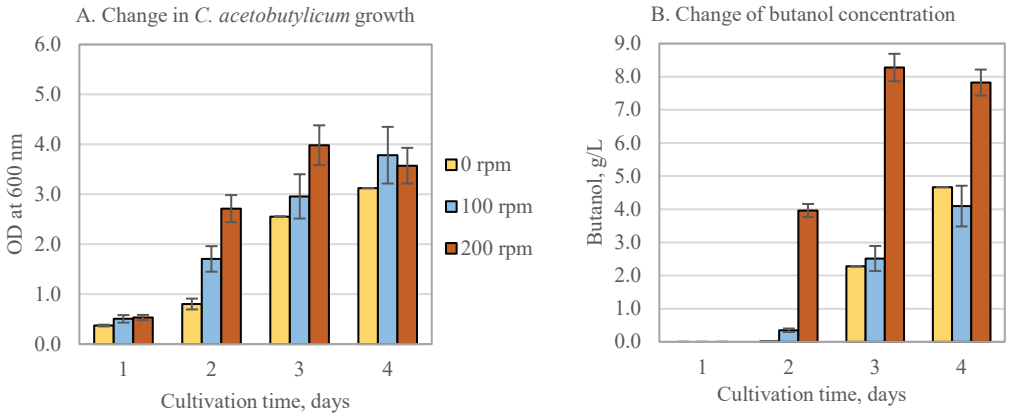

Fig. 9. Agitation effect over time on *C. acetobutylicum* growth (change in OD values) (A) and butanol concentrations (B). Error bars – standard deviation. Cultivation temperature 37 °C. Cultivation was done in 100 mL sealed serum bottles, final medium volume was 50 mL, triplets.

Compared to other tested cultures, agitation had little effect on cell growth and butanol production on *C. saccharoperbutylacetonicum* (Fig. 8). The highest OD was achieved on day 2, but the highest butanol concentration was measured on day 3. When culture was cultivated at 0 rpm and 100 rpm it had very similar results (12.09 g/L and 12.64 g/L), lower values were observed when *C. saccharoperbutylacetonicum* was cultivated at 200 rpm (11.49 g/L). Further assessments could not be drawn from these results as the standard deviations are quite broad for these results.

In the studies done with *C. saccharoperbutylacetonicum* usually, agitation is used during fermentation, however, agitation speed may differ. In one study it was mentioned that agitation was used, but without further elaboration of the actual RPM applied [27]. Since an agitation speed of 150 rpm is used widely among different experiments without comparing it with other speeds [28], [29], [30], it might be considered as standard for this culture. In research done by Al-Shorgani *et. al.,* [19] different agitation speeds were compared to optimize butanol production using palm oil mill effluent, and it was concluded that 100 rpm was the most suited for this experiment [19].

The agitation had a positive effect on *C. acetobutylicum* (Fig. 9). When cultivated at 100 rpm and 200 rpm, it reached similar OD results (3.78 and 3.57 on day 4), but butanol concentrations differed more. When *C. acetobutylicum* was cultivated at 200 rpm, butanol concentration reached much higher values than at 100 rpm or no agitation. At 200 rpm the highest value was reached on day 3 and it was 8.28 g/L.

Different effects of agitation rate during the cultivation of *C. acetobutylicum* have been reported. When lactose was used as a carbon source, higher solvent concentration was obtained in fermentors without agitation [31]. However, Yerushalmi and Volesky [32] determined that anaerobic solvent production increased when fermentor impellor speed was increased from 190 rpm to 340 rpm [32] and Ranjan *et. al.* [33] determined that when rice straw hydrolysate media is used, the optimal agitation rate is 150 rpm [33]. Interestingly, Doremus *et. al.* [34] found that the agitation rate's effect on butanol productivity depends on the pressure applied to the culture. When there was no pressure, the lower was agitation rate, the higher was butanol productivity. However, the effect of agitation was not observed when 1 bar pressure was applied to the culture during cultivation [34]. In this study, the

fermentation was done under pressure that was created by the cultures. The pressure was normalized once per day when samples were taken to measure OD and butanol concentration.

4. CONCLUSIONS

One of the most important steps of biobutanol production is the optimization of external factors, which include fermentation temperature, pH, and agitation. While there is information on recommended values for these factors, it can differ from one study to another. In this study, the optimal temperature, pH, and agitation rate were determined on butanol-producing bacteria *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. acetobutylicum*.

The optimal temperature for *C. beijerinckii* growth and butanol production was 32 °C, which is lower than recommended in other sources. Since it was the lowest tested temperature in this study, even lower temperatures should be evaluated in future tests. While the best agitation rate for *C. beijerinckii* growth is 0 rpm, a higher butanol concentration was obtained when it was cultivated at 200 rpm. pH from 6 to 7.5 did not affect *C. beijerinckii* growth and butanol production. Similarly, little effect of pH was observed on *C. saccharoperbutylacetonicum*, however, pH 7.5 was slightly better, and an agitation rate of 100 rpm provided slightly better results. Due to poor growth in the temperature test, it was difficult to determine the optimal temperature for *C. saccharoperbutylacetonicum*. With the obtained results, 30 °C seems to be optimal, but further testing should be done in the future to confirm it. For *C. acetobutylicum* cultivation optimal temperature, pH, and agitation rate were respectively 37 °C, 6.5, and 200 rpm. It should be emphasized that quite wide standard deviation values were obtained for a large part of the results, while there were also several triplets with very small standard deviation values. This indicates that the test methodology should be further improved to reduce the sources of errors and make the significant differences between various factors easier to distinguish.

It should be emphasized that in this optimization study, relatively high butanol concentrations were achieved in comparison to other studies. In the tests, butanol concentrations above 10 g/L were repeatedly attained, and the *C. beijerinckii* culture even reached 13.66 g/L, which is one of the highest reported results for this strain in the scientific literature. This suggests that without incorporating *in situ* butanol recovery during fermentation, it may not be possible to obtain higher butanol yields because the already high butanol concentration in the culture medium is likely inhibiting the strains.

In future research, it would be recommended to test these parameters in combinations as they might influence each other. As one of the main problems of biobutanol production is its economic viability, different cost-reduction solutions should be explored more, for example, industrial by-product usage as carbon and nitrogen sources.

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