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Intensification of Acetone-Butanol-Ethanol Fermentation via Products Recovery: Thermopervaporation Assisted by Phase Separation

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Inexp the present work, we propose the techniques providing a way to enhance biobutanol production via the acetone-butanol-ethanol (ABE) fermentation process. The proposed techniques employ highly efficient butanol-producing strains and extended range of renewable substrates. Also, an efficient approach to butanol removal from the culture liquid is presented – the novel technique of membrane thermopervaporation with porous condenser, coupled with phase separation (TPV-PC), which uses the low-grade heat (50-80˚C) only to create the driving force.
In order to increase the butanol yield in the fermentation process, the reducing agent (glycerol) was introduced into the studied substrates based on grain crops flour and beet molasses. It was found that glycerol enhances the fermentation efficiency with almost all the clostridia strains used, providing an increase in butanol fraction in the total ABE content – from 60 to 80%.
It was shown that the TPV-PC technique provides stable butanol removal from the fermentation broths up to concentration of 0.5% and lower, regardless on the microorganisms strain and the fermentation broth composition.

1. Introduction

Butanol is a large-capacity product of chemical industry with many applications in paints, polymers, plastics, food and flavor industries (Green, 2011). Furthermore, biobutanol is considered as promising liquid fuel due to its high energy content, low vapors pressure and ability to mix with gasoline and diesel fuel in high ratio (Jin et al., 2011). Acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* bacteria is employed as principal technology for biobutanol production (Lee et al., 2008).
The competitive ability of biobutanol production depends, particularly, on the selectivity of carbohydrates transformation into target product. In conventional acetone-butanol-ethanol fermentation process, along with butanol, acetone and ethanol are formed in the ratio 60:30:10, respectively (Zverlov et al., 2006).
Attempts were made to enhance the target product yield. Nielsen et al. (2009), designed the recombinant strains containing pathway genes for butanol biosynthesis from Clostridia. Atsumi et al. (2008), developed the microbial platforms for butanol biosynthesis, using the microorganisms which are able to ferment wide spectrum of substrates. These microorganisms have established genome, studied metabolism and well-developed gene engineering tool. However, the butanol yield in all cases was significantly lower than in stock strains *Clostridium acetobutylicum*.
Carbon bonded to oxygen in acetone has higher oxidation degree than that in butanol. Therefore, it can be assumed that acetone accumulation can be promoted in media having higher oxidative potential, whereas reducing media tend to accumulation of butanol.
Logotkin (1958) studied the effect of various substances on the solvents yield and butanol to acetone ratio. He proposed to shift the butanol to acetone ratio via introduction of the substances, acting as reducing agents, into the medium. It was shown that fermentation of mannitol ($C_6H_{14}O_6$), which is the stronger reducing agent

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than glucose, results in relatively higher amounts of butanol and hydrogen formed. In contrast, fermentation of calcium gluconate, the milder reducing agent, produces mainly acetone (Logotkin, 1958). The reducing substances which can be employed are both organic (glycerol, formic acid, reduced methylene blue or neutral red) and inorganic (hydrosulfite $Na_2S_2O_4$, sodium bisulphite and bisulphite). Glycerol is the most attractive reducing additive due to its high feedstock availability (Sotoft et al., 2010).
Another severe drawback for biobutanol commercialization is the problem of fermentation products separation, since butanol content in ferm-broth is low (< 20 g/L) (Jones, 2001).
Various approaches can be used to increase the amount of butanol obtained from 1 L of ferm-broth. These approaches were tested, and all of them somehow involve partial separation of organic solvents from the ferm-broth and recovery of the latter for further fermentation (Qureshi and Ezeji, 2008).
Generally, the most attractive process is that involving continuous *in situ* extraction of fermentation products with periodical reactor feeding. This process provides the maximum fermentation performance, since ABE concentration in the ferm-broth is always below inhibition level. However, in such a long-term process, it is difficult to maintain the sterility, because feeding and ABE extraction processes raise up the risk of culture contamination with inimical microorganisms. It is also challenging to find materials and conditions for butanol extraction without affecting the fermentation process (Jones, 2001).
We propose a novel membrane method of TPV-PC. The method allows to separate ABE fermentation products using only low-temperature heat (50-80°C) to maintain the process driving force (Borisov et al., 2017; Elkina et al., 2013). The proposed technique provides a way to avoid contamination of the fermentation culture during the separation process, since the operation temperature destroys the majority of microorganisms. After ABE removal to level below inhibition, the culture liquid may be reused for fermentation. The technique also increases the overall process productivity, as the production technology excludes the fermenter cleaning stage as well as ferm-broth sterilization, which takes place during the ABE separation at elevated temperatures.
The aim of this work is to enhance the ABE fermentation process by means of: 1) increasing the butanol selectivity via introduction of glycerol as the culture medium component; 2) separation of ABE fermentation products using the TPV-PC technique to decrease the ABE concentration below the threshold inhibition value, thus providing a way to reuse the culture liquid in fermentation process and to decrease the separation expenses.

2. Experimental part

2.1 ABE fermentation

Bacterial Strains
The butanol producers used were *Clostridium acetobutylicum* strains, Bioresource Center Russian National Collection of Industrial Microorganism: B-10535, B-4361, B-10935, B-5358, B-10937, B-10939, B-10940.
Conditions and media composition for Bacterial Strains growth:
-grain crops flour, concentration in medium - 6%.
-synthetic medium, g/L: KH_2PO_4 – 0.7; K_2HPO_4 – 0.7; $MgSO_4$ x $7H_2O$ – 0.1; $MnSO_4$ x H_2O – 0.02; $FeSO_4$ x $7H_2O$ – 0.015; NaCl – 0.01; glucose – 20, ammonium acetate – 3, yeastrel – 1.0; peptone – 1.0; cysteine – 0.5; soluble starch – 1.0; resazurin – 0.001; agar – 15, distilled water up to 1L.
Conditions and media composition for fermentation:
- Grain crops flour: wheat, rye or corn with concentration in media – 9-10%.
- Beet molasses – 11.6%, ammonium sulphate – 0.06%, superphosphate extract 0.26%, wheat bran – 0.01%, CaCO3 – 1.0%.
- Molasses-flour medium with substrate concentration – 9-10%, flour to beet molasses ratio 35/65.
- Starch substrate: corn starch – 6.0 %, yeastrel – 0.1 %, 1 mL of microelements solution (1): 0.5 g K_2HPO_4 + 0.5 g KH_2PO_4 + 2.2 g CH_3COONH_4 to 100 mL; mL of microelements solution (2): 2.0 g $MgSO_4$ x $7H_2O$ + 0.1 g $MnSO_4$ x H_2O + 0.1 g NaCl + 0.1 g $FeSO_4$ x $7H_2O$ to 100 mL; 0.1 mL of vitamins solution: 100 mg p-aminobenzoic acid, 100 mg thiamine, 1 mg biotin to 100 mL.
Fermentation temperature 37°C, fermentation time 48-66 hours. During the fermentation process, the culture liquid was analyzed for key parameters of the process: residual sugar content, amount of the organic solvents synthesized and butanol fraction in the solvents.
Cultivation of *C. acetobutylicum* was carried out in a 3 L bioreactor (KF 103/4 Prointeh, Russia).To create anaerobic conditions, the culture medium was sparged with nitrogen for 1 h, under constant stirring, immediately after sterilization (121°C, 20 min). After sterilization, the feeding medium was sparged with nitrogen until it reached room temperature (for several hours, depending on the volume). During the experiment, the feed tank was maintained under nitrogen pressure (30 mbar [1 bar = 105 Pa]) to avoid oxygen entry. The bioreactor was inoculated directly with the spore suspension (10% [vol/vol]). Laboratory stocks of *C. acetobutylicum* were routinely maintained as spore suspension in sterile double distilled water at 40°C. Spores (200 mL) were heat shocked for 10 min at 800 °C followed by cooling in the anaerobic chamber for 5

min. The culture was inoculated into 20 mL medium and was incubated anaerobically for 15–16 h at 370°C until an optical density of 1.0–1.2 was achieved at 600 nm (Beckman Du 640 spectrophotometer).

2.2 Thermopervaporation with a porous condenser (TPV-PC)

The TPV-PC experiments were carried out using the lab scale setup described in Borisov et al. (2017). The hot feed mixture (60°C) was circulated in the membrane module. Cooling agent circulated with a constant flow rate of 0.3 L/min through the porous condenser compartment of the module at a temperature of 10°C. The flat sheet commercial composite membrane MDK-3 was used.
The composition of feed and permeate mixtures was analyzed using the gas chromatography technique. A Crystallux-4000M gas chromatograph was equipped with a TCD detector with the following parameters: injection port temperature was 230°C, column temperature was 180°C, and detector temperature was 230°C. The mixtures were analyzed on a Porapak Q packed column. To estimate the concentration of water–butanol permeate which is a two-phase system, the sample was homogenized by the addition of water.
The TPV-PC capacity by ferm-broth, Q_f, was calculated as follows:

$$Q_f = \frac{V_f}{S \cdot t} \quad (1)$$

, where V_f – ferm-broth volume, S – membrane area, t – separation time.
The TPV-PC capacity by butanol, Q_{BuOH}, was calculated as follows:

$$Q_{BuOH} = \frac{V_{f1} \cdot C_{f1} - V_{f2} \cdot C_{f2}}{S \cdot t} \quad (2)$$

, where V_{f1} and V_{f2} – feed volume before and after separation, C_{f1} and C_{f2} – butanol concentration in the feed and treated ferm-broth, S – membrane area, t – separation time.

3. Results and discussion

3.1 Increasing of butanol yield by glycerol addition

In order to enhance the butanol yield in the fermentation process, first experiments were carried out with addition of glycerol as reducing agent to the substrates studied: grain crops flour (corn, wheat, and rye), beet molasses and their mixtures. The results illustrating the glycerol effect on the process are given in Table 1. For comparison, the data obtained without glycerol addition are also provided.
As can be seen from Table 1, glycerol significantly enhances the fermentation process. In almost cases, the process shifts towards increase in carbohydrates to butanol conversion. It was shown that glycerol enhances the fermentation efficiency for almost all of the clostridia strains used.

Table 1: Effect of glycerol addition on the strains productivity for different substrates

Strain	Substrate for fermentation	Concentration, g/L		Carbohydrates conversion,%	
		Butanol	Sum of the products	By sum	By butanol
B-10535	Corn flour	13.5	20.1	40.2	27.0
	Corn flour + Gl*	14.6	21.8	43.6	28.4
B-4361	Corn flour	12.2	19.1	38.2	24.4
	Corn flour + Gl	14.4	20.8	41.6	28.8
	Wheat flour	7.1	10.6	34.2	21.2
	Wheat flour + Gl	14.6	21.5	43.0	29.2
B-10935	Molasses + wheat flour	13.4	16.3	32.6	26.8
	Molasses + wheat flour + Gl	15.7	19.3	38.6	30.2
B-5358	Rye flour	13.7	19.9	39.8	27.4
	Rye flour + Gl	13.5	17.9	35.8	27.0
B-10937	Molasses	13.6	17.2	34.4	27.2
	Molasses + Gl	15.5	19.6	39.2	30.2
B-10939	Corn flour	13.6	20.5	41.0	27.2
	Corn flour + Gl	13.9	19.9	39.8	27.8
	Corn starch	13.5	17.9	35.8	27.0
	Corn starch + Gl	14.8	18.8	37.6	29.6
B-10940	Molasses	14.0	17.2	34.4	28.0
	Molasses + Gl	14.5	18.3	35.8	28.4

*Gl-Glycerol

Based on the experimental results, the *C. acetobutylicum* strains B-10535, B-10935 and B-10937 were selected, as they showed increased butanol resistance (2 and more %) as well as synthesis rate and final butanol concentration in feed. Fermentation of the optimized media by the strains mentioned above showed

that process parameters (concentration of the solvents sum, degree of carbohydrates conversion to solvents and butanol, and butanol fraction in the solvents sum) were close to theoretically possible values.
The fermentation processes for *C. acetobutylicum* strains B-10535, B-10935 *и* B-10937 were studied over time in the periodical system in 3 L laboratory fermenters.
Figure 1 shows the results of reducing sugars utilization and butanol biosynthesis for the fermentation process with glycerol addition.

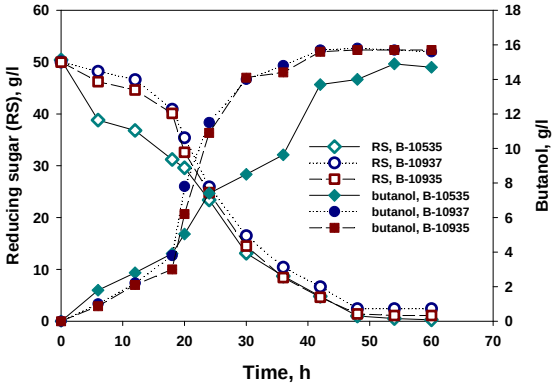


Figure 1: Time curves for carbohydrates and butanol concentrations under fermentation with glycerol addition

When using the B-10535 strain, the maximum butanol concentration was achieved already in 40 h, whereas for the B-10937 and B-10935 strains, this time was 30-35 h of cultivation, which is 5-10 h earlier. For all the experiments performed, the residual sugars concentration to the moment of 50 h of fermentation did not exceed 3 g/L. After 55 h, the butanol concentration remained constant.
Table 2 provides the comparative data illustrating fermentation productivity for the selected strains. These data indicate the fact that the percentage ratio of the solvents (acetone:butanol:ethanol) with addition of glycerol shifted towards increased butanol content compared to the “conventional” 3:6:1. The biobutanol concentration in all cases was higher than 14.7 g/L. The maximum yield and concentration of butanol in ferm-broth was observed for the B-10937 and B-10935 strains. Therefore, the ferm-broths obtained on the basis of these strains were selected to investigate the ABE extraction via the TPV-PC technique.

Table 2: The comparative data for key parameters of the strains (butanol producers) cultivation with addition of glycerol

Strain	Organic components concentration, g/L				Solvents yield, %		
	Sum of the products	N-butanol	Acetone	Ethanol	By n-butanol	By acetone	By ethanol
B-10535	22.0	14.7	6.4	0.92	66.8	29.0	3.9
B-10937	19.5	15.7	3.4	0.4	80.5	17.4	2.1
B-10935	19.6	15.7	3.0	0.88	80.1	15.3	4.5

3.2 Extraction of acetone, butanol and ethanol from the ferm-broth via the TPV-PC technique

Figure 2 shows the effect of butanol concentration in the ferm-broth on the fermentation productivity by butanol. It can be seen that the maximum fermenter productivity by butanol is achieved at butanol concentration in the ferm-broth 5-6 g/L. It is known that the butanol removal rate in the TPV-PC process decreases with a decrease in butanol concentration, as the process driving force (transmembrane difference of butanol vapors pressure) declines.
In view of this, it was concluded that butanol removal from the ferm-broth is performed to threshold value of 0.5%, which corresponds to the increased clostridia productivity and provides maintaining of the TPV-PC process driving force. The experiments were carried out for butanol removal from the ferm-broths containing both molasses and flour medium. It was shown that the TPV-PC provides stable butanol removal from the ferm-broths up to concentration of 0.5% and lower, depending on the microorganisms strain and ferm-broth composition (growth medium, concentrations of acetone, butanol and ethanol). The separation temperature was 60°C and the permeate condensation temperature was 10°C.
The process was estimated by the following parameters: the TPV-PC capacity by feed mixture – ferm-broth volume with the preset butanol concentration at which the TPV-PC module with membrane area of 1 m^2 in time of 1 h will provide butanol concentration decrease to 0.5%; and the TPV-PC capacity by sum of the organic components (acetone, butanol and ethanol) – volume of the organic components passed through the

membrane having area of 1 m^2 in time of 1 h at butanol concentration decrease in ferm-broth from the given value to 0.5% (see Eq(1) and Eq(2) in experimental part).

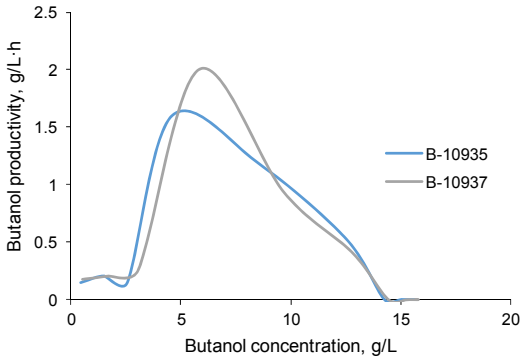
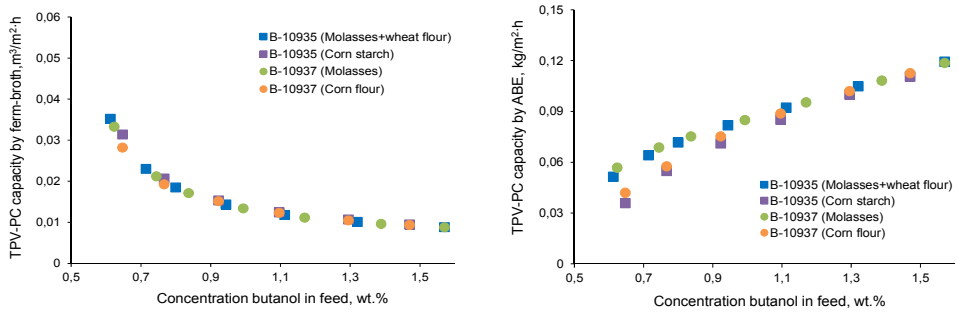


Figure 2: Effect of butanol concentration on butanol productivity

Figure 3 shows the correlation between the TPV-PC capacity by ferm-broth and the butanol concentration in ferm-broth. It can be seen that the TPV-PC capacity by ferm-broth decreases 4-fold (from 80 to 20 L/m^2·h) with an increase in butanol concentration in the range of 0.6-0.9%; further, it declines smoothly in the concentrations range of 0.9-1.5% and reaches the value of 10 L/m^2·h. This is accounted for by the fact that the amount of butanol, which has to pass through the specified membrane area, increases. However, the fermentation performance increases with an increase in butanol concentration in the ferm-broth, which is preconditioned by more full conversion of carbohydrates into the target product. In view of this, the separation process has to be started at butanol concentrations close to 15 g/L.



a b

Figure 3: Effect of the butanol concentration in ferm-broth on the TPV-PC capacity by feed ABE ferm-broth (a) and ABE (b)

The TPV-PC capacity by butanol seems to be a more conclusive value, as the aim of the process is not only to remove butanol rapidly to the level below inhibition, but also to choose a less consuming way for removal, i.e., to use smaller membrane area and lower energy for heating and liquid circulation in the system. The mentioned parameters decrease with an increase in butanol concentration in the ferm-broth. Indeed, as shown at Figure 3 (b), the TPV-PC capacity by butanol normalized to the membrane area unit rises monotonically with an increase in butanol concentration. It is also clear that, provided the constant fermenter productivity, an increase in butanol concentration will result in decline of the overall ferm-broth volume which is to be separated, and therefore, the energy consumption for heating and pump work will also decrease.

Based on the experimental data, it was calculated that under separation of ferm-broth with initial concentration of 15 g/L, the TPV-PC capacity by butanol is approx. 0.1 kg/m^2·h. Taking into account the value of fermentation productivity by butanol achieved in the present work (0.2-0.37 g/L·h), the ratio between the feed mixture volume and the membrane area is sufficient for continuous butanol removal when it is within the range of 0.27-0.5 m^3/m^2.Therefore, the industrial membrane module having membranes area of 100 m^2 is able to maintain the fermenter of 27-50 m^3 volume. Such values correspond to industrial biotechnology processes, which indicate the TPV-PC process viability for intensification of the biobutanol production.

4. Conclusions

In the present work, an attempt was made to enhance the ABE fermentation process. The butanol selectivity was increased by glycerol introduction in the growth medium.To decrease the ABE concentration below the inhibition level and to recycle the culture liquid in the fermentation process, the TPV-PC technique was employed for removal of ABE fermentation products.

It was shown that addition of glycerol to the substrates (grain crops flour: corn, wheat, rye, beet molasses and their mixtures) results in increased carbohydrates to butanol conversion. It was found that glycerol enhances the fermentation efficiency with almost all clostridia strains used. With introduction of glycerol, the percentage ratio of the solvents – acetone:butanol:ethanol – shifted towards increased butanol content compared to the "conventional" 3:6:1. The butanol fraction in the total ABE content was up to 80%, and the end concentration of butanol was higher than 14.7 g/L in all cases.

The TPV-PC technique was used to remove butanol from the ferm-broths, containing both molasses and flour medium. It was concluded that butanol removal from the ferm-broth is performed to threshold value of 0.5%, which corresponds to the increased clostridia productivity and provides maintaining of the TPV-PC process driving force. It was shown that the TPV-PC technique provides stable butanol removal from the ferm-broths up to concentration of 0.5% and lower, regardless on the microorganism strain and ferm-broth composition (growth medium, concentrations of acetone, butanol and ethanol).

The calculations performed on the basis of the experimental data shows that the industrial membrane module having membranes area of 100 m^2 is able to process the fermenter of 27-50 m^3 volume. This indicated the TPV-PC process viability for intensification of the biobutanol production.

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