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Investigation of Effective Immobilization Method for Ethanol Producing *E. coli* Strain

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Abstract

There is a growing interest to find alternative cheap carbon sources for bioethanol production. Whey powder is a by-product of cheese industry with high lactose content. Immobilization of bacteria is an effective method for enhancing production of their metabolites including bioethanol. In this work, a new and effective immobilization method for bioethanol production from whey powder as a cheap/renewable carbon source was investigated. For this, different immobilization methods were evaluated to avoid disruption of bead structures during ethanol fermentation. The best results were obtained when sodium alginate was sterilized under the UV lamp instead of heating for immobilization, and the fermentation media was supplemented with $CaCl_2$ as a stability factor. In addition, the beads were successfully reused in at least three 48 h batch fermentations for ethanol production.

Keywords: *E.coli*, ethanol, immobilization, repeated batch fermentation, whey powder, lactose.

1. Introduction

Cheese whey is a dairy industry waste and contains lactose (5-6%), protein (1%), fat (0.06%), several minerals and vitamins [1]. Cheese whey is a useful, inexpensive and alternative carbon source for microbial fermentation. Some countries such as New Zealand, the United States, and Denmark use cheese whey for producing ethanol [1, 2]. Whey powder is also concentrated form of cheese whey and contains more lactose. For ethanol production, *Saccharomyces* and *Kluyveromyces* are used as fermentative organisms. However, *S. cerevisiae* cannot utilize lactose and pentose sugars like xylose [3]. *Kluyveromyces* sp. ferments lactose to ethanol but it is not effective at moderate sugar concentrations compared with *S. cerevisiae* [4]. *Escherichia coli* can ferment various sugars such as lactose, glucose, fructose and xylose [5]. In previous studies, *E.coli* FBR5 strain was an alternative ethanol producer using different carbon sources including pure sugars, corn stover, potato processing waste water, corn and potato processing waste, sugar beet molasses and whey/ whey powder [6-11]. In addition, some strategies such as cell immobilization, VHb technology, co-culturing and evolutionary adaptation have been conducted for enhancing bioethanol production (reviewed by Akbas and Stark [12]). Especially immobilization technology provides improvement of ethanol production through preventing contamination [13], reducing fermentation times [14] and allowing to reuse the organisms in repeated batch fermentations [11]. The common method for immobilization is cell entrapment in natural or synthetic polymers. For bacterial immobilization, sodium alginate has been evaluated as a natural and safe biopolymer by several researchers [10, 11, 14]. Therefore, in this work, an effective Ca-alginate immobilization method was investigated for bioethanol production.

2. Materials and Methods

2.1. Bacterial Strain

In this work, *E.coli* FBR5 strain [5] was used for ethanol production.

2.2. Immobilization

For immobilization, *E.coli* FBR5 strain was incubated in LB media (peptone 10g/L and yeast extract 5g/L; pH 7.0) at 180 rpm for 24 hours at 37 °C. After incubation, the cell culture OD 600nm was adjusted to 0.5, and then 40mL of cell culture was centrifuged at 4000 rpm for 15 min at 4 °C. The supernatant was removed and the cell pellet was used for immobilization. 5 different immobilization methods (A-E) were used for determining the best immobilization applications for ethanol production. The beads were approximately 3.0 mm in diameter.

After the immobilization, the beads were added into LB media and incubated at 180 rpm for 24 hours at 37 °C for ethanol production.

Method A

Sodium alginate (Sigma Aldrich, Germany) was mixed with distilled water to give 6% (w/v) alginate solution.

Alginate solution was heated at 121 °C for 15 min for sterilization.

The bacterial cell pellet was mixed with 20 mL NaCl (0.9%, w/v) and 20 mL alginate (6%, w/v) solutions, respectively. The obtained cell-alginate mixture solution was dropped into 2% (w/v) $CaCl_2$ solution and stirred for 30 min. After the bead formation process, the beads were collected and washed with sterile distilled water. The beads were transferred into the 5 different storage solutions (Table 1) and stored at +4 °C for 7 days.

Method B

Alginate was sterilized under the UV lamp in a Biosafety cabinet level II for 30 min and then mixed with sterile distilled water at a final concentration of 6% (w/v). Alginate solution was stirred until homogeneous using by a magnetic stirrer. *E.coli* FBR5 strain was immobilized as described in method A procedure and then transferred into 5 different storage solutions (Table 1) at +4 °C.

Method C

Bead preparation was performed as in Method B procedure. The obtained beads were covered with 0.2% chitosan (w/v) in a beaker for 60 min. Then, the beads were collected and stored in 5 different storage solutions (Table 1).

Method D

Beads were obtained according to method B and dried on a filter paper at room temperature for 60 min. The dried beads were stored in storage solutions (Table 1).

Method E

Alginate was sterilized using UV lamp for 30 min as performed in method B and then mixed with sterile distilled water at the final concentration of 3% (w/v). The alginate solution was mixed with Tween20 to give 0.1% (v/v) final concentration of alginate. In addition, 0.65% CMC (carboxymethylcellulose; w/v) was added into the 2% $CaCl_2$ (w/v) solution.

For immobilization, the cell pellet was mixed with 40 mL alginate-Tween20 solution and then dropped into the $CaCl_2$-CMC solution, and stirred for 30 min. The beads were transferred into the 5 different storage solutions (Table 1) and stored at +4 °C.

Table 1. Different storage solutions (SS1-SS5) for immobilized beads.

Storage Solutions (SS)	Ingredient
SS1	2% $CaCl_2$ (w/v)
SS2	0.2% Glucose (w/v), and
SS3	10% Glycerol (v/v)
SS4	0.9% NaCl (w/v)
SS5	PBS (Phosphate Buffer, pH at 7.0)

2.3. Preparation of Fermentation Media and Fermantation

In this study, cheese whey powder was obtained by Bahçıvan Gıda (Kırklareli, Turkey) and used as a carbon source (78-80% lactose, w/v). For preparing the cheese whey powder solution, whey powder (128g) was diluted with distilled water (400mL) and heated at 121°C for 15 min for sterilization. The obtained solution was centrifuged at 10 000 rpm for 10 min and then pH adjusted as 7.0 using with 10N NaOH or 1N HCl [10-11].

In this work, 6 different fermentation media were used (Table 2). The lactose contents of the media were supplied from whey or lactose solution. Initial lactose concentration of fermentation media was set at 5% (w/v). These media were supplemented with $CaCl_2$ (5g/L), or $CaCl_2$ (5g/L) and yeast extract, or $CaCl_2$ (5g/L), yeast extract and peptone (Table 2).

All of the fermentation media (40 mL) were transferred into 50 mL Erlenmeyer Flasks. The free cells (initial cell concentration was set as OD_{600nm} 0.04) or immobilized cells (10 beads) were incubated at 37 °C, 100 or 200 rpm for 48 hours.

3. Results and Discussion

3.1. Immobilization

When immobilized cells were stored with different types of storage solutions, the structures of beads were maintained in the SS1, SS2, SS3, and SS4 for 7 days at 4°C. However, the beads were disrupted in the SS5 (PBS Buffer) solution after they were stored.

After storage, the beads prepared according to method A and stored for 7 days were disrupted in the fermentation media after 24 hours at 37 °C, 100 or 200 rpm. This could be due to the heating step during sodium alginate sterilization. The beads obtained by method B and stored for 7 days were maintained in the fermentation media when they were incubated at 37 °C with shaking at 100 or 200 rpm for 48 hours. Thus, the beads were collected and washed with sterile distilled water and then transferred into the fresh media. During the second fermentation the beads were disrupted in 48 hours. Therefore, the beads were reprepared as described in method B and stored for 7 days at 4 °C. These beads were incubated in LB media enriched with $CaCl_2$ (5g/L, w/v). In $CaCl_2$ containing LB medium no morphological change on beads was observed during the second and then the third repeated batches during 48 hour fermentation period. The beads attained from method C were disrupted in LB medium enriched $CaCl_2$ after 24 hour incubation. The beads prepared by using method D retained their structures in the LB media enriched with $CaCl_2$ in 48 hours, at 37 °C with shaking at 100 or 200 rpm. The beads obtained by using method E were maintained in the LB media enriched with $CaCl_2$ after the same incubation conditions.

Table 2. The contents of different fermentation media.

Substrate	Media	Contents of Media
Whey powder	**WP1**	5 g/L $CaCl_2$
	WP2	5 g/L $CaCl_2$ + 5 g/L yeast
	WP3	5 g/L $CaCl_2$ + 5 g/L yeast + 10 g/L peptone
Lactose	**ML1**	5 g/L $CaCl_2$
	ML2	5 g/L $CaCl_2$ + 5 g/L yeast
	ML3	5 g/L $CaCl_2$ + 5 g/L yeast + 10 g/L peptone

It was determined that $CaCl_2$ as a supportive supplement necessary for the improvement of bead structure.

3.2. Repeated Batch Fermentations

The beads prepared by method B and stored in SS2 were selected since they were physically the best with no swelling or shrinking behavior after 7 days storage compared to other storage solutions. These beads were successfully reused in repeated batch fermentations with the lactose-containing media (Table 3) enriched with $CaCl_2$. The calcium ions could improve the calcium-alginate structure during the fermentations and prevented beads from swelling [15]. Therefore, all fermentation media were supplemented with $CaCl_2$ [16].

In the repeated batch fermentations, the lowest cell growth profiles were observed in the low shaking conditions (100 rpm). In addition, the OD_{600nm} values were much higher at 200 rpm shaking with whey powder, yeast and peptone supplementations (Table 3). However, the beads were reused at least 3 successive fermentation runs both with lactose (ML) and whey powder (WP) media.

According to OD_{600nm} values of free cells, the cell growth in both WP1 and ML1 were too low after 48 hours at 37 °C with shaking at 100 or 200 rpm (Table 3). Therefore, the fermentation media were supplemented with yeast extract or combination of yeast extract and peptone were found to be more effective for cellular growth.

Table 3. The OD_{600nm} values of free cells and immobilized cells in different media after three successive batches fermentations.

	Media	Free cells	Immobilized Cells		
			1. Batch	2. Batch	3. Batch
100 rpm	**WP1**	0.73	0.95	0.79	0.59
	WP2	2.61	2.14	1.80	2.02
	WP3	2.36	2.27	3.60	2.23
	ML1	0.14	0.06	0.06	0.08
	ML2	4.27	1.40	1.45	1.71
	ML3	4.82	2.38	2.36	2.50
200 rpm	**WP1**	0.73	2.14	1.90	2.10
	WP2	1.84	6.23	4.66	6.23
	WP3	1.91	7.23	5.38	7.39
	ML1	0.19	0.12	0.06	0.09
	ML2	2.80	1.62	2.51	2.72
	ML3	3.67	3.25	3.02	2.94

4. Discussion

The food industry wastes have been intensively investigated for ethanol production [6-11, 17]. Cheese whey and whey powder were effectively used for metabolite production [8, 18]. New and efficient strategies needed to be performed for improving bioethanol production. Immobilization of cells has many advantages like using cells in repeated batch fermentations for several times, improving the yield and reducing the fermentation times. During efficient fermentation, immobilized bead structures should be maintained and successfully used several times. Some of the ingredients like $CaCO_3$ [19] or $CaCl_2$ [16] were added into the fermentation media for stabilizing the structure of calcium-alginate beads. It was also reported that calcium supplementations ($CaCO_3$ and $CaCl_2$) improved the ethanol yields [20].

It can be concluded that the structure of beads was maintained by using UV for sterilization of sodium alginate instead of heating treatment. In addition, $CaCl_2$ supplementation into the fermentation media had beneficial effect on bead stability. The beads were also reused at least 3 repeated batch fermentations.

In this work, an effective immobilization method (method B) was determined for repeated batch fermentations. It was also shown that the immobilized beads could be stored at different types of storage solutions except PBS buffer. These results could be useful for immobilization of ethanol producing organisms for large scale fermentations in industrial use.

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Author's Contributions

Taner Sar: Performed the experiment, analyzed data, and wrote the manuscript.

Meltem Yesilcimen Akbas: Analyzed data, supervised the experiment's progress, and wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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