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Ethanol Production from Desizing Wastewater using Co-Culture of *Bacillus subtilis* and *Saccharomyces cerevisiae*

Sukon Tantipaibulvut[a]*, Anawat Pinisakul[b], Phannee Rattanachaisit[b], Kadsarin Klatin[a], Benjaporn Onsriprai[a], Kanokwan Boonyaratsiri[a]

[a]*Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, 10140,Thailand,*
[b]*Department of Chemistry, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, 10140, Thailand,*

Abstract

This research studied the production of ethanol from desizing wastewater of dye-bleaching industry by using co-culture of *Bacillus subtilis* D (*B*) and *Saccharomyces cerevisiae* TISTR 5160 (*S*). This work began with the study of the growth of single culture of *B* and *S* in wastewater by varying incubation temperature. The optimum temperature for the growth of *B* and *S* was 37ºC and 30°C, respectively. For co-culture, the order of *S* inoculation, the inoculum ratio of *B* to *S*, shaking effect, incubation temperature after *S* addition, and the effect of nitrogen sources were studied. The optimum condition for ethanol production of co-culture was shaking at 150 rpm and 37ºC with the inoculation of *B* 1 day before *S* and the inoculum ratio of *B* to *S* was 5:10. This gave the ethanol content of 5.8 g/L after 48 h. Addition of nitrogen sources prolonged the period the ethanol production going to the maximum.

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Keywords: Ethanol; dye-bleaching industry; wastewater; *Bacillus subtilis*; *Saccharomyces cerevisiae*

1. Introduction

Textile plants, particularly those involve in dyeing and finishing process, consist of numerous wastewater streams from various operations. Among those wastewater streams, the one from the sizing and desizing operations was the main sources of pollution [1]. In the sizing operation, the fibers are coated with a layer of sizing agents, mainly consisting of biopolymers like starch and other polysaccharides. After a weaving operation, the sizing agents are removed by washing with hot water. This washing step is

* Corresponding author. Tel.: +0-662-470-8938; fax: +0-662-470-8891.
E-mail address: sukon.tan@mail.kmutt.ac.th.

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called the desizing step. Desizing wastewater makes up approximately 50% of the organic load in wastewater discharged from the textile finishing industry [2, 3].

Bioethanol is currently considered as one of the best substitutes for petroleum-derived fuels in many countries to solve their energy requirements in an environmentally friendly way [4]. It can be produced from sugar, starch or lignocellulosic material. The production of ethanol from starch requiring two-step process where the starch is first converted to glucose by hydrolysis; the resulting sugar can in turn be converted to ethanol by fermentation [5].

Considering the substantial availability of high content of readily biodegradable matter at very low price by local dye-bleaching processing plants, the use of desizing wastewater as a low cost substrate for ethanol production could provide a benefit for the cost reduction. However, there is little information available on the use of desizing wastewater for the production of ethanol.

The aim of this study was to use *Bacillus subtilis*, which produces α-amylase, to co-cultivate with *Saccharomyces cerevisiae* to study the effect of yeast inoculation at different time point, shaking condition, fermentation temperature and the addition of nitrogen sources on the ethanol production.

2. Materials and Methods

2.1. Microorganisms and the source of desizing wastewater

Bacillus subtilis D (*B*) was obtained from Department of Microbiology, King Mongkut's University of Technology Thonburi, and *Saccharomyces cerevisiae* TISTR 5160 (*S*) was obtained from Thailand Institute of Scientific and Technology Research (TISTR), Thailand. They were maintained at 4°C on a nutrient agar (NA) slant and a yeast-malt (YM) agar slant, respectively; and subcultured monthly.

The desizing wastewater, provided by a dye-bleaching plant in Samutprakarn Province, was kept at 4°C less than 20 days. The composition and characteristics of the effluent are indicated in Table 1.

Table 1. Composition and characteristic of the effluent from the desizing process of dye-bleaching industry.

Parameter	Unit	Value range
pH		4.7 – 6.5
Temperature	°C	26 – 31
Total solids	mg/L	15,800 – 34,800
Total suspended solids	mg/L	1,620 – 2,180
Total dissolved solids	mg/L	13,620 – 33,260
BOD	mg/L	7,160 – 8,954
COD	mg/L	73,260 – 79,540
Starch	μg/ml	573 – 1881
Reducing sugar	μg/ml	1,700 – 10,325

2.2. Growth of monoculture in desizing wastewater

The cells of *B* from NA slant and *S* from YM agar slant were first grown in nutrient broth (NB) and YM broth, shaking at 150 rpm for 24 h, at 37°C and 30°C, respectively. Then, 10-ml of this culture (OD_{660} = 0.8) was transferred to 100 ml of desizing wastewater and shaking at 150 rpm, at 30 or 37°C, to investigate which temperature is suitable for their growth. Samples were withdrawn at 24-h interval and the spread plate counts on NA (for *B*) and YM agar medium (for *S*) was used for counting the viable cells.

2.3. Ethanol production by co-culture

For ethanol production by co-culture of *B* and *S*, cells from NA slant of *B* and from YM slant of *S* were inoculated into 100 ml of NB and YM broth; shaking at 150 rpm, 37°C and 30°C for 24 h, respectively. The amount of *B* and *S* cell suspension (OD_{660} = 0.8) were varied in both the order of inoculation and the ratio of each culture inoculated into wastewater (100 ml). Co-culture was incubated

under static condition at either room temperature (RT, 23-26°C) or 37°C for ethanol fermentation. At 24-h interval, culture broths were analysed for ethanol content.

Fermentation was also carried out at static or shaking condition with the addition of various nitrogen sources (ammonium nitrate, sodium nitrate, peptone and yeast extract) at 2% final concentration and incubated at 37°C. All data are averages of two experiments, each with two replicates.

2.4. Analytical Methods

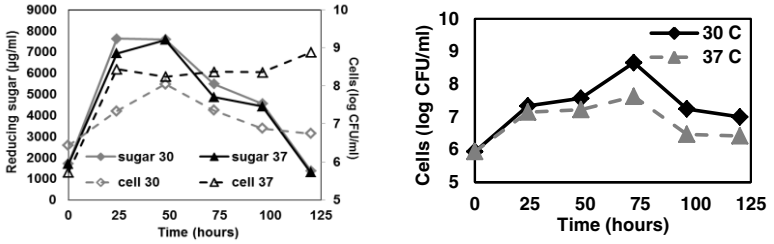
Reducing sugar was estimated by the DNS method and measuring the absorbance at 540 nm [6]. Residual starch was determined by measuring the blue colour of starch-iodine complex at 620 nm [7].

Total solids, total suspended solids, total dissolved solids, COD and BOD were measured according to procedures described in Standard Methods for the Examination of Water and Wastewater [8]. Ethanol concentration in fermented wastewater was analysed by a gas chromatograph.

3. Results and Discussion

3.1. Growth of monoculture in desizing wastewater

B. subtilis (*B*) grew better at 37°C than at 30°C with the maximum cell number of 8.44 log CFU/ml at 24-h fermentation and it was stable around this until the end of fermentation (Fig 1(a)). The optimum temperature for maximum growth of *B* was reported to be 40°C [9, 10]. However, the concentration of reducing sugar at both temperatures was not significantly different. On the other hand, *S. cerevisiae* (*S*) grew better at 30°C than at 37°C. The maximum cell number was 8.66 log CFU/ml at 72-h fermentation compared with 7.62 log CFU/ml in the culture grown at 37°C (Fig 1(b)). Salvadó et al. [11] reported that the optimum temperature for the growth of *S. cerevisiae* T73 was around 32°C. In addition, *S. cerevisiae* TISTR 5160 cannot produce ethanol when it was grown alone in desizing wastewater. Because Thailand is a hot country, the fermentation temperature chosen for next experiments was 37°C.



(a) *B. subtilis* (b) *S. cerevisiae*

Fig. 1. Growth and reducing sugar profile in desizing wastewater of *B. subtilis* (a) and growth profile at either 30 or 37°C of *S. cerevisiae* TISTR 5160 (b), shaken at 150 rpm for 5 days.

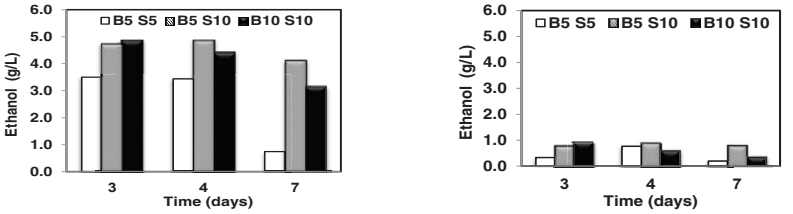
3.2. Ethanol production by co-culture

3.2.1. Effect of yeast inoculation at various time points on ethanol production

Simultaneous saccharification and fermentation of non-hydrolysed starch involves the enzymatic hydrolysis of starch to fermentable sugars and their conversion to ethanol in the same fermenter, thereby preventing the inhibitory effect of sugar on amylase activity [12]. Direct fermentation of starch to ethanol was carried out using co-culture of *B* and *S*. To determine which time of *B* growth that gave the best ethanol production by *S* in the co-culture, the stages of *S* inoculation in the desizing wastewater containing *B* was investigated. *B* may be inoculated together with *S* in the wastewater (simultaneous inoculation, SI) and grown at 37°C, 150 rpm; or inoculated and grown for 1 day (B1S) or 2 days (B2S) before *S* inoculation in the wastewater. Furthermore, the inoculum ratio of each culture was also studied, that was 5% of *B* to 5% of *S* (B5S5), 5% of *B* to 10% of *S* (B5S10) and 10% of *B* to 10% of *S* (B10S10).

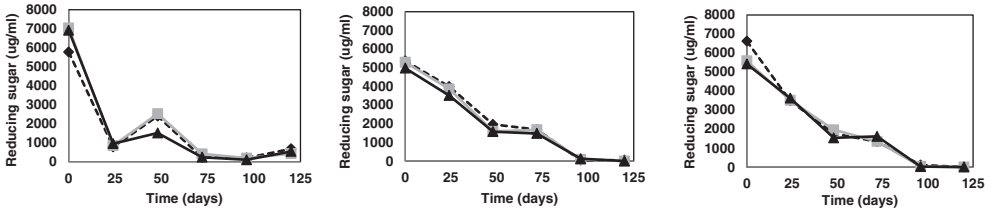
As can be seen from Fig 2, the production of ethanol can be investigated only in the co-culture of B1S and B2S. This may be due to the inability of *S* to degrade starch into glucose and SI cannot provide enough glucose for *S* to produce ethanol. The ethanol content for B1S at the 3rd day of fermentation was higher than 3.5 g/L; while that of B2S was lower than 1 g/L. The ethanol content of B5S10 was increased to 4.8 g/L on the 4th day before reduced to 4 g/L at the 7th day of fermentation. On the other hand, the ethanol content of B10S10 gradually reduced from 4.8 g/L to 3.1 g/L on the 7th day of fermentation.

As shown in Fig 3, reducing sugar concentration decreased sharply for the first 48 hours of fermentation in B1S and B2S without any sugar remaining at 120 h. From these results, the inoculum ratio of B5S10 and the inoculation time of B1S were chosen for further studies.



(a) inoculation of B one day before S (b) inoculation of B two day before S

Fig. 2. Effect of yeast inoculation time and co-culture inoculum ratio on ethanol production; B = *B. subtilis*, S. = *S. cerevisiae*.



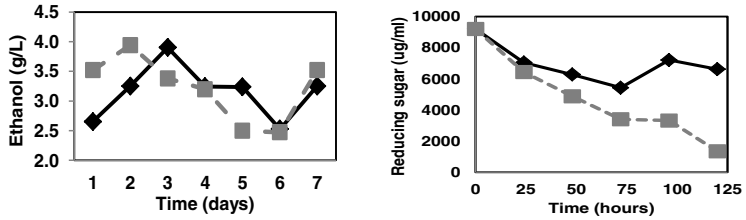
(a) simultaneous inoculation (b) inoculation of B one day before S (c) inoculation of B two day before S

Fig. 3. Effect of yeast inoculation time and co-culture inoculum ratio of *B. subtilis* (B) and *S. cerevisiae* (S) on reducing sugar consumption; B5S5 ◆, B5S10 ■, B10S10▲.

3.2.2. *Effect of temperature after yeast inoculation on ethanol production in static condition*

The influence of temperature on ethanol production by *S* was evaluated in static condition with the inoculum ratio of B5S10 and inoculation time of B1S at 37°C and 150 rpm. After *S* was inoculated in the wastewater, the co-culture was incubated at either RT or 37°C statically. Fig 4 shows the time course of ethanol production and sugar consumption at two temperatures studied. It appeared that when the temperature increased, the time the ethanol concentration going to the maximum was shortened. This was similar to that found by [13]. The maximum concentration of ethanol produced was 3.9 g/l at the 2nd day and 3rd day of fermentation at 37°C and RT, respectively. [14] found that the optimum temperature for maximum ethanol production using starch in co-culture of *S. diastaticus* and *S. cerevisiae* 21 was 30°C and there was no remarkable loss in ethanol yield up to 40°C. [15] reported that maximum ethanol was produced at 38°C by *S. cerevisiae* in a fermentation of glucoamylase treated starch.

The sugar consumption of co-culture grown at 37°C was also higher than that grown at RT. Therefore, the temperature used for ethanol production in next experiment will be 37°C.

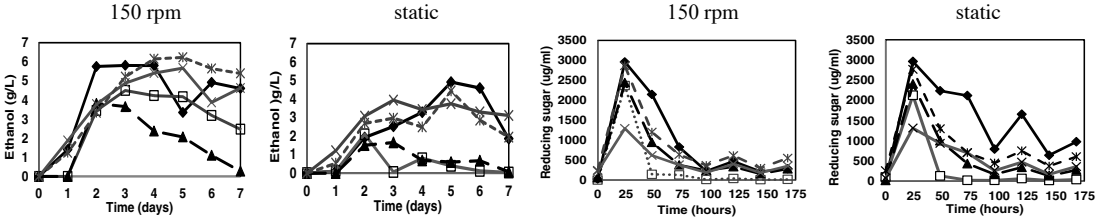


(a) ethanol (b) reducing sugar

Fig. 4. Effect of alcoholic-fermentation temperature on ethanol production and sugar consumption of B1S with B5S10, after the inoculation of yeast, the incubation temperature was either room temperature (23-26°C, ♦) or at 37°C (■) in static condition.

3.3.3. *Effect of nitrogen sources and shaking condition after yeast inoculation on ethanol production*

Ethanol production depends on the fermentation mode [16]. In order to find a suitable mode for higher ethanol production, the fermentation was carried out in static and shaking condition. Suitable mixing of nutrients and cells greatly affects the biochemical reaction. Mixing provided by a proper shaking of the vessel results in a homogenous supply of nutrients to the cells and as a result a higher fermentation rate [17]. Furthermore, under the fermentation condition a small amount of O_2 is required for yeast cells to synthesize the unsaturated fatty acids which are essential for plasma membrane integrity [18]



(a) ethanol content (b) sugar concentration

Fig. 5. Effect of nitrogen sources on ethanol production and sugar consumption in shaking (left) and static condition (right) at 37°C with co-culture of B1S; non-supplemented culture ◆, ammonium nitrate □, sodium nitrate▲, peptone X, yeast extract ✳

The content of ethanol reached 5.8 g/L on the 2nd day in shaking condition compared to 4.9 g/L on the 5th day in static condition (Fig 5). This was similar to that found by [19]. A slight increase in ethanol production in shaking condition may be due to the more effective contact of yeast cells with the nutrients in shaking compared to static condition. This can be supported by the fact that biochemical reactions during fermentation are greatly affected by the mixing and mass transfer of substrates to microorganisms [17]. Breisha [18] observed that the optimum condition for ethanol production was the addition of a small amount of oxygen at the beginning of fermentation.

Furthermore, the addition of nitrogen sources in the wastewater caused the decrease of ethanol content for approximately 33% compared to those of non-supplemented one, especially in shaking condition for the first 48-h after fermentation. It also prolonged the period the ethanol production going to the maximum. For example, in shaking condition the addition of yeast extract into wastewater obtained the maximum ethanol (6.2 g/L) on the 5th day, while the non-supplemented one gave the maximum ethanol (5.8 g/L) on the 2nd day and it was around this level until the 4th day of fermentation. [20] found that when sucrose was used as an adjunct in sweet sorghum juice, 3 g yeast extract l^{-1} and 5 g peptone l^{-1} gave the maximum ethanol production efficiency with the concentration, productivity and yield of 120.68 g l^{-1}, 2.01 g l^{-1} h^{-1} and 0.51 g g^{-1}, respectively. The amount of sugar remaining was also dependent on supplemented nitrogen sources. Not all sugars in the media were completely utilized by the yeast.

4. Conclusion

It is concluded that desizing wastewater is possible to be used as an economic source for bio-ethanol production for fuel by direct fermentation of starch to ethanol with co-culture system**.** The optimum condition for ethanol production of co-culture was shaking at 150 rpm and 37ºC with the inoculation of *Bacillus subtilis* (B) 1 day before *Saccharomyces cerevisiae* (S) and the inoculum ratio of B5S10. This gave the ethanol content of 5.8 g/L after 48 h. Addition of nitrogen sources prolonged the period the ethanol production going to the maximum.

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