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Project: Ethanol Productivity Optimization by Molasses -Cassava Waste Pulp Mixture Fermentation

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Project: Ethanol Productivity Optimization by Molasses -Cassava Waste Pulp Mixture Fermentation

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Co-fermentation of Molasses with Cassava Waste Pulp Hydrolysate to Ethanol for Economic Optimization

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บทคัดย่อ ไฮโดรไลเซส (hydrolysate)ของกากมันสำปะหลังถูกนำมาหมักร่วมกับกากน้ำตาลเป็นเอทานอลเพื่อให้ค่าการผลิตมีความคุ้มทุน โดยใช้เชื้อ *Saccharomyces cerevisiae* พบว่าสภาวะที่เหมาะสมที่สุดของการหมักคือ น้ำตาลทั้งหมดเริ่มต้น 245 กรัม/ลิตร เติม KH_2PO_4 8 กรัม/ลิตร หมักที่ 30 ° ซ นาน 48 ชม. กวนผสมที่ 100 รอบ/นาที ภายใต้สภาวะจำกัดออกซิเจน ได้เอทานอล 70.60 กรัม/ลิตร (0.31กรัม เอทานอล/กรัม น้ำตาลทั้งหมด) และเมื่อเติมกากใยของกากมันสำปะหลัง ที่เหลือหลังการย่อยกากมันฯด้วยเอ็นไซม์ (residual fiber) ลงไปหมักร่วมด้วย 30 กรัม น้ำหนักแห้ง/ลิตร เอทานอลที่ผลิตได้จะเพิ่มขึ้นเป็น 74.36 กรัม/ลิตร (0.32 กรัม เอทานอล/กรัม น้ำตาลทั้งหมด) การนำไฮโดรไลเซสของกากมันสำปะหลังมาหมักร่วมกับกากน้ำตาลนี้ มีข้อดีคือทำให้เป็นการหมักที่ไม่จำเป็นต้องเติมรีดิวซ์ไนโตรเจนให้แก่เชื้อ

คำสำคัญ: กากน้ำตาล กากมันสำปะหลัง การหมักร่วมกัน เอทานอล *S. cerevisiae*

Abstract Molasses was co-fermented with cassava waste pulp (CWP) hydrolysate to ethanol by *Saccharomyces cerevisiae* TISTR 5606 (SC 90) for economic optimization. The optimal condition found for ethanol production from the mixture of molasses and CWP-enzymatic hydrolysate (molasses/CWP-EH) was 245 g/l initial total sugar supplemented with 8g/l KH_2PO_4 when fermented for 48 h at 30 °C under an oxygen limited condition with agitation at 100 rpm, to give an ethanol of 70.60 g/L (0.31 g ethanol/g total sugar). The molasses/CWP-EH containing cassava tuber fiber (CTF, solid residue of CWP after enzymatic hydrolysis) at 30 g/l dry weight increased the ethanol production to 74.36 g/L (0.32 g ethanol/g total sugar). Co-fermentation of molasses with CWP-EH had the advantage of not requiring any supplementation of the ferment with reduced nitrogen.

Key words: Molasses · Cassava waste pulp · Co-fermentation · Ethanol · *S. cerevisiae*

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1. Introduction

Molasses, a waste product from the sugar industry, is a major substrate for ethanol production in Thailand. Because it contains a high concentration of sucrose, at 30–40% (w/w), and other nutrients necessary for microbial growth and ethanol production [1], pretreatment and saccharification steps are not required for fermentation with molasses. However, it was reported that supplementation of molasses with urea, MgSO4, MnCl2 or soy bean powder could improve the ethanol production from molasses. Urea and soy bean powder provide a reduced nitrogen source for yeast growth, Mg2+ is a cofactor of some enzymes in the ethanol fermentation pathway and Mn2+ increases the activity of invertase, an enzyme that hydrolyses sucrose to glucose and fructose, which is necessary for sucrose fermentation of *Saccharomyces cerevisiae* [2, 3].

Cassava waste pulp (CWP), a waste product from the cassava starch industry, is produced at ~7 million tons annually in Thailand [4]. Its major composition is starch at ~56% (w/w dry weight; DW) [5], but it is not suitable as an animal feed because it has a low nitrogen content, while it is not economically viable as a substrate for ethanol production on its own because its saccharified sugar content is too dilute.

Although there has been an effort to improve the technology to extract as much starch from cassava tubers as possible, at present a considerable amount of starch remains in the CWP. The dumping of the CWP leads to environmental problems, including smell pollution, from the microbial deterioration of the residual starch in CWP. Here, the value addition of CWP was performed by the co-fermentation of its saccharified starch with molasses to ethanol using *S. cerevisiae*. The nutrient supplementation, and their optimal concentration, required for maximizing the ethanol production were determined.

2. Materials and methods

2.1. Materials

The molasses sample was collected from the Khonburi Sugar Co., Ltd., Nakhon Ratchasima province, Thailand, and was kept at 4 °C until use. The CWP was collected from the Sa-nguan Wong Industry Co., Ltd., Nakhon Ratchasima province, Thailand and was kept at -20 °C. Chemical composition of CWP is shown in Table 1. Just before use it was thawed to room temperature.

The enzymes, cellulase (AccelleraseTM1500; 2500 Carboxymethylcellulose (CMC) Units (U)/g and 650 p-Nitrophenyl-glucoside (pNPG) U/g), α-amylase (Spezyme alpha; 13,775 U/g) and glucoamylase (GC 147; 580 U/g) were obtained from Genencor, Danisco US Inc., USA.

The *S. cerevisiae* TISTR 5606 (SC 90) was obtained from the Thailand Institute of Science and Technology and was maintained on a yeast/peptone/dextrose (YPD) agar slant (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose and 20 g/l agar, pH 5.5) at 4°C.

2.2. Hydrolysis of the CWP

2.2.1. Acid hydrolysis

The CWP at the designated substrate loading level of 100 g/l was hydrolyzed at 1g CWP (DW) in 9 mL of 1 N HCl (1g CWP/0.33g HCl) and autoclaved at 121°C, 15 lb/in2 (0.1034 MPa) for 15 min and filtered [5]. The filtrate was harvested, and is hereafter defined as the CWP-acid hydrolysate (CWP-AH), and its reducing sugar content was analyzed by the Somogyi-Nelson method [6].

2.2.2. Enzymatic hydrolysis

The CWP at a 250 g/l substrate loading was suspended in deionized water (1 g CWP (DW)/4 mL deionized water) and autoclaved at 121°C, 15 lb/in2 for 15 min. Then it was sequentially hydrolyzed by cellulase (1.41 CMC U/g) at 50°C for 24 h, α-amylase (48 U/g) at 85°C for 1 h and glucoamylase (4.8 TGA U/g) at 60°C for 3 h [5]. The resultant CWP slurry was filtered, the filtrate hereafter being defined as the CWP-enzymatic hydrolysate (CWP-EH), and its reducing sugar content was analyzed. In some experiments the solid residue of the CWP separated from the above filtrate, designated here as cassava tuber fibers (CTF), was dried at 65°C and remixed into CWP-EH at 0, 25, 30, and 35 g/l.

2.3. Ethanol production from CWP-AH and CWP-EH

2.3.1. Inoculum preparation

A single colony of *S. cerevisiae* TISTR 5606 grown on YPD agar at 30°C for 48 h was inoculated into 50 mL YPD broth in a 250-mL Erlenmeyer flask and incubated at 30°C on a gyrotary shaker (100 rpm) for 24h. The culture was then transferred into 100 mL fresh YPD broth in a 500-mL Erlenmeyer flask at 1% (v/v) and incubated at the same condition until late log phase (18 h). The cell number/mL of the culture was determined using a haemacytometer under a light microscope before harvesting by centrifugation (4°C, 8000 rpm, 15 min), and used as the inoculum by suspending in fermentation medium.

2.3.2. Fermentation medium preparation

The CWP-fermentation medium was prepared by adding 2 g/l (NH4)2SO4 into 35 mL of the CWP-AH or the CWP-EH in a 50-mL Erlenmeyer flask, adjusting the medium to pH 5.5 and sterilizing it by autoclaving at 110°C (10 min).

The molasses/CWP-EH fermentation medium was prepared by mixing molasses (26.1 g) into CWP-EH (100 mL). The resultant supernatant analysed for the initial total sugar content by the phenol sulfuric acid method (Dubois et al. 1956) was 265 g/L. The molasses/CWP-EH mixture was supplemented with 2g/L (NH4)2SO4, 2g/L KH2PO4, 0.75 g/L MgSO4.7H2O and 10 g/L yeast extract; and then sterilized by autoclaving (110 °C, 10 min).

2.3.2. Ethanol fermentation

The fermentation medium (CWP-AH, CWP-EH or molasses/CWP-EH (standard or modified)) was inoculated with the inoculum cells to a final 108 cells/mL and incubated at 30°C with agitation at 100 rpm for 48 h under an oxygen limited condition. The oxygen limited condition was obtained by capping the 50-mL Erlenmeyer flask with a rubber stopper connected to an airlock containing saturated copper sulfate solution. After the 48-h fermentation period the culture medium was clarified by centrifugation and the ethanol concentration in the supernatant was analyzed by gas chromatography method [7].

2.4. Nutrient requirement for maximum ethanol production

Composition of the molasses/CWP-EH medium was varied by adding or not adding the nutrient supplements as shown in Table 2, and then ethanol fermentation of the modified molasses-CWP-EH was performed and analyzed for the ethanol content as above. The CWP-EH fermentation media was then further optimized, in terms of the highest ethanol production, by univariate sequential analysis of the optimal concentration of KH2PO4, initial total sugar and CTF in the ferment. The molasses/CWP-EH was supplemented with various concentrations of KH2PO4 (0, 2, 4, 6, 8 and 10 g/L final), and then fermented for 48 h prior to the analysis of the ethanol level. Then the initial concentration of total sugar in the molasses/CWP-EH mixture supplemented with the determined optimal level of KH2PO4 was varied at 205, 225, 245 and 265 g/L and fermented for 48 h prior to the analysis of the ethanol level. Finally the CWP-EH containing the determined optimal level of KH2PO4 and molasses for the optimal initial total sugar level was supplemented with CTF 0, 25, 30 or 35 g/L and fermented for 48 h prior to the analysis of the ethanol level.

3. Results

3.1. Acid and enzymatic hydrolysis of CWP

Hydrolysis of CWP at a 100 g/L substrate loading by HCl yielded 31.6 g/l reducing sugar or 0.28 g reducing sugar/g (DW) CWP. When the CWP was hydrolyzed by cellulase, α-amylase and glucoamylase at a 250 g/L substrate loading level, 34.9 g/l reducing sugar (0.14 g reducing sugar/g CWP (DW)) was obtained.

3.2. Ethanol production from the CWP-AH and CWP-EH

Ethanol fermentation was performed using the CWP-AH or the CWP-EH fermentation medium and inoculating with *S. cerevisiae* TISTR 5606 (108 cells/mL final) and incubating under an oxygen-limited condition at 30°C for 48 h with agitation at 100 rpm. The ethanol yield obtained was 0.149 g ethanol/g reducing sugar and 0.242 g ethanol/g reducing sugar for the CWP-AH and the CWP-EH, respectively. Accordingly, the CWP-EH was selected. Recently, the potential of CWP containing 67.8%w/w (DW) starch as a raw material for ethanol production was evaluated. It was found that the starch hydrolysate (22.6 g/L glucose) obtained from 30 g CWP (DW) gave an ethanol production of 9.9 g (0.43 g ethanol/ g glucose) without (NH4)2SO4 supplementation at 48 h [8]. The ethanol production from a CWP-EH by *Rhizopus oryzae* gave a higher ethanol yield and productivity than with glucose at the same carbon content, which may reflect that the organic nitrogen in the CWP-EH promoted the *R. oryzae* population growth and caused a more rapid onset of oxygen limitation in the medium and so increased the ethanol production [5].

The CWP-EH contained a low concentration of reducing sugar (34.9 g/l). This would not be economically viable to recover and so the CWP-EH was co-fermented with molasses, another abundant and sustainably renewable waste product in Thailand.

3.3. Ethanol production from the molasses/CWP EH mixture

The level of some nutrients present in the molasses and in the CWP-EH that are known to potentially influence ethanol production by *S. cerevisiae* [1, 9, 10] as analysed by Food research and testing laboratory (FRTL), Faculty of Science, Chulalongkorn University are shown in Tables 3 and 4, respectively.

3.3.1. Nutrient requirement for a more optimal ethanol production level

Various modified molasses/CWP-EH media were prepared as described in Table 2 and then fermented to ethanol for 48 h as described in the methods section. The lowest ethanol concentration was obtained in the media with all four supplements (media No. 1), some 1.05-fold less than the unsupplemented molasses/CWP-EH (Fig. 1). A slight reduction in the obtained ethanol concentration was also noted with the (NH4)2SO4 + KH2PO4, (NH4)2SO4 + yeast extract and the KH2PO4 + MgSO4 supplemented media, suggesting no clear single inhibitory nutrient. High ethanol concentrations were obtained with media No. 2, 10–12 and 15, the highest (62.67 g/L) being was found in the modified molasses/CWP-EH that was supplemented with only 2g/l KH2PO4 (medium No.15), again showing no clear pattern of a single optimal single nutrient supplement.

3.3.2. Optimal concentration of KH2PO4for ethanol fermentation in the molasses/CWP-EH

Various concentrations of KH2PO4 were then added into the molasses/CWP-EH mixture and then fermented for 48 h to ethanol as described in the methods. The highest ethanol concentration (70.92 g/L) in the 48-h-old ferments was found when the

molasses/CWP-EH mixture was supplemented with 8g/l KH_2PO_4 (Fig. 2), although this was not significantly greater than that at 6 or 10g/l KH_2PO_4, but was 1.12-fold higher than that at 2 g/l KH_2PO_4.

3.3.3. Optimal initial total sugar concentration in the molasses/CWP-EH fermentation

The level of molasses in the molasses/CWP-EH supplemented with 8 g/l KH_2PO_4 was varied to give a final initial total sugar concentration from 205-265 g/L and then fermented to ethanol for 48 h. With an initial total sugar content of 245 and 265 g/L a similar ethanol concentration in the 48-h-old ferment was obtained at 70.6 and 70.92 g/L, respectively, which was higher than that obtained with the two lower (225 and 205 g/L) total sugar levels, respectively (Fig. 3). Thus, an initial total sugar concentration of 245 g/L was used in the subsequent trials.

There was no marked effect on the ethanol production level when the 8 g/L KH_2PO_4 was replaced by 8 g/L NaH_2PO_4 (data not shown). The depletion of phosphate in the CWP-EH, and so the need for its supplementation in the ferment, might be caused by the formation of insoluble calcium phosphate. Molasses contains a high concentration of Ca^{2+} because calcium oxide was used to clarify the sugarcane juice in the sugar production process. The molasses used in this study contained 6.8 g/L of Ca^{2+} (Table 3). In accord, a Ca^{2+} ion concentration of 5.47 and 6.6 g/L has been reported in molasses previously by Takeshige and Ouchi [3] and Chotineeranat et al. [1], respectively. Thus, the requirement for phosphate supplementation would depend on the concentration of Ca^{2+}ions in the molasses.

3.3.4. Effect of the addition of CTF on the ethanol production from the molasses/CWP-EH

The inclusion of CTF at 0-35 g/L in the molasses/CWP-EH increased the net ethanol production level in the 48-h-old ferment up to 1.05-fold (Fig. 4), with the highest ethanol concentration (74.36 g/L) being found with the addition of 30 g (DW)/L of CTF. However, further increasing the CTF from 30 to 35 g/L (DW) caused a decrease (1.04-fold) in the obtained ethanol concentration.

The reason that CTF enhanced the net amount of ethanol produced during the fermentation is unclear but several low cost plant materials have previously been reported as biomaterials for cell immobilization by natural adsorption in ethanol fermentation, including sugarcane bagasse [11], sugar beet pulp [12], corn cob and grape pomance [13]. During the fermentation process yeast cells were exposed to several stresses, such as ethanol, CO_2 and oxidative stresses, amongst others [14]. Thus, the CTF might protect yeast cells from these stresses and so result in an increased ethanol production. Regardless, the advantages of the natural adsorption technique are that the yeast population growth is less affected and there is adsorption of new cells and wash out of old cells [15]. In addition, the adsorption technique is simple and easy to operate [16].

4. Conclusion

Based on this study, co-fermentation of molasses and CWP-EH (cassava waste pulp-enzymatic hydrolysate) mixture for ethanol production had the advantage of not requiring any reduced nitrogen supplementation. The addition of CTF (cassava tuber fiber) into the molasses/CWP-EH increased the net ethanol production.

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Table 1 Chemical composition of CWP

Composition	% (w/w DW)
Carbohydrate	67.8
Protein	2.1
Fat	1.5
Moisture	80.0
Ash	3.7

Analysed at Food research and testing laboratory (FRTL), Faculty of Science, Chulalongkorn University

Table 2 Nutrient supplementation in the modified molasses/CWP-EH mixture

Nutrients (w/v)	Medium No.															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2 g/L (NH4)2SO4	+	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-
2 g/L KH2PO4	+	+	-	+	+	+	-	-	+	+	-	-	-	-	+	-
0.75 g/L MgSO4·H2O	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-	-
10 g/L Yeast extract	+	+	+	+	-	-	-	+	-	+	+	+	-	-	-	-

Table 3 Selected nutrient levels in the molasses

Component	Content (g/100 g) *	Analytical method
Inorganic nutrients:		
Nitrogen (N)	3.00 x 10-1	Inhouse method based on AOAC(2012),991.20
Phosphorus (P)	1.20 x 10-1	
Potassium (K)	1.27	Inhouse method based on AOAC(2010),984.27,975.03
Magnesium (Mg)	2.30 x 10-1	
Trace elements:		
Calcium (Ca)	0.68	
Copper (Cu)	< 3.60 x 10-4	Inhouse method based on AOAC(2010),984.27,975.03
Zinc (Zn)	1.70 x 10-4	
Manganese (Mn)	4.43 x 10-3	
Sugars:		
Sucrose	31.69	
Glucose	8.73	Asean Manual of Food Analysis (2011) p.27-32
Fructose	8.87	
Volatile acid:		
Acetic acid	1.00	AOAC(2010),935.57,942.15
Non-volatile acid:		
Lactic acid	1.50	AOAC(2010),935.57,942.15

Based on a specific gravity of molasses of1.38.

Table 4 Selected nutrient levels in the CWP-EH

Component	Level (g/100 g) *	Analytical method
Inorganic nutrients:		
Nitrogen (N)	Not detectable	Inhouse method based on AOAC(2012),991.20
Phosphorus (P)	6.82 x 10-3	
Potassium (K)	1.67 x 10-2	Inhouse method based on AOAC(2010),984.27,975.03
Magnesium (Mg)	2.66 x 10-3	
Trace elements:		
Calcium (Ca)	1.79 x 10-3	
Copper (Cu)	< 3.60 x 10-4	Inhouse method based on AOAC(2010),984.27,975.03
Zinc (Zn)	1.10 x 10-4	
Manganese (Mn)	1.20 x 10-5	
Sugars:		
Sucrose	< 0.10	
Glucose	2.84	Asean Manual of Food Analysis (2011) p.27-32
Fructose	< 0.10	

Based on a specific gravity of the CWP-enzymatic hydrolysate of 1.01.

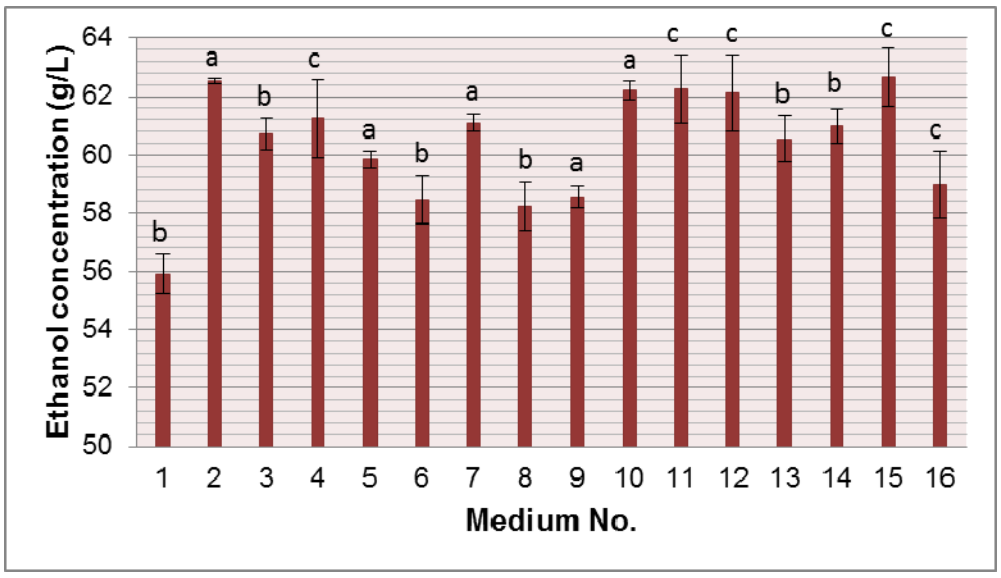

Fig. 1 Effect of nutrient supplementation on the net ethanol production level in the 48-h ferment of the molasses/CWP-EHs. Medium number refers to composition shown in Table 2. Data are shown as the mean ± 1 SD, derived from 3 independent replicates. Means with a different lowercase letter are significantly different ($p < 0.05$; Duncan's multiple means test).

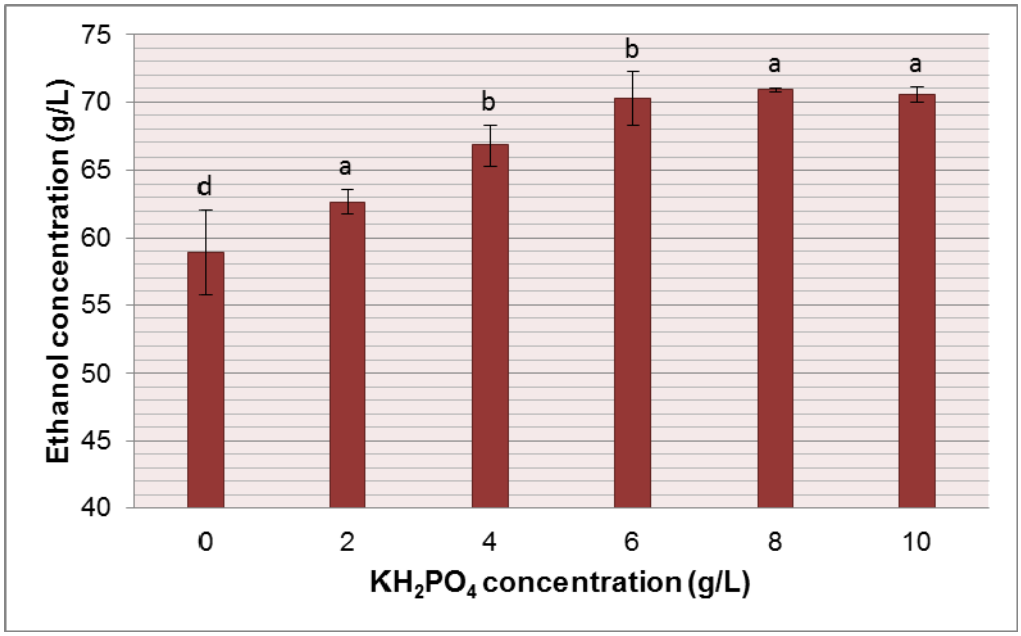

Fig. 2 Effect of the KH_2PO_4 concentration on ethanol production in the molasses/CWP-EH mixture. Medium number refers to composition shown in Table 2. Data are shown as the mean ± 1 SD, derived from 3 independent replicates. Means with a different lowercase letter are significantly different ($p < 0.05$; Duncan's multiple means test).

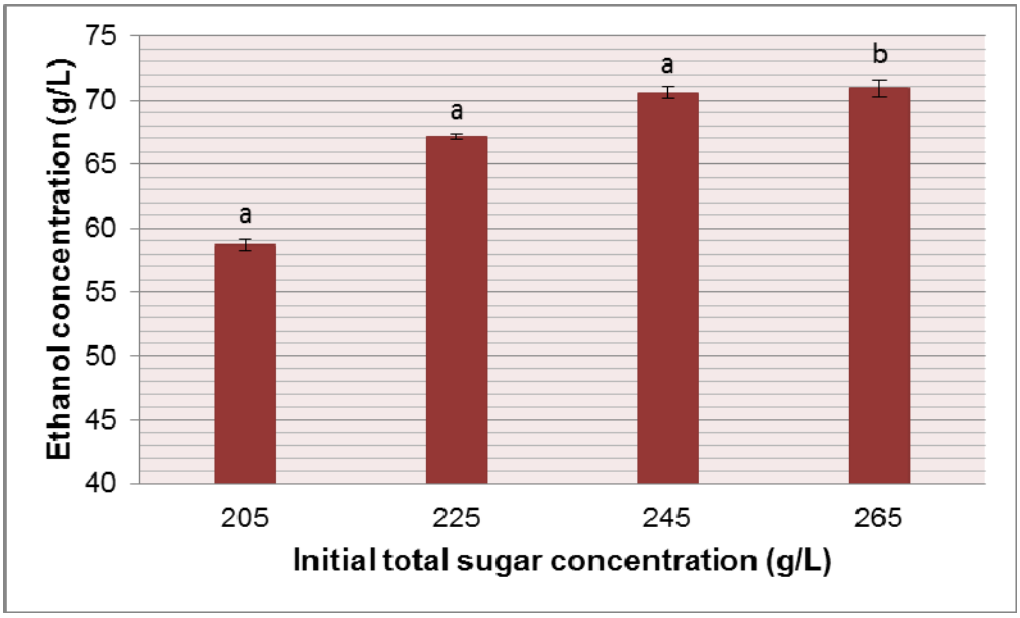

Fig. 3 Effect of the initial total sugar concentration on the net ethanol production in the molasses/CWP-EH mixture supplemented with 8g/L KH_2PO_4. Data are shown as the mean ± 1 SD, derived from 3 independent replicates. Means with a different lowercase letter are significantly different ($p < 0.05$; Duncan's multiple means test).

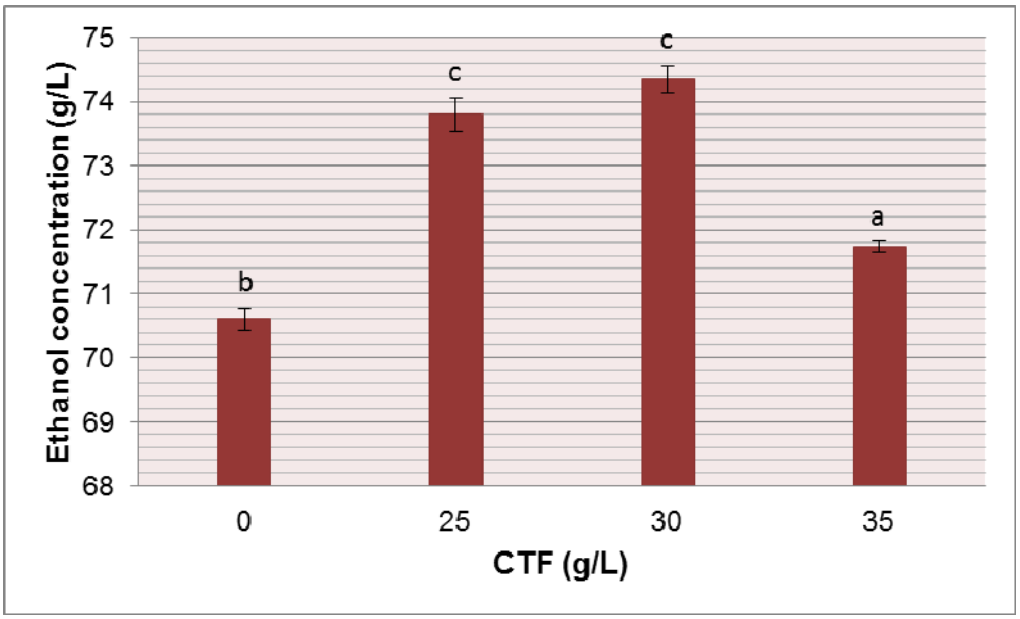

Fig. 4 Effect of added CTF in the molasses/CWP-EH mixture on the ethanol production after 48 h of fermentation. The molasses/CWP-EH mixture contained 8g/L KH_2PO_4 at an initial total sugar level of 245 g/L. Data are shown as the mean ± 1 SD, derived from 3 independent replicates. Means with a different lowercase letter are significantly different ($p < 0.05$; Duncan's multiple means test).

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