Utilization of Sugarcane Syrup and Non-Lysed Dried Spent Brewer's Yeast for Cost Effective Succinate Production by *Actinobacillus succinogenes*

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Abstract

In this study, non-lysed dried spent brewer's yeast (DSB) was evaluated as a low-cost nitrogen source for succinate production by *Actinobacillus succinogenes* 130ZT using sugarcane syrup as an inexpensive source of carbon. The results indicated that the DSB could promote the succinate production efficiency. Succinate concentration at 8.32 g/L with a yield of 0.82 (g/g glucose) from 15 g/L initial glucose that were supplemented with 30 g/L of DSB were achieved. This result was comparable to that obtained using commercial yeast extract at 5 g/L in which succinate concentration at 8.37 g/L was obtained. The highest yield and productivity of succinate at 0.74 g/g glucose and 0.53 g/L/h in glucose-based medium were obtained, respectively by controlling pH at 6.8 and continuously supplying external CO_2. The optimized concentration of sugarcane syrup was observed at 10% (w/v) in which the succinate concentration at 35.75 g/L with a yield of 0.76 (g/g glucose) were attained. The present study suggested that DSB and sugarcane syrup could be used as nitrogen and carbon sources for industrial succinate production.

Keywords: Succinate, sugarcane syrup, dried spent brewer's yeast,

Actinobacillus succinogenes, CO_2

Introduction

Succinate is considered as one of the top 12 building block chemicals that could be manufactured from renewable feedstocks [1]. It is a potential precursor for the synthesis high value products of commercial importance including polymers, surfactants, green solvents, detergents, flavors and fragrances [2]. Up to now, succinate is mainly produced commercially from n-butane through the hydrogenation of petroleum-derived maleic anhydride. However, the increase in price of oil and petroleum derivatives has made the microbial production of succinate from cheap carbon substrates as an economically attractive option for succinate as a renewable commodity chemical [3]. In addition, the microbial succinate production incorporates CO_2, a primary greenhouse gas, providing further incentive for production by white biotechnology [4]. Therefore, the succinate fermentation from low-cost substrates offers the opportunity to be both greener and more cost effective than petroleum-based alternative products.

It has been reported by the U.S. Department of Agriculture (USDA, 2008) that raw cane sugar and sugarcane molasses were sold at prices less than 0.50 US$/kg, and therefore many fermentation industries have used refined sucrose and sugarcane molasses as target substrates to produce lower price bio-based products. In Thailand, sugarcane is cultivated in various parts of the country, but the entire crop is used in sugar production. Up to now, many fermentation industries in Thailand, especially ethanol industries have used molasses, a by-product in the production of sugar as the substrate [5]. As the increasing industrial demand for bio-fuel and bio-based chemicals production coupled with an unstable supply of sugarcane molasses, this resulted in the increasing industrial demand for bio-based production. Hence, this carbon source is becoming limited. Moreover, using molasses in fermentation causes serious problems in the downstream processing and in the final waste treatment. Unlike sugarcane molasses, sugarcane syrup containing 55% inverted sugar is not only renewable and abundant but also a cheap source of carbon as compared with another refines carbohydrate like glucose or sucrose [6]. Utilization of sugarcane syrup in the fermentation industry would reduce the use of molasses. An

alternative renewable carbon source is required to sustain the fermentation industries thus sugarcane syrup seems to be the most promising one.

A few literatures have been reported the replacement of a commercial yeast extract in succinate production by the low-cost nitrogen source such as corn steep liquor solution [7] or dried corn steep liquor [8], and spent brewer's yeast hydrolysate [9]. However, the different strategies for using spent brewer's yeast hydrolysate along with various preparation process have used for succinate production. In a previous study, our laboratory has accomplished the development of an inexpensive nitrogen source, which is spent brewer's yeast extract (SBE) for replacement of commercial yeast extract in succinate production by *A. succinogenes* 130ZT [10]. Nevertheless, the preparing of SBE is quite difficult, and needs to be prepared in the large volume of reaction in order to make it more economically as an alternative nitrogen source. Sridee et al. [11] showed that dried spent brewer's yeast cell (DSB), a by-product from brewery industry, contained high nitrogen and many essential mineral salts. Therefore, it may be used as a low-cost nitrogen supplementation for succinate production instead of commercial yeast extract. To the best of our knowledge, there is no report on directly used DSB for succinate production. In this report, we investigate the feasibility of using sugarcane syrup as an alternative carbon source supplemented with DSB as a low-cost nitrogen source with aim to develop an economical succinate production. We also evaluate the effect of sugarcane syrup concentrations, pH values as well as CO_2 supply on the production of succinate in titer, yield, and productivities by *A. succinogenes* 130ZT.

Materials and methods

Materials

Sugarcane syrup containing the total soluble solids of 76.19 ºBrix, 55.79% inverted sugar kindly supported from Mitr Phol Sugar Corp., Ltd., Thailand. The DSB (*Saccharomyces uvarum*) with a moisture content of 10% (dry basis) was kindly provided by Khon Kaen Brewery Company Ltd., Thailand. The SBE was prepared as described previously [10].

Microorganism and inoculum preparation

A. succinogenes 130ZT (DSM 22257) was purchased from the German Type Culture Collection. The strain was pre-culture in 100 mL sealed-anaerobic bottles containing 75 mL of the culture medium with the composition (g/L): $KHCO_3$ 10.0, NaH_2PO_4 8.5, K_2HPO_4 15.5, $MgSO_4.7H_2O$ 0.05, NaCl 1.0 and yeast extract 10.0. The pH of the medium was adjusted at 7.5 with concentrated NaOH before sterilization (15 min at 121ºC). For inoculum preparation, five grams per liter of glucose (autoclaved separately) was added to the medium after sterilization. Aseptic CO_2 was sparged for 2 min to make an anaerobic environment before inoculation. The seed medium was inoculated with 0.75 mL of stock culture and incubated at 37 ºC for 16-18 h with intermittent gentle shaking.

Anaerobic fermentation

Fermentation in anaerobic bottles

Fermentation in anaerobic bottles was carried out in sealed 100-mL anaerobic bottles containing 50 mL culture medium. The fermentation medium was the same as described above. The initial pH of the medium was adjusted at 7.5 before sterilization. Fifteen grams per liter of glucose (autoclaved separately) was added to the medium after sterilization. Aseptic CO_2 was sparged for 2 min to make an anaerobic environment. The medium was incubated at 37 ºC with 150 rpm shaking speed. Succinate production was examined up to 24 h at a regular interval of 12 h.

Anaerobic fermentation in stirred bioreactors

Batch fermentation was performed in 2-L stirred bioreactor. The different pH controlled was estimated (pH 6.2, 6.8, and 7.2 using 3M KOH as a neutralizing base). Further, the effect of carbon dioxide gas was also investigated at different levels (non-limiting CO_2 supply, limiting CO_2 supply for 1 h at the beginning, and without supply of CO_2). Subsequently, production of succinate was optimized at different concentrations of sugarcane syrup (1, 5, 10, 15 and 20% w/v) in the fermentation medium maintained at the optimized pH and performing at the optimized CO_2 supply obtaining from the previous experiment.

Analytical methods

Biomass concentration was determined by measuring the optical density at 550 nm using a spectrophotometer (Spekol-1500, Anatytik Jena, Thailand). The optical densities were then converted to dry cell weight (OD 1.0 = 0.333 mg of cell dry weight/L) and defined as biomass concentration. Organic acids (succinate, formate, and acetate), ethanol, and total sugars were quantified by high performance liquid chromatography (Agilent technology, Japan) equipped with an ion exclusion column (BIO RAD, Aminex, HPX-87H, USA) with a column temperature of 45 ºC using 4 mM H_2SO_4 as a mobile phase with a flow rate of 0.4 mL/min). Concentrations of the three sugars (sucrose, glucose and fructose) were combined and reported as total sugars concentration. The total nitrogen (TN) content in samples was measured by Kjeldahl method [11].

Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL). The differences among mean values were established using Duncan's multiple-ranges test (DMRT) at 95% significance level.

Results and discussions

Effect of yeast concentration on succinate production

In order to improve the efficiency of succinate production DSB was used as nitrogen source, DSB was dissolved in sterilized water with 1:1 solid-liquid ratio. The yeast particles of DSB were then removed by membrane filtration. The suspension after removing of yeast particles was subsequently used as a nitrogen source for succinate production by *A. succinogenes* 130ZT. As shown in Fig. 1, succinate production was increased with increasing of DSB concentration from 5 to 30 g/L. Beyond this concentration, a constant in succinate production was observed. The succinate production at 8.37 g/L was obtained from 15 g/L glucose supplemented with 5 g/L of commercial yeast extract. The succinate level obtained here was comparable to those of succinate formation using 30 g/L DSB or 5 g/L SBE) in which succinate production at 8.32 and

8.14 g/L, respectively. According to the comparison of nitrogen content in various nitrogen sources, DSB has about 2.5 times lower in total nitrogen content (31.78) as compared with commercial yeast extract (85.32) or SBE (83.90) (Table 1). Thus, the higher amount of nitrogen source in succinate production supplemented with DSB was required. Based on the obtained results, supplementing of 30 g/L of DSB provide the equivalent amount of succinate production from either 10 g/L YE or 10 g/L SBE. Sridee et al. [12] compared some nutrients and trace elements in commercial yeast extract, (HiMedia laboratory, India) and DSB (Beerthip Brewery (1991) Co., Ltd., Thailand). They found that DSB contained much higher amount of trace elements such as calcium, magnesium and iron, whereas sodium and chloride contents were lower than that of commercial yeast extract. These higher of trace elements content in DSB may benefit to the growth microorganism and therefore significantly accelerated the rate of biomass formation and succinate production [12]. As for biomass formation, it was revealed that the biomass formation was increased by the increased of DSB concentration from 5 to 50 g/L. This result is acceptable since DSB contained high amount of protein, defined as total nitrogen content (Table 1). It has been shown that *A. succinogenes* is a fastidious microorganism and nitrogen source supplied important growth factors for succinate production [13]. It is implied that DSB contains various amino acids, vitamins, minerals, and growth factors thus promoting growth of microorganisms. Similar result was observed by Jiang et al. [14] who used fresh spent brewer's yeast hydrolysate as extra nitrogen source in succinate production by *A. succinogenes* NJ113. They found that the fresh spent brewer's yeast hydrolysate significantly accelerates the rate of biomass formation and succinate production. In Thailand, the cost of YE (Bio-basic INC, bacteriological grade, Canada), and DSB (Khon Kaen Brewery, Co., Ltd., Thailand) was approximately 160.5 and 0.36 US$/kg, respectively. Moreover, the application of DSB is still limited, being basically used as animal feed [15]. From this result, it is likely that the high cost of commercial yeast extract could be reduced when the fermentation medium was supplemented by DSB. This finding indicated that supplementing of culture medium with DSB as nitrogen source at concentration as 30 g/L was the most promising substrate for commercial succinate production.

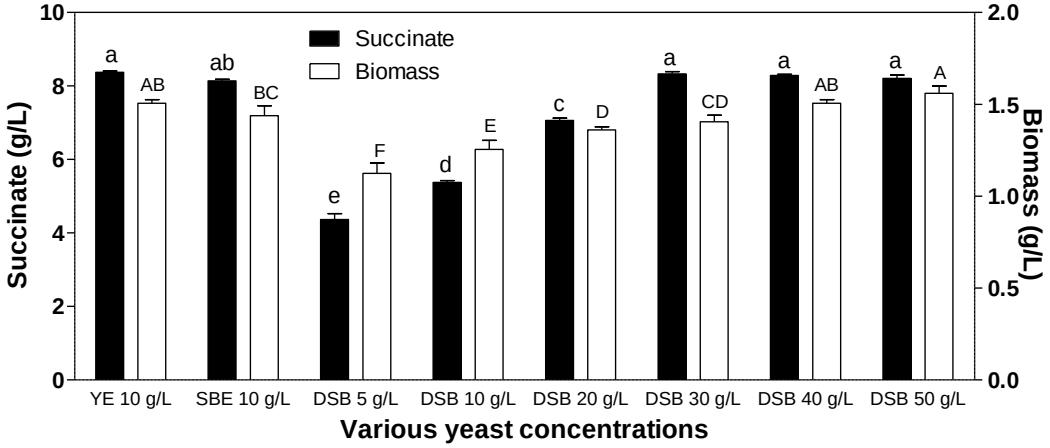

Fig. 1 Effect of various concentration of yeast extract on succinate production by *A. succinogenes* in 100-mL anaerobic bottle. Abbreviations: YE, commercial yeast extract, SBE, spent brewer's yeast extract, DSB, dried spent brewer's yeast cell. Different superscripts are significantly different (p<0.05).

Table 1. Specifications for nitrogen sources.

Nitrogen sources	Total nitrogen (%, dry basis)
Commercial yeast extract (YE)	85.32±0.53[a]
Spent brewer's yeast extract (SBE)	83.90±0.74[a]
Dried spent brewer's yeast (DSB)	35.87±0.77[b]

[a-b]Mean values with different superscripts are significantly different (p<0.05).
Data are means ± standard deviation from three replications.

Succinate production with different external carbon dioxide supply

The effect of different levels of external CO_2 supply on cell growth and succinate production by *A. succinogenes* 130ZT under pH-controlled batch fermentation was shown in Fig. 2. Under these conditions, the higher succinate production and glucose utilization rate were observed in the fermentation with non-limiting CO_2 supply which resulted in 32.11 g/L succinate production with a yield of 0.76 g/g glucose and productivity as 0.45 g/L/h. The reductions in succinate production and glucose utilization rate were found in the fermentation without providing of CO_2, which was about 5 times lower when compared to that of the fermentation with non-limiting CO_2 supply (Fig. 2A

and 2B). Similarly, Samuelov et al. [16] and Lu et al. [17] reported that succinate production was enhanced by adding of CO_2 gas during the fermentation of rumen bacteria. Also, Xi et al. [18] found that higher succinate production by *A. succinogenes* NJ113 was obtained at a higher supply of CO_2 level. They suggested that CO_2 is not only a substrate for succinate production, but it is also necessary for cell growth. On the other hand, external CO_2 supply had a negative effect on *Anaerobiospirillum succiniciproducens* growth, while it had a somewhat positive effect on succinate production [19]. Additionally, Vemuri et al. [20] explained that succinate is principally formed through two pathways: the reductive arm of the TCA cycle and the glyoxylate shunt. Through the reductive arm of the TCA cycle CO_2 is incorporated into the final product succinate via the enzyme phosphoenolpyruvate carboxylase. This gas is therefore necessary for succinate production, and CO_2 availability impacts substrate utilization and succinate accumulation rates. Furthermore, CO_2 functions as an electron acceptor and alters the flux of phosphoenolpyruvate, which metabolizes to pyruvate and lactate/ethanol at low CO_2 levels but makes succinate at high CO_2 concentration [2].

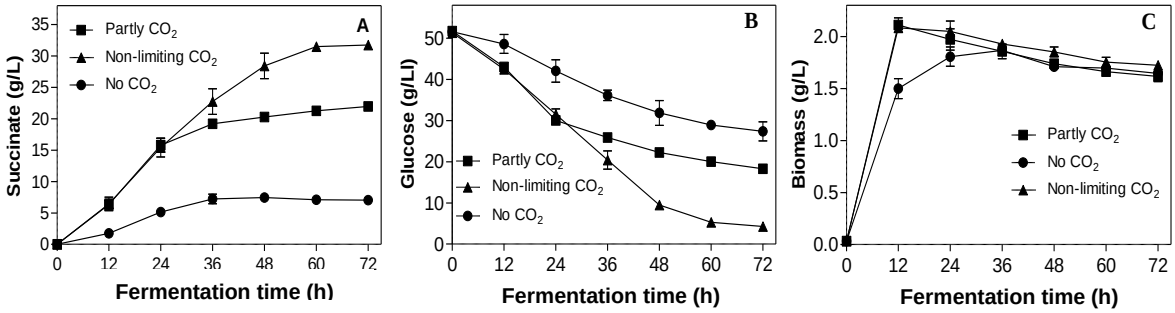

Fig. 2 Effect of carbon dioxide gas on succinate production by *A. succinogenes* 130ZT under pH controlled at 6.8 in 2-L fermenter. Glucose concentration used was 50 g/L. (A) succinate production; (B) glucose utilization; (C) biomass production.

Effect of different controlled pH on succinate production

Since the culture pH can affect the activity of key enzyme for CO_2 fixation in succinate fermentation by succinate-producing rumen bacteria. Fermentations were performed at three pH levels (pH 6.2, 6.8, and 7.2) to confirm the optimum pH for succinate production and to investigate their effect on cell growth. As shown in Table 2, succinate production was maximized at pH 6.8. The concentration of succinate increased from 23.6 g/L to

31.11 g/L when pH increased from 6.2 to 6.8 and slightly decreased thereafter when a controlled pH 7.2 was applied. The highest succinate yields as 0.76 (g/g glucose) and productivity as 0.54 g/L/h, were also obtained at the controlled pH 6.8. Although, the succinate yield and productivity at pH 7.2 and 6.8 were not significantly different (p<0.05), while that at pH 6.2 were much lower. Since more alkaline is needed to maintain a higher pH during fermentation. Based on the obtained result, the preferred controlled pH for succinate production by *A. succinogenes* 130ZT was 6.8, and it was applied for further investigation. Wan et al. [21] reported that the optimized pH for succinate production of *A. succinogenes* 130Z was found at pH 6.8 In addition, Xi et al. [17] showed that *A. succinogenes* NJ113, produced the succinate at the maximal level at pH 6.8, but the succinate level was minimized when pH was increased to 7.4. This observation is consistent with the investigation of Agarwal et al. [7] which showed a pH of 6.5 is optimum for succinate production by *Escherichia coli*.

Table 2 Fermentation kinatics of glucose with different levels of controlled pH by *A. succinogenes* 130ZT in 2-L fermenter at 72 h incubation time under non-limitation CO_2 supply (0.5 vvm CO_2 sparging) supplemented with 30 g/L DSB.

Controlled pH values	Residual glucose (g/L)	*Maximum biomass (g/L)	Succinate (g/L)	Acetate (g/L)	Formate (g/L)	Succinate yield (g/g glucose)	Succinate productivity (g/L/h)
6.2	17.72±0.62	1.86±0.42	23.6±0.60	4.56±0.34	2.02±0.60	0.74±0.19	0.43±0.01
6.8	5.73±0.81	2.15±0.20	35.11±0.50	5.12±0.44	3.45±0.20	0.76±0.06	0.49±0.03
7.2	14.29±0.70	1.98±0.32	26.52±0.62	4.01±0.52	3.12±0.33	0.72±0.02	0.48±0.03

*Maximum biomass was obtained at 24 h. Data are means ± standard deviation from three replications.

Fig. 3 showed the fermentation kinetic of *A. succinogenes* 130ZT by controlling at the optimized pH 6.8 and non-limitation CO_2 supply that was obtained from the glucose base medium experiment. The growth of bacteria started as early as 12 h. However, it reached maximum within 24 h with the biomass of 2.05 g/L. Glucose was consumed remarkably at the first 12 h until 48 h of fermentation time after which it was slightly consumed. Moreover, the residue glucose was left (9.5 g/L) at the end of fermentation in which 40 g/L of glucose was consumed. At the end of fermentation, succinate concentration of 32.11 g/L was observed as a major fermentative product while 6.11 g/L acetate, 3.55 g/L formate, and 0.06 g/L ethanol were also detected.

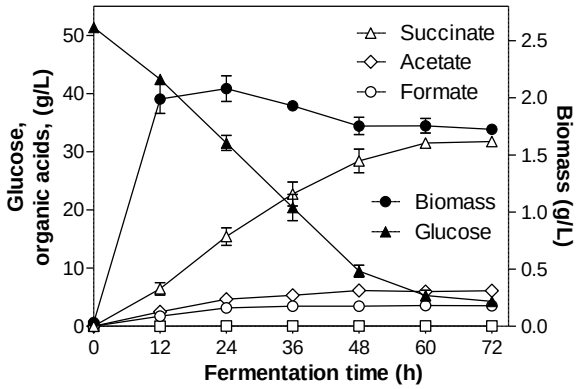

Fig. 3 Time course of glucose fermentation by *A. succinogenes* 130ZT in 2-L fermenter under non-limitation CO_2 supply (0.5 vvm CO_2 sparging) and controlled pH at 6.8. Glucose and DSB concentration used were 50 g/L and 30 g/L, respectively.

Succinate production from different concentrations of sugarcane syrup

Sugarcane syrup is an inexpensive and readily available carbon source for a next target bio-conversion of fermentative succinate. The production of succinate increased with increasing of sugarcane syrup concentration from 1 to 15% (w/v) (Fig. 4). The maximum values of succinate concentration (35.7 g/L) and biomass formation (2.08 g/L) were obtained in the culture with initial sugarcane syrup at the concentration of 15% (w/v). However, the succinate concentration along with biomass formation was decreased when initial concentration of sugarcane syrup at 20% (w/v) was used. Similar phenomenon was observed by Lin et al. [22] who demonstrated that the high level of glucose concentration over 100 g/L was lower the succinate level. Additionally, Kotzamanidis et al. [23] discussed that the decreased sugar utilization encountered with high sugar concentration was due to the osmotic effects during the lactic acid fermentation from beet molasses by *Lactobacillus delbrueckii* NCIMB8130. In this study, it is likely that the cell growth and succinate concentration were also inhibited when high concentration of sugarcane syrup as 20% (w/v) was used. It might be due to substrate inhibition. For biomass formation, the maximum biomass was obtained at 24 h in all fermentations of various sugarcane syrup concentrations. It also found that the titers of succinate at the end of fermentation from 10 to 15% (w/v) sugarcane syrup used were not significantly different. Even though, higher succinate (35.70 g/L) was produced from sugarcane syrup at 15%, w/v the high residual sugars (37.5 g/L) was obtained compared with the residue sugars of 5.8 g/L (from

the initial concentration of 10%, w/v sugarcane syrup) (Fig. 4). Since the higher amount of sugars leftover at the end of fermentation may increase the cost of succinate purification in downstream processing. Therefore, considering economically, the optimal concentration of sugarcane syrup would be at 10% (w/v).

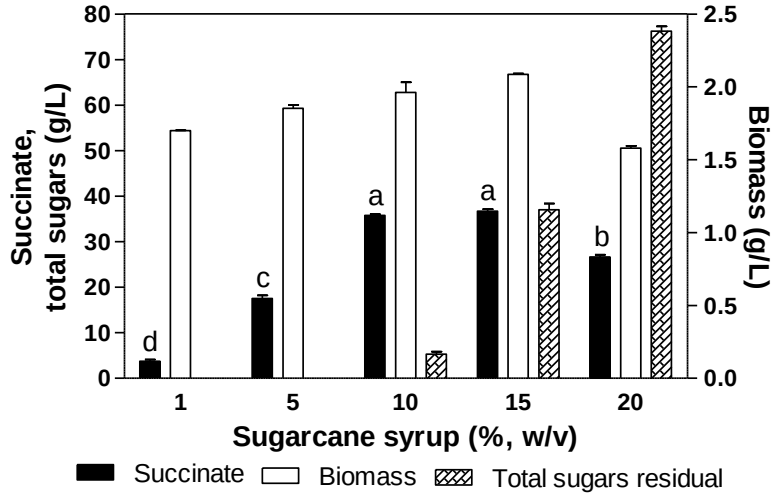

Fig. 4 Effect of different concentrations of sugarcane syrup supplemented with 30 g/L DSB on succinate production by *A. succinogenes* at 72 h incubation time under non-limitation CO_2 supply (0.5 vvm CO_2 sparging) at controlled pH 6.8. Biomass presented was at 24 h incubation time. *Total sugar residual is consisting of glucose, fructose, and sucrose.

Considering the price of sugarcane syrup (containing 76% total sugars) and glucose, they were about 0.53 and 2.67 US$/kg, respectively. Assuming the succinate yield of 80% (w/w) total sugar of sugarcane syrup (or glucose), the raw material cost of the bioprocess was therefore estimated to be 1.33 and 3.34 US$/kg succinate. According to the downstream purification cost accounts for 60-70% of the product cost [12], the total raw material cost of producing succinate from sugarcane syrup (or glucose) would be about 2.26 US$/kg succinate (or 5.68 US$/kg succinate), indicating the cost saving around 60.21%. Therefore, it is clear that the cost of succinate production from sugarcane syrup was much lower than that from glucose.

Conclusion

This study demonstrated that the by-product from brewery industry, DSB could be successfully used as a low-cost nutrient supplement instead of commercial yeast extract for batch succinate fermentation by *A. succinogenes* 130ZT. The succinate production by *A. succinogenes* 130ZT was enhanced by supplying of CO_2 during fermentation in which the succinate fermentation was 5 times higher than that of those without CO_2 supplementation. In term of the controlled pH investigation, the highest succinate yield of 0.76 (g/g glucose) was obtained at controlled pH at 6.8. Further, the optimized concentrations of sugarcane syrup, was observed at 10% (w/v) in which the succinate production at 35.75 g/L was obtained. Based on these results, the cost effectiveness of succinate production could be obtained from sugarcane syrup fermentation supplemented with the DSB.

Acknowledgements

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