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Progress of fermentation methods for bio-succinic acid production using agro-industrial waste by *Actinobacillus succinogenes*

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Abstract

Production of bio-succinic acid through microbial fermentation using agro-industrial waste has been observed extensively. Large scale production is now being considered since bio-succinic acid has broad range application in several industries including agricultural, food, chemical, metal, and pharmaceutical industry as a precursor, ion chelator, and or additive agent. One of promising microbes able to produce bio-succinic acid in large amount and recognized for industrial production is *Actinobacillus succinogenes*. However, several factors still need to be considered in order to optimize the fermentation performance. Therefore, this paper aims to review the progress of fermentation methods and discuss the fermentation strategy focused on the influences of different carbon sources, nitrogen sources, pH regulator, and fermentation configuration to the fermentation parameters including concentration, productivity and yield of bio-succinic acid. As a result, combinations of several fermentation strategies are needed to be performed to realize an efficient and effective bio-succinic acid industry.

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Keywords: *Actinobacillus succinogenes*; Agro-industrial waste; Bio-succinic acid; Fermentation strategies

1. Introduction

Bio-succinic acid – a term of succinic acid ($C_4H_6O_4$) produced from renewable resources – is one of top twelve value added chemicals from biomass based on report released by US Department of Energy [1]. As the most important C4 building-block, bio-succinic acid is widely used in several industries including agricultural, food, chemical, metal, and pharmaceutical industry as a precursor, ion chelator, and or additive agent [2–6].

Naturally, bio-succinic acid is formed by plants, animals, and microorganism as a fermentation product. However, highest production of bio-succinic acid is achieved by anaerobic microbial fermentation [7]. Various microbes can

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be utilized as bio-succinic producer including bacteria isolated from rumen, such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Basfia succiniciproducens* and *Bacillus fragilis* and certain fungi such as *Fusarium*, *Aspergillus* and *Penicillium* species [8–12].

Fortunately, *A. succinogenes* is recognized as one of the most promising microorganisms for industrial production, due to it produces relatively large amount of bio-succinic acid [13,14] and its able to use a broad range of carbon sources [15] including lactose, xylose, arabinose, cellobiose, and other reduced sugars, to produce succinic acid as a major end product [9,16,17]. Besides, various renewable and inexpensive resources have been utilized by *A. succinogenes* to produce bio-succinic acid, including empty fruit bunch (EFB), sugarcane bagasse, corn stover, carob pods, corn fiber, sake lees, rapeseed meal, wheat bran, waste bread, corn stalk, cotton stalk, cane molasses, duckweed, fresh cassava root, cheese whey, corn cob, and corn straw.

This paper aims to review the progress of bio-succinic acid production by *A. succinogenes* as the most promising microorganisms using agro-industrial wastes. Fermentation strategy as the key success of microbial production of bio-succinic acid will be discussed.

2. Bio-succinic acid production by microbial fermentation

Microbial fermentation of bio-succinic acid was conducted for the first time by Georgius Agricola in 1546 [5,7]. Currently, large scale microbial fermentation in order to produce bio-succinic acid is expected, since bio-succinic acid has wide application in several industries. In order to meet the needs, different fermentation strategy of bio-succinic acid production has been observed by many researchers considering several factors affect to the fermentation performance, including carbon sources, nitrogen sources, pH regulator, and fermentation configuration. As the promising microorganism, the summary will be focused on the use of *A. succinogenes* as the producer of bio-succinic acid, as shown in Table 1.

3. Production of bio-succinic acid using different carbon sources

Various carbon sources have been employed by researchers to produce bio-succinic acid using *A. succinogenes*. In order to meet industrial needs by reducing production cost, carbon sources were originated from several agro-industrial wastes as the renewable and inexpensive resources which abundantly available. Empty fruit bunch (EFB) of palm oil has been utilized to produce bio-succinic acid. By using autoclave-alkali pretreatment, reducing sugar of EFB was used as the fermentation substrate obtaining 33.4 g/L bio-succinic acid concentration with the productivity of 1.69 g/L/h and yield of 0.47 g/g substrate [36]. In the other side, sugarcane bagasse has been observed as the carbon sources of bio-succinic acid fermentation. By using batch fermentation mode, concentration of bio-succinic acid obtained was 22.5 g/L, with productivity of 1.014 g/L/h and yield of 0.43 g/g substrate [18]. Meanwhile, 70.81 g/L of bio-succinic acid concentration by using fed-batch fermentation mode, with productivity of 1.42 g/L/h and yield of 0.815 g/g sugar [23].

Corn stover has also been utilized as the carbon sources by several researchers. Bio-succinic acid concentration obtained was 42.8 g/L, productivity of 1.27 g/L/h, and yield of 0.74 g/g sugar by using yeast extract and corn steep liquor as the nitrogen sources [29]. Moreover, other study has been attempted to improve the production of bio-succinic acid by supplementing yeast extract and yeast cell hydrolysate as the nitrogen sources, obtaining 56.4 g/L of bio-succinic acid concentration with the yield of 0.73 g/g glucose [27]. Another strategy has been conducted by performing simultaneous saccharification and fermentation and obtaining 47.4 g/L of bio-succinic acid concentration, 0.99 g/L/h of productivity and yield of 0.72 g/g substrate [35].

Other types of corn waste has also been observed as the carbon source to produce bio-succinic acid via fermentation route, including corn fiber, corn stalk, corn cob, and corn straw. Corn fiber has been used in two different studies. Using different types of nitrogen sources, there is slight difference against the value of bio-succinic acid concentration. 35.4 g/L of bio-succinic acid concentration was obtained by using yeast extract and corn steep liquor as the nitrogen source with the productivity of 0.98 g/L/h and yield of 0.725 g/g total sugar [19], while 35.5 g/L of bio-succinic acid concentration was obtained using yeast extract and yeast cell hydrolysate as the nitrogen sources, with 0.63 g/L/h of productivity and 0.677 g/g glucose [22]. Corn cob has also been used obtaining 23.64 g/L of bio-succinic acid concentration, 0.49 g/L/h of productivity, and 0.58 g/g sugar of yield [33]. In the other study, corn straw has been utilized as the carbon sources by conducting different mode of fermentation, obtaining higher value of bio-succinic acid concentration, productivity, and yield at 53.2 g/L, 1.21 g/L/h, and 0.825 g/g sugar respectively, by using fed-batch mode than by using batch mode [34].

Table 1. Production of bio-succinic acid by *A. succinogenes* in different strategy.

Fermentation operating factors				Conc.	Prod.	Yield	Ref.
Carbon sources	Nitrogen sources	pH regulator	Configuration	(g/L)	(g/L/h)	(g/g)	
EFB	Yeast extract and corn steep liquor	MgCO ₃	SSF	33.4	0.69	0.47	[5,7]
Sugarcane bagasse	Yeast extract	NaOH	SHF	22.5	1.014	0.43	[18]
Carob pods	Yeast extract	NaOH	SHF	9.04	1.67	0.39	[13]
				18.97	1.43	0.94	
Corn fiber	Yeast extract and corn steep liquor	MgCO ₃	SHF	35.4	0.98	0.725	[19]
Sake lees	Yeast extract and corn steep liquor	MgCO ₃	SHF	48	0.94	0.75	[20]
Rapeseed meal	Yeast extract	Na ₂ CO ₃	SSF	15.5	0.22	0.12	[21]
				23.4	0.33	0.12	
Corn fiber	Yeast extract and yeast cell hydrolysate	MgCO ₃	SHF	35.5	0.63	0.677	[22]
Sugarcane bagasse	Corn steep powder	MgCO ₃	SHF	39.9	1.37	0.82	[23]
				70.81	1.42	0.815	
Wheat bran	Yeast extract	MgCO ₃	SHF	50.6	1.04	0.73	[24]
Waste bread	Yeast extract	NaOH and H ₂ SO ₄	SHF	47.3	1.12	0.55	[25]
Corn stalk Cotton stalk	Yeast extract and urea	NaOH	SHF	17.8	0.56	0.66	[26]
				15.8	0.62	1.23	
Corn stover	Yeast extract and yeast cell hydrolysate	Mg(OH) ₂ and NaOH	SHF	56.4	N/A	0.73	[27]
Cane molasses	Yeast extract	Na ₂ CO ₃	SHF	46.4	0.97	0.79	[28]
				55.2	1.15	N/A	
Corn stover	Yeast extract and corn steep liquor	Na ₂ CO ₃	SHF	42.8	1.27	0.74	[29]
Duckweed	Corn steep liquor powder	MgCO ₃	SHF	62.12	1.04	0.81	[30]
			SSF	52.41	0.87	N/A	
			SSSF	65.31	N/A	0.85	
Fresh cassava root	Yeast extract	MgCO ₃	SHF	93.34	1.87	0.77	[31]
				151.44	3.22	1.51	
Cheese whey	Yeast extract and peptone	NaOH	N/A	21.2	0.44	0.57	[32]
Corn cob	Yeast extract	MgCO ₃	SHF	23.64	0.49	0.58	[33]
Corn straw	Yeast extract	MgCO ₃	SHF	45.5	0.95	0.81	[34]
				53.2	1.21	0.825	
Corn stover	Corn steep liquor	MgCO ₃	SSF	47.4	0.99	0.72	[35]

SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; SSSF: Semi-Simultaneous Saccharification and Fermentation; Conc.: concentration of bio-succinic acid; Prod.: productivity of bio-succinic acid.

Carob pods have also been used as the carbon sources for fermentation. A study showed 9.07 g/L and 18.97 g/L of bio-succinic acid concentration has been obtained by employing batch and fed-batch fermentation mode, respectively [13]. Sake lees have also been utilized as carbon sources and obtaining 48 g/L of bio-succinic acid concentration [20]. Rapeseed meal as the carbon source, indicates concentration of bio-succinic acid at 23.4 g/L by employing fed-batch fermentation mode [21], while wheat bran as the carbon sources, shows the concentration of bio-succinic acid at 50.6 g/L by employing batch fermentation mode [24]. Waste bread [25] and cotton stalk [26] has also been studied as the carbon sources for the production of bio-succinic acid, resulting the concentration of bio-succinic acid at 47.3 g/L and 15.8 g/L, respectively. Cane molasses [28] and cheese whey [32] has also been investigated, obtaining concentration of bio-succinic acid of 55.2 g/L and 50 g/L, respectively. Moreover, duckweed [30] as the carbon sources has been investigated by employing different configuration of fermentation and obtaining the highest bio-succinic acid concentration at 65.31 g/L by applying semi-simultaneous saccharification and fermentation (SSSF). From all the carbon sources, fresh cassava root [31] exhibits the highest value of bio-succinic acid concentration at 151.3 g/L by applying fed-batch fermentation mode. Thus, the use of agro-industrial waste has to be considered as the carbon sources of microbial fermentation to produce bio-succinic acid in large

scale since agro-industrial waste abundantly available throughout the year, renewable, inexpensive, environmentally friendly, and does not compete with food needs.

4. Production of bio-succinic acid using different nitrogen sources

One of the significant factors affecting the fermentation performance and bio-succinic acid production is the presence of proper nitrogen sources. There are two types of nitrogen sources, including organic and inorganic sources. However, in the production of bio-succinic acid by *A. succinogenes*, organic nitrogen sources are extensively used by researchers due to inorganic nitrogen sources are not proper for *A. succinogenes* growth [28]. The organic nitrogen sources commonly used are yeast extract, meat extract, peptone, tryptone, corn steep liquor, and nutrient broth.

Different types of nitrogen sources has been employed including wheat bran, beef extract, soy peptone, soy bean powder, yeast extract, and corn steep liquor. Among them, yeast extract exhibits the highest result for bio-succinic acid production with 22.09 g/L of bio-succinic acid concentration. Besides, by applying yeast extract as the nitrogen sources, ratio of bio-succinic acid is also high comparing with other by products [33]. Yeast extract as the nitrogen source has also been extensively used by researchers in order to produce bio-succinic acid by microbial fermentation. In the other hand, a mixture of yeast extract and corn steep liquor has also been employed obtaining various concentration of bio-succinic acid.

The effect of supplementing yeast cell hydrolysate to the production of bio-succinic acid has also been observed considering yeast cell containing high amounts of protein, lipid, RNA, vitamin and mineral [37]. Based on the result, by supplementing yeast cell hydrolysate, cell growth and glucose consumption is increase 32.5% and 49%, respectively, obtaining 53.24 g/L of bio-succinic acid concentration and total sugar utilization of 97.5%.

5. Production of bio-succinic acid using different pH regulator

pH level is one of key parameter in the microbial fermentation since the maintenance and regulation of cell activities is highly dependent to the pH [38]. Most bacterial fermentation require near neutral pH level to optimize the performance. However, in the production of bio-succinic acid through fermentation route, acidification of medium will be absolutely occurred. Therefore, pH regulator should be provided to neutralize the pH condition of fermentation cultures.

Study about the effect of pH regulator to the fermentation performance in order to produce bio-succinic acid has been conducted. MgCO_3 , NaHCO_3 , and Na_2CO_3 have been tested as the pH regulator of the fermentation culture. From the result, MgCO_3 exhibited higher bio-succinic acid production and less by-product comparing with the use of NaHCO_3 and Na_2CO_3 [33]. This is due to Mg^{2+} has several effects on the cellular metabolism including controlling pH, providing CO_2 which is required to form succinic acid, and acts as the cofactor of PEP carboxykinase as the key enzyme used to synthesize succinate [39].

Other study also revealed that glucose consumption in the fermentation using Na_2CO_3 , NaHCO_3 , $\text{Mg}(\text{OH})_2$, and MgCO_3 as the pH regulator were much higher compared with $\text{Ca}(\text{OH})_2$, CaCO_3 , NaOH , and $\text{NH}_3\cdot\text{H}_2\text{O}$ [27]. However, when $\text{Ca}(\text{OH})_2$ and CaCO_3 were used as pH regulator, the cell growth was severely suppressed obtaining low amount of bio-succinic acid. Meanwhile, among the three Na^+ pH regulator, NaOH obtained higher concentration of bio-succinic acid than Na_2CO_3 and NaHCO_3 . Based on the study, the use of MgCO_3 as pH regulator also resulted the best cell growth, glucose utilization, and succinic acid production parameters [27]. Due to its good performance as pH regulator, MgCO_3 has been widely used by researchers to produce bio-succinic acid using *A. succinogenes* obtaining various concentration, productivity, and yield of bio-succinic acid as shown in the Table 1. Besides, NaOH has also been used by researchers considering relatively cheap cost.

6. Production of bio-succinic acid using different fermentation configuration

In order to enhance the production efficiency and reduce the cost, configuration of hydrolysis and fermentation step is able to be adjusted. There are three configurations that have been investigated, including separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and semi-simultaneous saccharification and fermentation (SSSF) [30]. SHF occurred by operating enzyme hydrolysis and fermentation in different stage, therefore optimization of each stage can be achieved. However, SHF process produces high glucose concentration and poses high contamination risk [40]. To overcome this problem, different configuration

of fermentation was proposed, called SSF. SSF occurred by operating enzyme hydrolysis and fermentation in the same stage and maintain low sugar concentration, thus decreasing the contamination risk. Several production of bio-succinic acid through SSF has been attempted by several researchers. Unfortunately, optimal condition of enzymatic hydrolysis and microbial fermentation is mismatched. Therefore, another strategy was proposed by combining SHF and SSF, called SSSF. SSSF occurred by operating pre-saccharification in short period with a faster hydrolytic rate before the main SSF process occurred. With the combined advantages of SSF and SHF, SSSF produces higher yields than SHF and SSF [41]. A study has been proven that SSSF produces higher concentration and yield of bio-succinic acid than SHF and SSF. Based on the results, by operating SHF, SSF, and SSSF, bio-succinic concentration obtained is 62.12 g/L, 52.41 g/L, and 65.31 g/L, respectively and yield of bio-succinic acid obtained by SSSF reach 0.85 g/g sugar [30].

7. Conclusion

Bio-succinic acid as the most important C4 building-block has to be industrially produced by microbial fermentation considering the wide application of this compound. As the most promising microbes, *A. succinogenes* has been investigated applying several fermentation strategies to improve the production of bio-succinic acid. Different carbon sources have been attempted utilizing several agro-industrial wastes since it is renewable, inexpensive, abundantly available, and environmentally friendly. Nitrogen sources as one of the significant factors has also been observed to improve cell growth. To maintain the pH condition of the fermentation cultures, pH regulator also play an important role to be chosen. Besides, configuration of fermentation also contribute to improve bio-succinic acid production. However, as the most recent strategy, fermentation configuration seems important to be more considered in order to realize an efficient bio-succinic industry, since the recent configuration improved the yield of bio-succinic acid.

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