

Use of corn steep liquor as an economical nitrogen source for biosuccinic acid production by *Actinobacillus succinogenes*

J P Tan¹, J M Jahim², T Y Wu³, S Harun², T Mumtaz²

¹Chemical and Process Engineering Department, Faculty of Engineering and Built Environment, *Universiti Kebangsaan Malaysia*, 43600 UKM Bangi, Selangor, Malaysia.

²Research Centre for Sustainable Process Technology (CESPRO), Faculty of Engineering and Built Environment, *Universiti Kebangsaan Malaysia*, 43600 UKM Bangi, Selangor, Malaysia

³School of Science, *Monash University*, Jalan Lagoon Selatan, Bandar Sunway, 46150, Selangor Darul Ehsan, Malaysia

E-mail: ¹jasonjptan2@hotmail.com

Abstract. Expensive raw materials are the driving force that leads to the shifting of the petroleum-based succinic acid production into bio-based succinic acid production by microorganisms. Cost of fermentation medium is among the main factors contributing to the total production cost of bio-succinic acid. After carbon source, nitrogen source is the second largest component of the fermentation medium, the cost of which has been overlooked for the past years. The current study aimed at replacing yeast extract- a costly nitrogen source with corn steep liquor for economical production of bio-succinic acid by *Actinobacillus succinogenes* 130Z. In this study, a final succinic acid concentration of 20.6 g/L was obtained from the use of corn steep liquor as the nitrogen source, which was comparable with the use of yeast extract as the nitrogen source that had a final succinate concentration of 21.4 g/l. In terms of economical wise, corn steep liquor was priced at \$ 200 /ton, which was one fifth of the cost of yeast extract at \$ 1000 /ton. Therefore, corn steep liquor can be considered as a potential nitrogen source in biochemical industries instead of the costly yeast extract.

1. Introduction

The environmental impact and sustainable issues have drawn the attention of the petrochemical industries to rely on biochemical methods which are environmentally benign and sustainable. In 2004, US Department of Energy (DOE) had identified 12 chemical building blocks which had the potential to be commercially produced via biological approach [1]. Some of the listed chemical building blocks including bioethanol and biosuccinic acid had moved into commercialization [2]. Since 2012, biosuccinic acid has been commercialized with at least 12 production plants around the world are already in operations by 2014 [2]. According to a life cycle analysis by BioAmber [3], bio-succinic

¹ To whom any correspondence should be addressed.



acid production had a net zero carbon footprint. Apart from that, a reduction of 60 % of total energy consumption compared to conventional petrochemical approach was also reported in their study [3]. Recent works in the literature had shown that succinic acid production by fermentation from renewable resources and a greenhouse gas, CO₂ are more cost-effective than the petroleum-based process [4]. Biotechnological production of succinic acid using by-products as a carbon source such as: sugar cane molasses, glycerol and others have been the major focus by many researchers. One of the key factors to be considered in the commercialization of bio-succinic acid is the cost of the fermentation medium. Carbon source in this case, is the major component and it has been the center of focus in bio-succinic acid research. Several cheap carbon sources for example flour, sake lees, cotton straw, rice straw, corn straw, corn core, wheat straw, waste bread, rapeseed meal, sugarcane bagasse, soybean meal, soybean solubles, sugarcane molasses, whey, glycerol and wood hydrolysate had been investigated and reported as substrate for bio-succinic acid production [5]. However, nitrogen source which is the second largest medium components, in this regard, had been given much lesser attention. The present work had an objective to study the potential of corn steep liquor as a nitrogen source in bio-succinic acid to replace the costly yeast extract. *Actinobacillus succinogenes* is used in this study since it is one of the highest yield and intensely researched microbe for bio-succinic acid production [6; 7]. Several aspects are investigated in this study including the yield of succinate, cost of the nitrogen sources as well as the nitrogen content of the corn steep liquor and yeast extract.

2. Materials and methods

A. succinogenes 130Z was purchased from DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany. The stock culture was maintained in Brain heart Infusion (BHI) media. For the fermentation of succinic acid, the mineral media consisted of: 0.2 g magnesium chloride hexahydrate (MgCl₂·6H₂O); 0.2 g calcium chloride (CaCl₂); 3 g potassium dihydrogen phosphate (KH₂PO₄) and 1 g sodium chloride (NaCl) per liter of medium. Inoculum (10% v/v) was prepared using BHI medium under aerobic condition at 120 rpm and 30 °C for 18 hours. Yeast extract and corn steep liquor with concentration of 15 g/L were employed for the fermentation of succinic acid. The performance of corn steep liquor and yeast extract in succinate fermentation were compared and discussed.

Succinate fermentation was performed in batch mode in 110 ml serum vials using 80ml working volume in sterile condition to study the profile of bacterial growth, succinic acid production, byproduct formation as well as consumption of sugars. Glucose was used as the sole carbon source in the fermentation at a concentration of 40 g/L.

Glucose was analysed using high performance liquid chromatography (HPLC), Agilent 1200 HPLC system (California, USA) equipped with Rezex RPM (Phenomenex, USA) 300 mm x 7.8 mm column and Refractive Index Detector (RID). Water was isocratically eluted at a flow rate of 0.6 ml/min while the column temperature was set at 60 °C. Soluble metabolites such as succinic acid, formic acid and acetic acid were analyzed using Agilent 1100 HPLC system (California, USA) equipped with Rezex ROA column (Phenomenex, USA) and Ultraviolet detector (UVD) at 210 nm. The column was eluted isocratically at a rate of 0.5 ml/min with 0.0025 M H₂SO₄ at 40 °C.

3. Results and discussion

Corn steep liquor is a by-product of corn wet-milling. It is a viscous liquid by-product consists of vitamins, minerals and nitrogen (including amino nitrogen) which is suitable for fermentation. It is an excellent source of organic nitrogen [8]. In the corn processing industry, the corn was steeped by counter current water for approximately two days. The steeped water was concentrated to a 50 % solid content liquid by evaporator. This liquid is called corn steep liquor [8]. On the other hand, yeast extract is the water soluble portion produced by extracting the cell contents from autolyzed yeasts. It is among the best growth stimulant for cell cultures and microbial fermentation. It is normally used with the concentration of 15 g/l in the fermentation.

In this study, corn steep liquor served well as the nitrogen source for *A. succinogenes*. As could be seen from Figure 1, Corn steep liquor was not only fermentable; the succinate production was, in fact, comparable with yeast extract. The final concentration of succinic acid using urea in fermentation was 12.61 g/l. Corn steep liquor reported a final succinate concentration of 20.6 g/L which was only 3.7 % lower than the fermentation using yeast extract as nitrogen source (21.4 g/L). Corn steep liquor was able to work as well as the costly yeast extract at the lower cost. This is one of the vital parameters to be considered when moving into industrial scale fermentation.

The nitrogen content of corn steep liquor was comparable to yeast extract. The reason that corn steep liquor has a close performance with yeast extract was because of their similarity in terms of total nitrogen and total amino nitrogen content. Total amino nitrogen is the available of small peptides chain (less than three amino acids). Total amino nitrogen could help to reduce the energy needed for the synthesis of proteins which are essential for the bacterial growth. Moreover, corn steep liquor is rich in minerals and growth factors since it is a byproduct of corn wet milling. In short, corn steep liquor has similar performance as yeast extract while cost approximately one fifth of the cost of yeast extract.

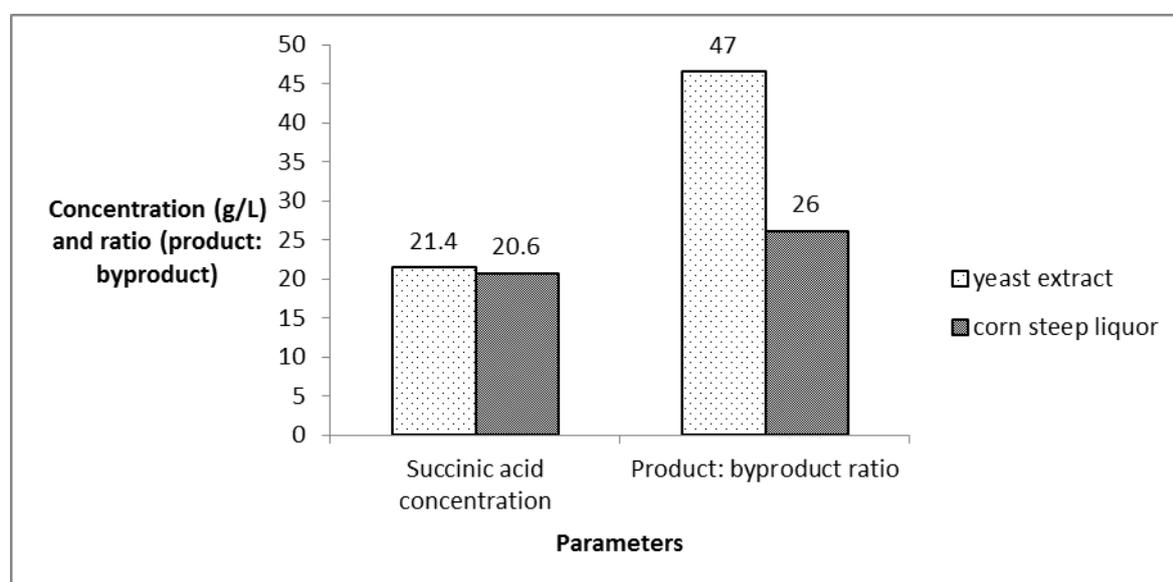


Figure 1. Comparison of succinic acid production and ratio of product: byproduct of yeast extract and corn steep liquor

The byproduct acetate and formate have a closer characteristics with succinate as these are all organic acids which lead to the difficulties during the purification of product succinate. 50-80 % of the cost of organic acid processing in industries is generally imputed to the product purification [9]. The ratio of succinate to by-product in this regard, acts as one of the primary indicator to predict the cost of product (organic acid) recovery, which is the reason it was considered in this study. Corn steep liquor in this case, had a lower product to byproduct ratio as compared to yeast extract.

Byproducts formation from the utilization of yeast extract and corn steep liquor is shown in Figure 2. The fermentation using corn steep liquor has 0.31 g/L of formic acid and 0.48 g/L of acetic acid after 72 hours of fermentation. On the other hand, yeast extract reported comparatively lower titers of byproducts which were 0.16 g/L of formic acid and 0.30 g/L of acetic acid. A lower concentration of byproducts allows an easier and cheaper downstream process. Corn steep liquor was found to be inferior in this regard.

The production of byproducts was also significantly affected by the concentration of total amino nitrogen. Higher amino nitrogen promotes higher flux into product instead of byproducts. Therefore,

the byproducts formation was slightly more in corn steep liquor compared to yeast extract. This was due to the higher amino nitrogen content in corn steep liquor as could be inferred from Table 1.

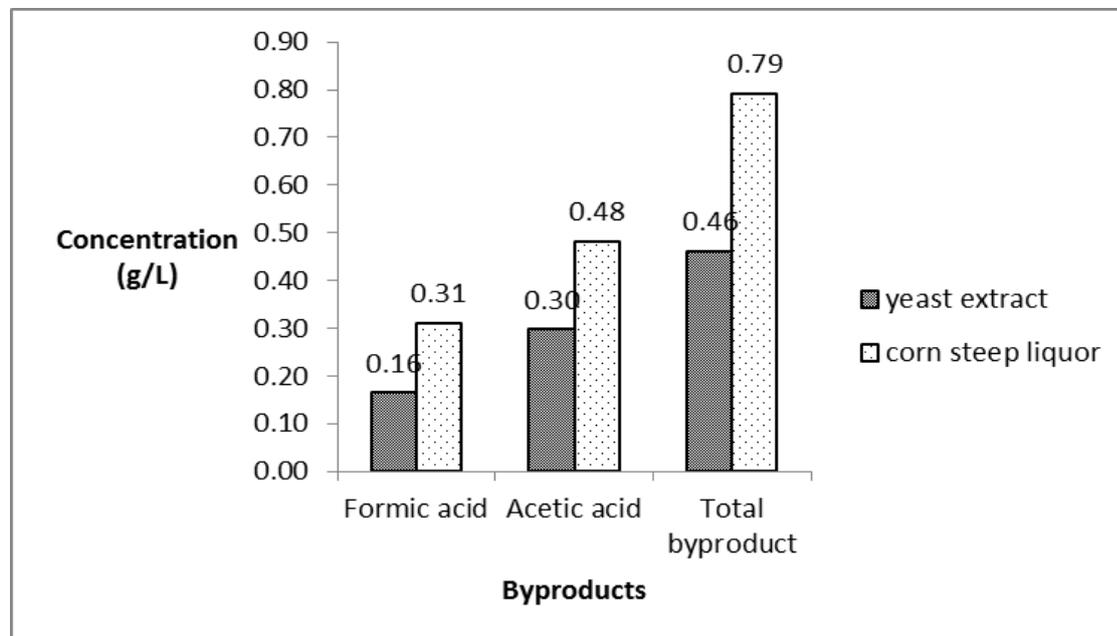


Figure 2. Byproducts formation comparison by yeast extract and urea

In spite of the production of products and byproducts, the nitrogen source content in corn steep liquor and yeast extract was compared in terms of its amount of total nitrogen content.

Table 1. Nitrogen content and cost comparison of corn steep liquor and yeast extract in dry basis

Nitrogen source	Yeast extract	Corn steep liquor
Total nitrogen, %	10-11.8	7.7-8.2
Total amino nitrogen, %	4.5-5.8	2.9-3.3
References	10	8
Cost \$/ton	1000	200
Reference	11	12

In order to make bio-succinic acid competitive, the cost of production is among the prime concerns in the stage of commercialization. The cost of raw material in this regard, plays a crucial role in determining the overall production cost of bio-succinic acid. Nitrogen source contributes directly to the cost of raw material. Therefore, the cost of nitrogen source is important to be considered in the selection of nitrogen source. Corn steep liquor in this regard, appeared as a better choice since the price in ton is only \$ 200, which was one fifth of the cost of yeast extract that was estimated to be \$ 1000 /ton by bbc research [11; 13]. Since nitrogen source is the second largest component in fermentation after carbon source, the cheaper cost of nitrogen will have a pronounced and direct effect on the production cost of succinic acid as a whole. This is especially significant during the commercialization stage where large amount of nitrogen source is needed.

4. Conclusion

In this study, corn steep liquor has been shown to be able to function as a nitrogen source to replace the costly yeast extract. Amino nitrogen content has an impact on succinate yield which were found abundant in corn steep liquor and yeast extract. Apart from having the comparable succinate production, corn steep liquor was estimated to cost at \$ 200 /ton, which was one fifth of the cost of yeast extract. Since nitrogen source is the second largest component in the fermentation medium, the utilization of corn steep liquor can significantly reduce the cost of bio-succinic acid production. This can further elevate the competitiveness of commercial bio-succinic acid.

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