Investigating the production of succinic acid via the fermentation of vegetable waste by *Actinobacillus Succinogenes*

Group 1-35

Clement Gwee, Lim Jin Sian, Ray Huang

Abstract

Succinic acid is a dicarboxylic acid with the chemical formula $C_4H_6O_4$ and is an essential chemical in the food, agricultural and pharmaceutical industry. Currently, most succinic acid is industrially produced using petroleum as a main ingredient, which unfortunately harms the environment by contributing to air pollution. The bacterium *Actinobacillus Succinogenes* is able to utilise food waste as a carbon source to produce succinic acid, and thus is more eco-friendly. In addition, food waste in Singapore remains a concerning issue as food waste generated in Singapore over the past decade has increased by 40% (Koh, 2019), leading to a higher amount of methane emissions. This study aims to investigate the production of succinic acid from different types of food wastes including cabbage, chye sim and sugarcane bagasse and the effect of glucose concentration in the food wastes on the concentration of succinic acid produced. DNS tests were done on local vegetable waste extracts to determine their glucose concentrations, while *A.succinogenes* was inoculated into fermentation mediums containing the waste extracts to synthesise succinic acid. The yield was analysed with a HPLC system. The results show that *A.succinogenes* was able to successfully synthesise succinic acid using glucose as a carbon source, as succinic acid yield sof 1603.5 ppm, 310.5 ppm and 1451.9 ppm was obtained for cabbage, chye sim and sugarcane bagasse extracts respectively. Local vegetable wastes could be concluded to be efficient sources of succinic acid production, as high succinic acid yields per g glucose at 1.4162 g, 1.2458 g, 1.1596 g were obtained for cabbage, chye sim and sugarcane bagasse extract respectively, showing that production of succinic acid using the mentioned food wastes is a viable alternative to the current production method and can be used to alleviate environmental pollution.

Introduction

Succinic acid, a dicarboxylic acid with the formula $C_4H_6O_4$, is a precursor for many chemicals which are essential in the food, agricultural and pharmaceutical industry. Chemicals such as

adipic acid, 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone are all derived from succinic acid and are used in pharmaceutical and food products, detergents and surfactants, as well as ingredients which help to stimulate animal and plant growth; succinic acid can also prevent corrosion and pitting of metals where it is used in electroplating, acting as an ion chelator (Zeikus, Jain, & Elankovan 1999). It also proves itself to be useful in the polymer industry, being a precursor to some polymers. According to Zeikus, Jain and Elankovan (1999), the market value of these existing uses of succinic acid exceeds $400 000 000 per year. Therefore, due to the significance of succinic acid in the consumer products industry, as a supplier for many intermediate and specialty chemicals, there is a need to conduct more research and development to improve the current production methods.

At the present time, a high percentage of commercial succinic acid is produced using petroleum oil or liquefied petroleum gas as a starting product via a chemical process (Song & Lee, 2006). More than 15 000 tonnes of industrial succinic acid is sold, and mostly via this petrochemical method (Zeikus, Jain, & Elankovan, 1999). However, research has shown that as compared to this petroleum-based process, which contributes to environmental pollution and requires using a non-renewable form of energy, production of succinic acid via fermentation of renewable resources is more cost-effective (Willke & Vorlop, 2004).

Actinobacillus succinogenes, a Gram-negative rod-shaped bacterium, which is facultatively anaerobic and pleomorphic, was originally isolated from the bovine rumen and belongs to the *Pasteurellaceae* family (Guettler, Rumler & Jain, 1999). Phosphoenolpyruvate (PEP) carboxykinase, malate dehydrogenase, malic enzyme, fumarase and fumarate reductase were found to be key enzymes responsible for the production of succinic acid in *A. succinogenes*, which utilises the PEP carboxylation pathway in the tricarboxylic acid (TCA) cycle to form the acid (Song & Lee, 2006). Moreover, due to CO2 fixation involved in the TCA cycle, *A. succinogenes* has high potential for CO2 sequestration, reducing greenhouse gas emissions (Pateraki et al., 2016). *A. succinogenes* has been the centre of attention in producing bio-based succinic acid. According to Song & Lee (2006), *A. succinogenes* has shown its ability to use a wide range of carbon sources such as arabinose, fructose, glucose or sucrose to produce a substantial amount of succinic acid under anaerobic conditions.

The bacterium produces succinic acid through fermentation of the carbon sources, and studies have already been made to test the limitations of *Actinobacillus succinogene*s. A study by Lin, Du, Koutinas, Wang, and Webb (2008) showed us that the production of succinic acid is affected by the glucose concentration. When the concentration was above 158 g/L, succinic acid yields dropped significantly. Another study by Wan, Li, Shahbazi, and Xiu (2008) showed that the optimum fermentation pH for *Actinobacillus succinogene*s is about 6.8.

Numerous food waste products have been evaluated for their capability to produce succinic acid via fermentation by *A. succinogenes*. Examples include cane molasses, cheese whey, crop stalk wastes, as well as straw hydrolysates (Li et al., 2010; Liu et al., 2008; Wan et al., 2007; Zheng, Dong, Sun, Ni, & Fang, 2009). However, not much research has been done on various types of locally available agricultural and vegetable wastes. Since these waste products may be an untapped feedstock for succinic acid production, they should be evaluated for their ability to do so. Bio-based succinic acid not only paves a way for a more effective and cost-friendly method for the commercial production of succinic acid, but also assists in ameliorating the issue of environmental pollution as a result of the petrochemical method.

Objective and Hypotheses

The objectives are to investigate the production of succinic acid from different types of food wastes and the effect of glucose concentration on concentration of succinic acid produced.

The hypothesis is that different types of wastes contain varying concentrations of glucose, and thus result in different yields of succinic acid produced by *Actinobacillus succinogenes*.

Experimental Procedures

Preparation of food wastes

3 different food wastes, Cabbage (*Brassica Oleracea*), Chye Sim (*Brassica parachinensis*) and Sugarcane Bagasse (*Sacharum Officinarum*) were used. 50 g of each food waste was blended with 250 ml of deionised water, and the residue removed via vacuum filtration while the filtrate was collected as the food waste extract.

Dinitrosalicylic acid (DNS) test to determine reducing sugar concentration

The DNS reagent is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, thus resulting in a colour change, corresponding to the concentration of reducing sugars present.

1.5 ml of DNS reagent was added to 1.5 ml of each food waste extract. The mixtures were heated in a boiling water bath for 5 min, before 0.5 ml of DNS stabiliser was added. The absorbance was read at 530 nm using a UV-vis spectrophotometer. A higher absorbance corresponded to a higher concentration of reducing sugars. The concentration of reducing sugars was read from a glucose standard curve.

Preparation of *A. succinogenes* preculture

*Actinobacillus succinogenes (*ATCC 55618) was inoculated in a trypticase soy broth while *Escherichia coli* (ATCC 25922) was inoculated in a LB broth. Both bacteria were incubated with shaking at 30°C for 24 hours.

Preparation of fermentation medium

The fermentation medium comprised (per 100 ml): 10 ml food waste extract as the carbon source, 1.6 g yeast extract, 0.3 g KH_2PO_4, 0.15 g K_2HPO_4, 0.1 g NaCl, 0.03 g $MgCl_2$, 0.03 g $CaCl_2$, 0.2 g $(NH_4)_2CO_3$.
It was autoclaved at 10 psi for 10 min.

Production of succinic acid

2 ml of *A. succinogenes* or *E. coli* preculture was added to 18 ml of fermentation medium containing 10% (v/v) food waste extract. The mixture was then incubated at 30°C with shaking for 72 hours. Five replicates were prepared for the test (*A. succinogenes*) and negative control (*E. coli*) samples. Bacterial cells were removed by centrifugation at 7000 rpm for 10 min and the supernatant containing succinic acid was collected. Yield of succinic acid was analysed by high

performance liquid chromatography (HPLC) using an organic acid column at 30°C with the solvent system 0.2% acetonitrile / 99.8% 0.05 M KH_2PO_4 pH 3 as the eluent. Succinic acid was detected using a UV detector at 210 nm.

Results and Discussion

DNS Test

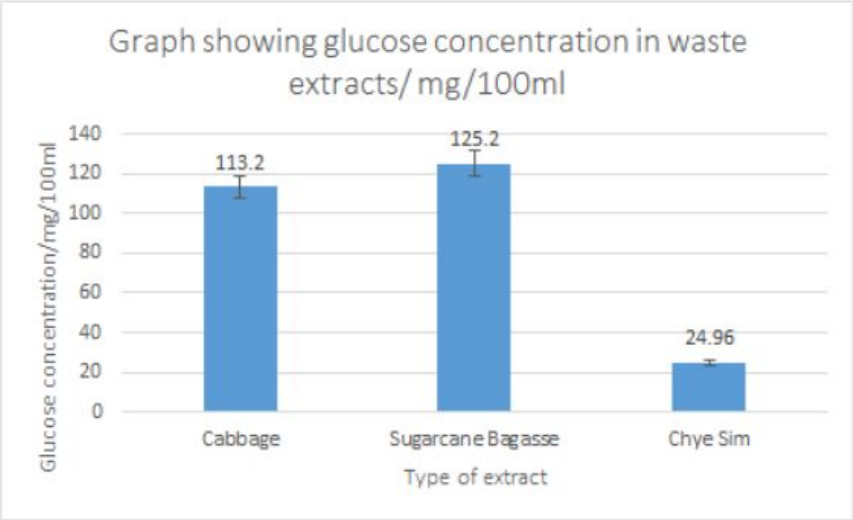

Fig 1.1: Graph showing glucose concentration in waste extracts/ mg/100ml

With reference to Fig 1.1, the glucose concentration of the sugarcane bagasse extract was the highest at 122.2 mg/100ml, followed by cabbage extract at 111.0 mg/100ml then chye sim extract at 22.2 mg/100ml.

The resulting p-value from the Kruskal Wallis test was 0.002, indicating a statistically significant difference among the 3 sets of data, as the vegetable wastes contained varying amounts of glucose.

Succinic acid yield of *A. succinogenes* on waste extracts

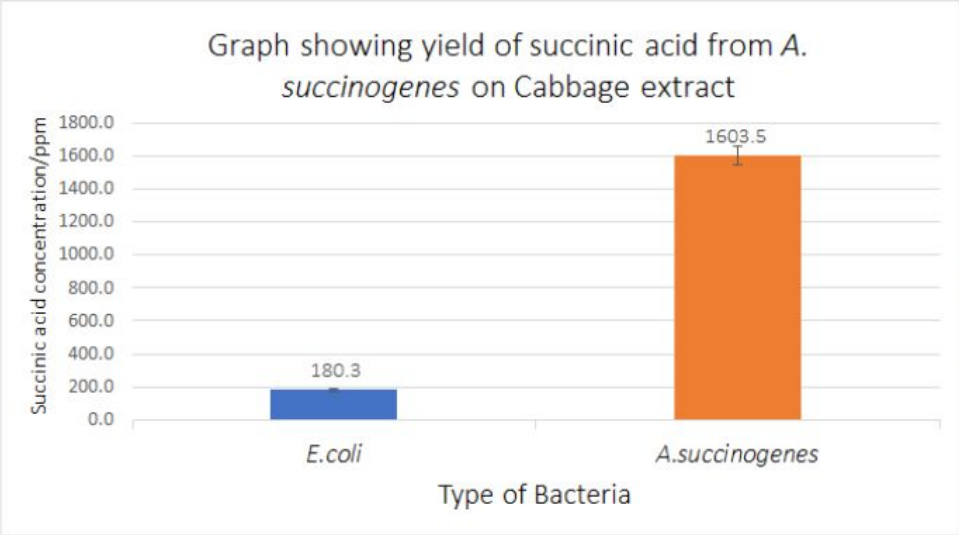

Fig 2.1: Graph showing yield of succinic acid from *A. succinogenes* on Cabbage extract

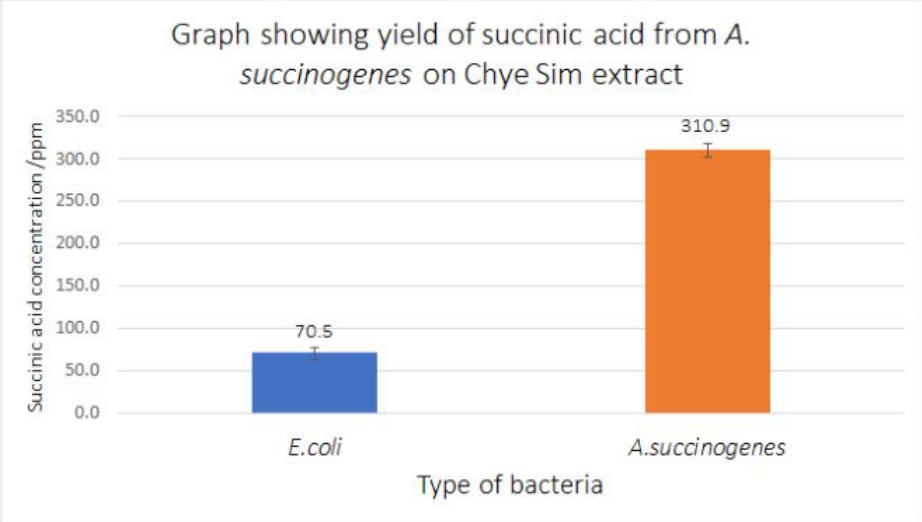

Fig 2.2: Graph showing yield of succinic acid from *A. succinogenes* on Chye Sim extract

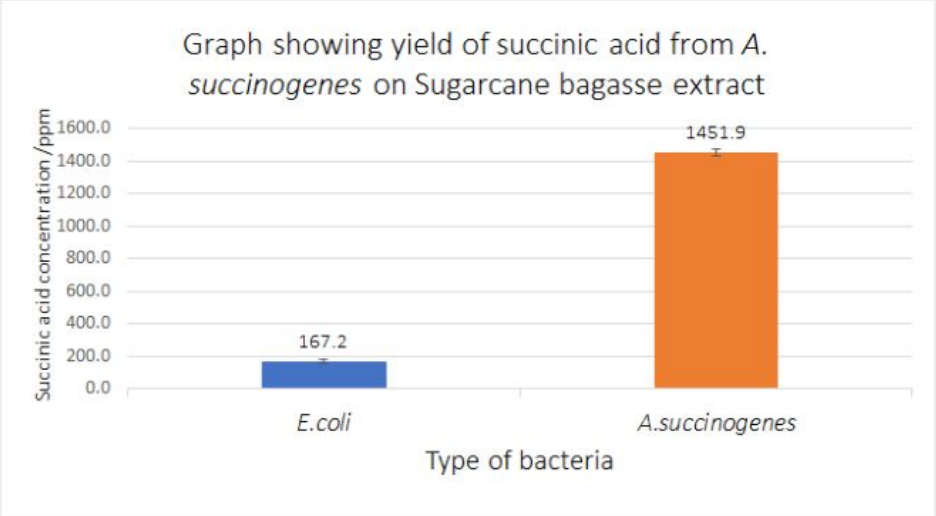

Fig 2.3: Graph showing yield of succinic acid from *A. succinogenes* on Sugarcane Bagasse extract

With reference to Fig 2.1, 2.2, and 2.3, a general trend can be observed that the succinic acid yield is higher when *A.succinogenes* preculture was added to the fermentation mediums.

The resulting p-value of 0.012 from the Mann-Whitney U test for all 3 wastes indicates a statistically significant difference between the succinic acid yield of *A.succinogenes* and *E. coli*., due to the ability of *A.succinogenes* of utilising the TCA cycle to synthesise succinic acid.

Succinic acid yield from different waste extracts

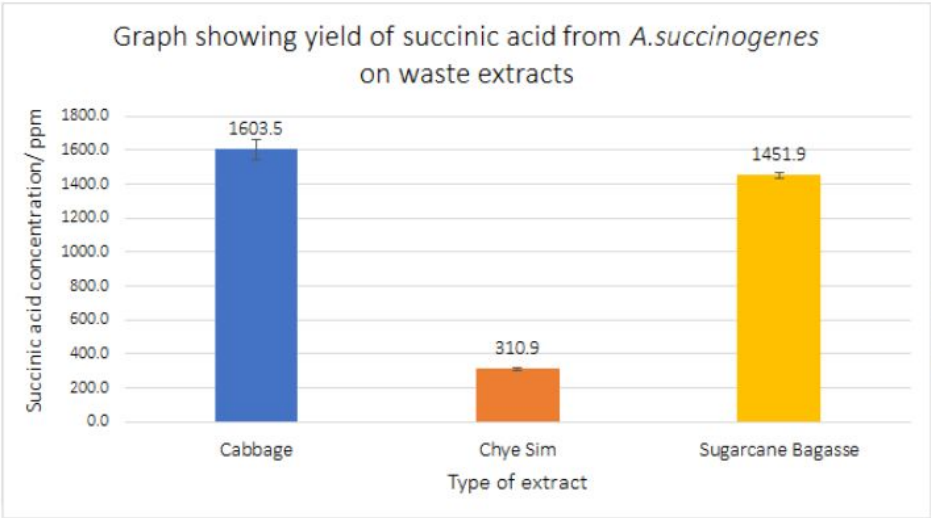

Fig 3.1: Graph showing yield of succinic acid from *A.succinogenes* on waste extracts

With reference to Fig 3.1, the succinic acid yield from *A.succinogenes* on cabbage extract was the highest at 1603.5 ppm, followed by sugarcane bagasse extract at 1451.9 ppm then chye sim extract at 310.9 ppm. This shows that cabbage extract was the most effective feedstock for succinic acid production, followed by then sugarcane bagasse extract then chye sim extract.

The resulting p-value of 0.004 from the Kruskal Wallis test indicates a statistically significant difference in succinic acid yield among the different waste extracts, as the waste extracts had varying glucose concentrations.

The yield of succinic acid from chye sim extract was the lowest as it had the lowest glucose concentration of 24.96 mg/100ml. Although sugarcane bagasse extract had a higher glucose concentration of 125.2 mg/100ml as compared to cabbage extract at 113.2 mg/100ml, it had a lower succinic acid yield. This could be attributed to the fact that there might have been substances within the sugarcane bagasse extract that inhibited the production of succinic acid by *A.succinogenes.* Alternatively, other carbon sources within cabbage extract could have increased the concentration of succinic acid produced.

Succinic acid yield from every waste extract was found to be lower than those obtained from other researchers who managed to attain 45.5g l^{-1} succinic acid from straw hydrolysates (Zheng, Dong, Sun, Ni & Fang, 2009), with this being likely due to the use of enzymatic hydrolysis as an intermediate step, resulting in much higher initial sugar concentration of 58 g l^{-1}.

Efficiency of succinic acid yield

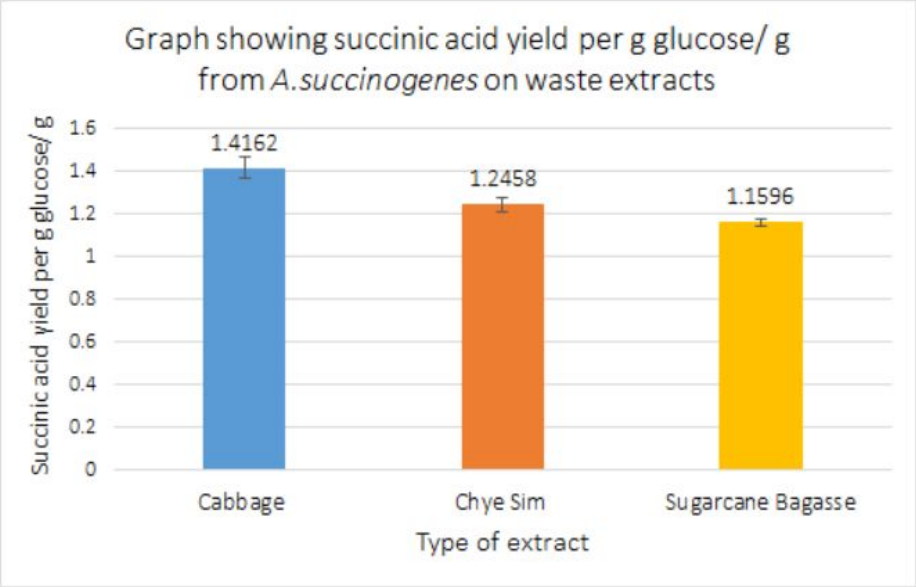

Fig 4.1: Graph showing yield of succinic acid per g glucose/g from *A.succinogenes* on waste extracts

With reference to Fig 4.1, the yield of succinic acid per g glucose was highest for cabbage extract at 1.4162 g, followed by chye sim extract at 1.2458 g, then sugarcane bagasse extract at 1.1596 g. Cabbage extract can be concluded to be the most efficient feedstock for succinic acid production, followed by chye sim extract then sugarcane bagasse extract.

The resulting p-value of 0.005 from the Kruskal Wallis test indicates a statistically significant difference in the efficiency of succinic acid yield among the different waste extracts, as other substances within the extracts could have boosted or inhibited succinic acid production.

The results were found to be comparable and even higher than other studies. A succinic acid yield of 1.23 g per g glucose/g was obtained using cotton stalk hydrolysates (Li et al., 2010). As compared to this, the succinic acid yield per g glucose from cabbage extract and chye sim extract were higher, showing that they can be considered efficient feedstocks for succinic acid production.

The results also exemplify that *A.succinogenes* is a more effective producer of succinic acid than other bacteria as the succinic acid yield per g glucose were found to be higher than other studies. Genetically engineered strains of *Mannheimia succiniciproducens* and

Corynebacterium glutamicum produced 1.16 g and 0.92 g succinic acid per g glucose respectively (Lee, Song & Lee, 2006; Okino et al., 2008).

Conclusion

From the HPLC analysis of the succinic acid yields of each waste extract, it can be concluded that *A.succinogenes* could synthesise succinic acid from the waste extracts far better than *E.coli* and that the cabbage waste extract had the best succinic acid yield to glucose concentration ratio. In addition, the succinic yield per gram glucose/g in each local vegetable waste extract was comparable to that of other waste extracts from other studies, demonstrating that the use of local vegetable waste as carbon sources and *A.succinogenes* is an efficient and cost-effective method for the production of succinic acid.

Implications

By using local food waste as a component in producing succinic acid, local food waste can be reduced, lightening a heavy burden on the total waste generated by Singapore as around 10 percent of the total waste generated in Singapore is food waste, yet only 18 percent of it is recycled with the rest being disposed of and incinerated (The National Environment Agency, 2020). This too will effectively reduce methane emissions from landfills and conserve energy and resources such as the space in landfills. The food waste can also bring economic value as the produced succinic acid can also fill in the rising demand of succinic acid in the recent years due to its cost-effectiveness and efficiency in pharmaceutical, agricultural and food industry.

Future Work

The ability of *A.succinogenes* to produce succinic acid using alternative carbon sources such as xylose or fructose can be investigated and evaluated further. Secondly, more research can be conducted to genetically engineer *E.coli,* a more abundant bacteria, to produce succinic acid. Thirdly, more research is needed to evaluate different types of locally available food wastes as sources for succinic acid production. Lastly but most importantly, much more research needs to be done to find more economical methods to retrieve and purify the succinic acid produced.

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