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Study the Effect of Different Renewable Carbon Sources on the Succinic Acid Biosynthesis by Optimization Culture Composition using 4-Liter Scale Bioreactor

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ABSTRACT

Background: The succinic acid demand accelerated through the years. Thus, the need to improve acid production economically is aggravated. The fermentation process by succinate bacteria showed promising acid production. However, due to different bacteria characteristics, mediums composition and operating conditions, a fixed quantity of succinic acid production cannot be determined or generalized. Recently, raising interest in examining cheap mediums in the fermentation process brought attention to utilizing different raw materials. Nevertheless, its influence on production is not fully comprehended.

Methods: This research aims to develop an evaluation of three succinate bacteria on succinic acid production. Also, it aims to demonstrate a comparative analysis of the Succinic acid production by three raw carbon sources (Corn fiber, Cane molasses and orange peel). The flask batch method and the 4-L Bioreactor were employed in the evaluation.

Result: The results of the study showed that the *Actinobacillus succinogenes* is the best succinate bacteria to provide commercial Succinic acid. The presence of 10 g of glucose was satisfactory in improving the fermentation among all bacteria. The highest Succinic acid production was 12.8 g/L provided by the corn fiber. The lowest succinic acid production was 18% less than the glucose medium that the orange peel gave. The results revealed a significant impact of the raw material composition on the production outcomes. At the bioreactor, the cells exhibited an exponential phase with rate growth of 0.175 [g/L.h] and the maximum Succinic acid produced was 25 g/L.

Key words: *Actinobacillus succinogenes*, Cane molasses, Corn fiber, Orange peel, Succinic acid.

INTRODUCTION

Succinic acid (SA) (succinate) (1,4-butanedioic acid) is a natural organic acid. Succinic acid can be found in plants, microorganisms and living creatures (Wang *et al*., 2014). SA is commonly used as a block-building chemical. SA is an active ingredient that helps reduce pain and integrate the components' deliverability to the human system. It is also used as food additive to enhance the cheese flavor (Ganesan and Weimer, 2017). The leading role of SA is in the production of many C4 family chemicals, such as esters, amides, 1,4-butanediol ($C_4H_{10}O_2$), adipic acid ($C_6H_{10}O_2$) and several green solvents (Meena *et al*., 2020, Carvalho, 2015) .

SA is naturally found in fruits during their early maturation. However, the acid tends to convert to succinate salt. Since this process cannot be controlled, it is considered ineffective for the production. The gap motivated researchers to find a better alternatives. To produce large acid quantities a complex chemical reaction is required which involve expensive petroleum and metal catalysis (Nghiem *et al*., 2017).

The microbial fermentation process is advantageous since it can provide commercial SA quantities from renewable sources and it consumes CO_2 gas in the process (Song and Lee, 2006). Technically, several pathways have been reported for SA production with alternative routes that can be expected. Yet, it is believed that the most agreed-on is SA is a final product in the metabolite of phosphoenolpyruvate carboxylase. The carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) is the key step in the production (Su *et al*., 2022). Using bacteria in SA production is the dominant SA source. The bacterial fermentation process is anaerobic with the advantage of

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genetic modification if required (Kaur and Kaur, 2018). Several researchers engineered Strains and modified the bacterial processes. By focusing on fermentation by bacteria, *Anaerobiospirillum succiniciproducens* (Ahn *et al*., 2016), *Actinobacillus succinogenes* (de Jong *et al*., 2012) and *Mannheimia succiniciproducens* (Song *et al*., 2008) were sought as effective since the SA production range varied between 32 g/L up to 105g/L (Nghiem *et al*., 2017) .

Utilizing fermentation gives more flexibility in selecting carbon sources. Commonly, mediums such as Glucose, Lactose, Arabinose, Xylose and Fructose can provide massive SA production (Podleśny *et al*., 2017). For instance, in preparing SA, the *Actinobacillus succinogenes* was fermented in a packed-bed reactor, where glucose was compared to different carbon sources. The optimal results were found at the glucose medium with a conversion of 88% and the volumetric productivity was 22 g L^{-1} h^{-1} (Ferone *et al*., 2018).

On the other hand, using *Actinobacillus succinogenes* and *Basfia succiniciproducens* to convert lactose resulted in the yield and concentration of SA dropping to 0.34 g g^{-1} and 13 g L^{-1} and the sugar consumption decreased from more than 99 to less than 55% (Terboven *et al*., 2021). Some studies considered further optimization by combining substrates of glucose and arabinose he succinate production reached to 10.2 mM succinate was produced. This is the highest succinate production under aerobic conditions in minimal medium (Joshi *et al*., 2019), (Liu *et al*., 2013). The *Basfia succiniciproducens* was investigated using Xylose as a carbon source. SA production reached 19 g/L with 0.62 g/g yield and 1.9 g/(L.h) productivity (Stylianou *et al*., 2021).

Despite the optimization process on the fermentation process, a cost reduction is still necessary. As a result, many researchers focused on investigating cheap renewable carbon sources to gain additional SA. The optimum nominated source should have specific properties such as conversion ability, low intermediate by-production, heavy tolerance and huge mass availability. Zhang *et al*. (2012) examined corn fiber as supplementary to the SA enhancement. The suggestion was motivated by the corn fiber hydrolysate constitutes. The corn fiber contains three sugars which are glucose, arabinose and xylose.

Despite the good results, the study served with different additives to comply with the bacteria (Zhang *et al*., 2012). Another cheap renewable source with massive controversy is cane molasses. The cane molasses was investigated and reported mediocre SA productivity of 0.57 g/L.h. This was not the only rising issue; the cane molasses was reported uncommercial due to the toxic involved with the sulfuric acid production. However, the improvement of purification technology has led to the consideration of the cane molasses again (Kuhnert *et al*., 2010). An effort to utilize the wasted orange peels resulted in an improvement of the SA production. However, it also unleashes a new question regarding the degradation of natural lignocellulosic Feedstocks by *succinogenes* (Zeikus *et al*., 1999).

Obviously, when a diverse group of bacteria, waste bioproducts and operating conditions were gathered, it became difficult to reach a holistic view of the success of the SA production. Thus, the current research evaluates the succinate bacteria's influence on SA production and other factors. A second aim is to demonstrate a comparative evaluation of the SA production by the corn fibre, cane molasses and orange peel.

MATERIALS AND METHODS

Materials

Three *Succinogenes* strains were under investigation, which are *Basfia succiniciproducens* (S01), *Actinobacillus succinogenes* (S02), *Mannheimia succiniciproducens* (S03). In 2022, The Succinogenes strains were received from the Biological Research Service Department at the Ministry of Science and Technology in Iraq and start searching directly. All media used in strains preparation and media for optimization were purchased from (Al Thiqa) company for Chemicals and laboratory supplies in Baghdad.

Strain preparation for succinic acid production

A mixture of 50 mL glycerol stock and 25 mL of culture medium was used for the cell incubation at a temperature of -80°C. For all the strains, the medium used was composed of 6.0 g glucose, 10.0 g yeast extract, 8.0 g $NaHCO_3$, 8.5 g $NaH_2PO_4 \cdot H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$ (Chen *et al*., 2011). The medium was prepared by stirring the yeast extract, $NaHCO_3$, $NaH_2PO_4 \cdot H_2O$, K_2HPO_4, $(NH4)_2SO_4$, at 121°C for 15 min.

Secondly, the Glucose and $CaCl_2$/$MgCl_2$ solution were sterilized separately and added aseptically. After inoculation, the flasks were incubated at 37°C in a rotary shaker at 200 rpm, for 12 h, until the late exponential phase was reached.

Strain evaluation at different mediums

At the initial screening step, the effect of the medium composition on the strain productivity was evaluated. The three mediums illustrated in Table 1 were repeatedly tested for each strain.

It is worth mentioning these selected media have been endorsed and examined in detailed in the literature (Cao *et al*., 2018 Li *et al*., 2010).

Succinic acid characterization

Determination of cell dry weight

Several samples were acquired at different time intervals during the cultivations period. To determine the biomass a 1ml sample was extracted then mixed with distilled water. The dilution process continued until a factor of 1000 was reached. The critical step was employing the optical density. So, the UV Spectrophotometer was sat at the wavelength of 600 nm. The cell dry weight is estimated by the mean of the absorbance measure. The absorbance measured was compared to the calibration curve and this was how the concentration was measured. Despite the measurement variation, the results were taken triple times to ensure reproducibility of the data.

Succinic acid determination

For SA determination, the cells were extracted from the fermentation broth. The recovering process used 6000 rpm centrifugation for 10 minutes at 4°C. This study required special extra supernatant utilization to estimate SA by using (HPLC) Waters 2690 Alliance Separations Module with a flow rate of (0.6) m/min, UV detection of (210) nm, Mobile Phase H_2SO_4 of (0.005N), Waters 996 Photodiode Array Detector and Column: Hi-Plex H, 300 × 7.7 mm with guard column and temperature at 40°C (Alsaheb *et al*., 2020).

Total sugar reduction measurement

The total sugar reduction is the best indicator for incremental acid production. Therefore, the procedure was utilized by decomposition of aqueous sugars. The sugar was diluted by sulfuric acid at elevated (35°C) for 48 h to obtain carbohydrates, according to Dionísio *et al.*, (2021). After the carbohydrates were obtained, a post-treatment with sulfuric acid and dries consisting of hydroxy methyl alfarfiral were done. The process resulted in a chemical reaction of orange color. The orange color intensity is resulted from the sugar content and could be determined by spectrophotometer. The Spectrophotometer (HACH Model DR/2500, Loveland, CO, USA) was used to determine the wavelength and calculate the sugar content. The method is known as Phenol- Sulphric Acid Method, which was steadily employed for similar purposes (Dubois *et al.*, 1956).

Succinic acid production optimization for flask scale

Cane Molasses, Corn Fiber and Orange peel were examined to maximize SA production. The investigation used the cheap materials as main carbon sources. The materials were purchased from a local market in Baghdad. Iraq. The main motivation behind the selection is the composition of the raw materials. Corn Fiber comprises approximately 30 to 50% arabinoxylan, 15 to 20% cellulose and 10 to 25% adherent starch-(Gáspár *et al.*, 2005). The Orange Peel composition consists of 12.6% hemicellulose, 11.1% cellulose and 4.5% starch and other components (Bampidis and Robinson, 2006). Cane molasses is composed of 31% of Sucrose and 15% of invert sugars and other components (Chan *et al*., 2012). The material composition reflected their importance in producing Succinic acid. The composition may slightly vary due to the location of farming and harvesting.

Bioreactor application for Succinic acid production

New Brunswick bioflo reactor of 4-L used for the continuous fermentation process. Additional adjustment was made to measure the biomass. The biomass concentrator reactor (BCR) was designed, then the model executed by Stainless Steel Welding Services Pty. Ltd in Australia.

For the selected medium, a 1000 ml flask was prepared separately. The solution added aseptically to the Bioreactor before inoculation.

The dissolved oxygen was set at 100% saturation before inoculation. A continuous adjustment to the speed maintained at 30% to control oxygen concentration. A silicon antifoam grade A was added to manage any expected foam occurrence. The silicon purchased from Sigma-Aldrich, USA (Abd *et al.*, 2022).

RESULTS AND DISCUSSION

Effect of medium composition on Succinic bacteria performance

During the pre-evaluation, the bacteria life cycle was about two days. When 36 h was reached, the three bacteria entered the stationary phase. Eventually, the population growth declined between 40 h to 48 h and reached the death phase. The death phase occurred even though the media component was still not fully consumed. All the results demonstrate the effect of mediums on the bacteria performance by four main indications 1, SA production, 2, cell dry weight, 3, biomass and 4, Total sugar. All the mediums in the study exerted a positive effect on SA production. The results indicate similar behavior, which supports reliable outcomes across the parameters.

Fig 1 shows the results of medium for all bacteria. *Actinobacillus succinogenes* (S02) has higher than the *Mannheimia succiniciproducens* (S03) then *Basfia Succiniciproducens* (S01). In (Fig 1-a), the SA produced by S02 is 6 g/L, which is around 2 g/L more than S01 and S03. Looking at (Fig 1-b), up to 25% additional dry cell weight was achieved by the S02 compared to S01 and S03. The biomass of S02 is more than the other two bacteria. This is because medium one contains 60 g of sugar as the primary source of carbon.

These findings confirm that the *Actinobacillus Succinogenes* have high sugar consumption during the fermentation. The findings came similar to the observation of the *A. succinogenes* in rumen. The microorganism was adapted to a symbiotic role in the rumen and produced 74 g of SA (Guettler *et al*., 1999). In the current study, the mechanism is expected to adhere to the glucose uptake by the A. succinogenes. The *A. succinogenes's* ability to

Table 1: The medium composition.

Media	Components formula
Medium 1	60 g total sugars, 20 g tryptone, 10 g yeast extract, 0.3 g $MgSO_4.7H_2O$, 0.45 g $Na_2HPO_4.12H_2O$, 0.6 g $NaH_2PO.2H_2O$, 0.3 g $(NH_4)_2SO_4.7H_2O$, 0.2 g $CaCl_2$ and 20 g $MgCO_3$.
Medium 2	Polypeptone 10 g, 5 g yeast extract, 3 g K_2HPO_4, 2 g $(NH4)_2SO_4$, 0.2 g $CaCl_2$, $2H_2O$. 0.2 g MgCl26H_2O and 10 g $MgCO_3$ $Na_2S.9H_2O$.
Medium 3	Glucose 10.00, 10.0 g yeast extract, 8.0 g $NaHCO_3$, 8.5 g $NaH_2PO_4 \cdot H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$.

produce large amounts of succinate is attributed to its genome, which encodes a sugar transport protein (McKinlay *et al*., 2010). However, it is still plausible that the total sugar consumed by *Actinobacillus succinogenes* can be increased under different condition (Almqvist *et al*., 2016).

The results reported in (Fig 2) shows that *Actinobacillus succinogenes* (S02) produced 3.50 g/L. So in order to judge the result, it is crucial to bear in mind that medium two does not contain sugar or glucose, but uses Polypeptone as the primary carbon source. The mediocre succinic production

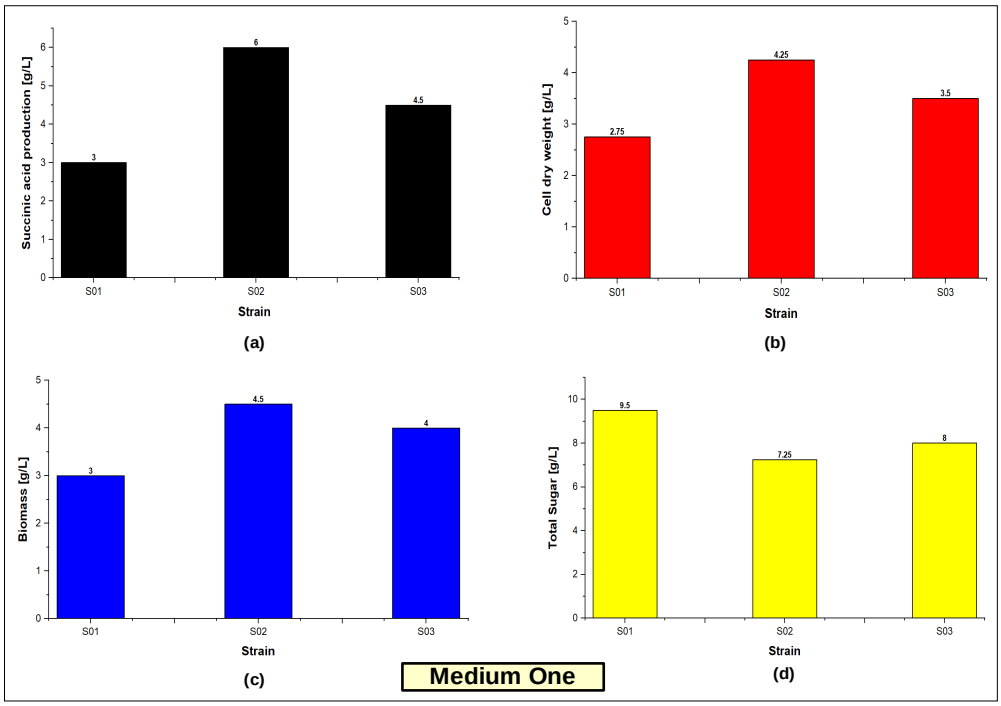

Fig 1: Succinic acid productions by 3 Strains selected using medium 1 (a) Succinic acid production, (b) Cell dry weight, (c) Biomass and (d) Total sugar.

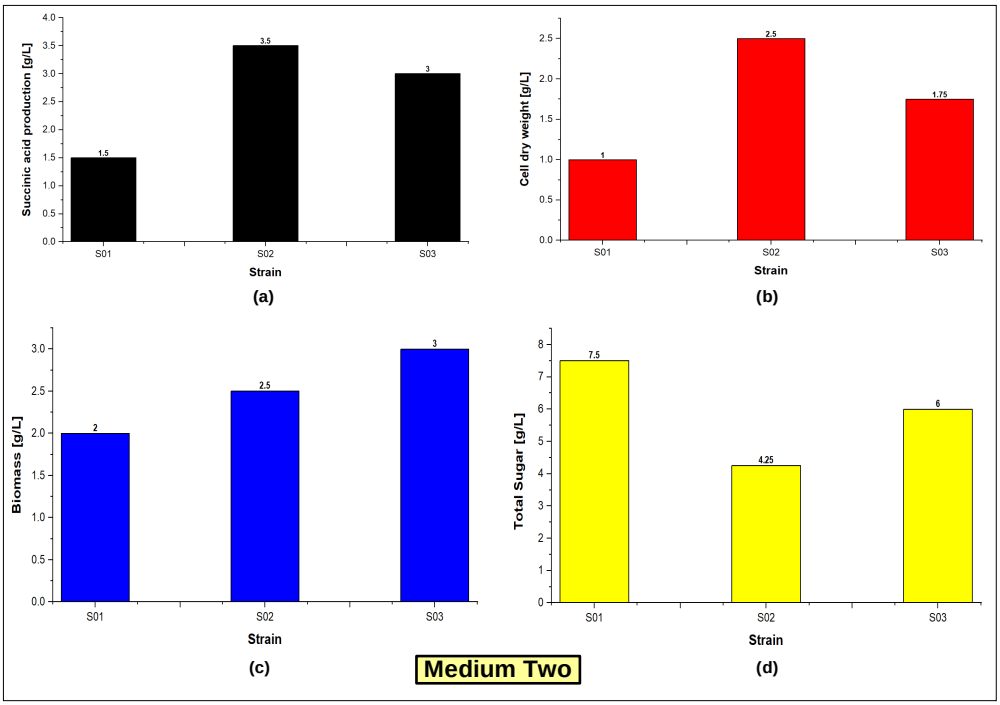

Fig 2: Succinic acid productions by 3 Strains selected using medium 2 (a) Succinic acid production, (b) Cell dry weight, (c) Biomass and (d) Total sugar.

for all the strains is due to the nature of Polypeptone. It is known that the biological constituents of Polypeptone have a mixture of casein peptone and meat peptone. The component of Polypeptone can increase cell density for several bacteria. Specific recent studies conducted on *Actinobacillus succinogenes* with Polypeptone proved that cell growth had accelerated and production increased (Pinkian *et al*., 2018; Kanchanasuta *et al*., 2020).

Regarding the cell dry weight, the recorded quantities of different strains seem far less than what has been obtained by medium one. For each strain, the results were lowered to 2.50 g/L, 1.75 g/L and 1.00 g/L. The total sugar consumed by S02 is 6.50 g/L compared to 4.25 g/L and 6.00 g/L for the other bacteria.

In Fig 3 the effect of glucose as the main component of the medium is demonstrated. The SA production exceeded what has been obtained by mediums one and two for all the strains. By analyzing S02, SA production is around 12.5 g/L, which is 60% more than S01 and S03. Furthermore, the cell dry weight recorded the quantities of the three types of strains as follow: 8.50 g/L, 5.00 g/L and 3.50 g/L for S02, S01 and S03, respectively. Regarding sugars utilization, the total sugar consumed by S02 is 7.50 g/L compared to 6.25 g/L for S03 and 5.00 g/L for S01.

S03 revealed weakness in attaining satisfactory SA production in all the mediums. S01 performance was moderate with potentiality at medium three. S02 had the utmost SA production quantity. The S02 has great performance in medium three especially, which attributes to the superiority of glucose over sugar and Polypeptone. Because glucose better absorbed in the reaction and accelerated bacteria growth (Liu *et al*., 2008; Ferone *et al*., 2019; Lin *et al*., 2020).

Our findings appear to be well substantiated by the pathway of the *Actinobacillus succinogenes in* Glucose medium (McKinlay *et al*., 2007), (Bradfield *et al*., 2014). It has been proved that sufficient Glucose utilization as a substrate, can enhance the succinate yield and improves the quality (Liu *et al*., 2013). In fact that associated with the low glucose concentration, so the SA would not be affected by cell fluctuation or inhibitors (Li *et al*., 2010).

S01 and S03 could be beneficial only if further investigations of carbon source utilization were conducted. Lastly, the second part of the study will only focus on S02 optimum at medium three.

Effect of raw materials on Succinic acid production

At this point, different types of sugars were involved in the process and were used as a carbon sources. By scrutinizing the typical raw material components, the corn fiber consists of 35% hemicellulose, 18% cellulose and 20% remaining starch (Gáspár *et al*., 2007). In contrast, Cane Molasses has glucose and fructose with an average concentration of 8.1 and 5.3%, respectively (Palmonari *et al*., 2020). the orange peel contains 16.9% soluble sugars, 9.21% cellulose and 10.5% hemicelluloses (Badaró *et al*., 2020). This variation in the composition revealed an impact on the magnitude of SA production. As seen in (Fig 4), the SA production vs material concentration manifests different SA levels. There is no question that the corn fiber produced the

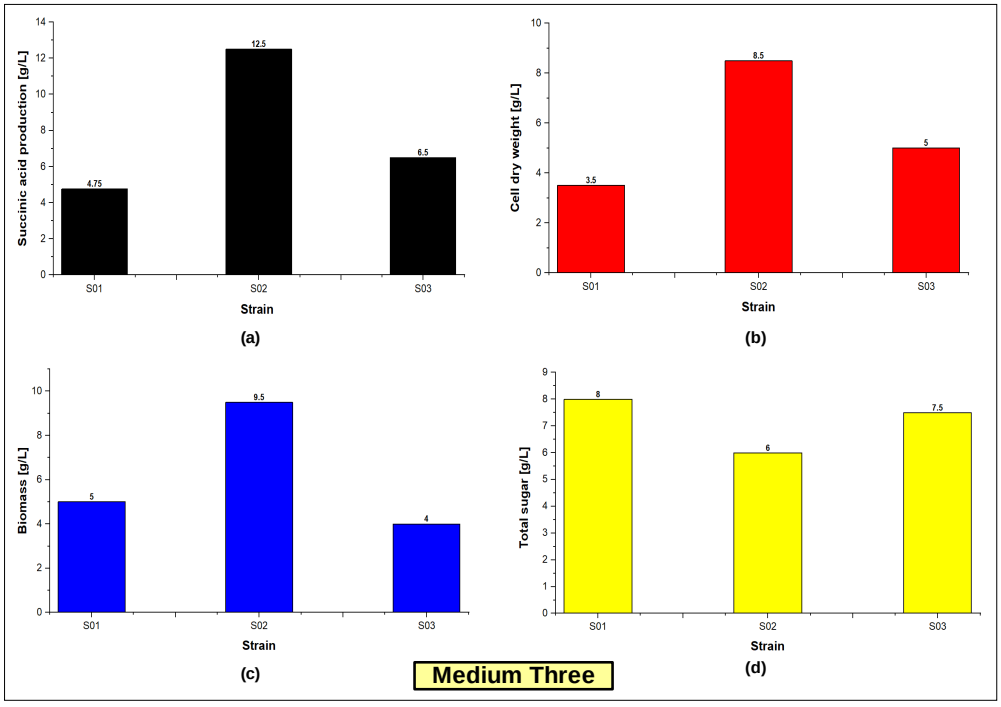

Fig 3: Succinic acid productions by 3 Strains selected using medium 3 (a) Succinic acid production, (b) Cell dry weight, (c) Biomass and (d) Total sugar.

highest Succinic acid. The total SA production increased by 6 g/L, approximately more than medium three. For the Cane Molasses, an addition of 1 g/L was observed. Surprisingly, the orange peel did not show any addition, but it seems to produce less than medium three.

Fig 4 elucidates a semi linear relation between the Orange Peel concentration and SA production. The second observation is that cane molasses stabilized production when the concentration reached 8 g/L. This means that any further addition of cane molasses will not be fermented. These results imply that S02 could efficiently ferment the combination of sugars in corn fiber.

Effect of raw materials on cell dry weight

In Fig 5, cane molasses and corn fiber are showing inconsistency between the increasing concentration and the cell dry weight. The results show that the intermediate content of sugar in cane molasses resulted in a slight drop in the middle concentrations. The lowest dry cell weight obtained is for the orange peel. When the corn fiber was used as a carbon source, the operation changed and the cell dry weight is 33% more than the cane molasses and 100% more than the orange peel. By comparing the previous cell dry weight obtained by medium three and the current carbon sources, 60% and 18% increase by corn fiber and cane molasses, respectively. The results in Fig 6 show high biomass retained from all the examined cultures.

Effect of raw materials on total sugar

The specific sugar consumption is a significant indication of cell growth. As much sugar is consumed SA production increases. The initial total sugar content varies as the carbon sources vary in the examination. However, at the end of the 48 h a significant trend appears, as seen in Fig 6.

Fig 6 illustrates the relationship between the sample concentration and the sugar consumed by S02. A clear consumption pattern, especially for the corn fiber, supports the possibility of high SA. The corn fiber reached the total sugar content of zero; this was interpreted by the S02 entirely

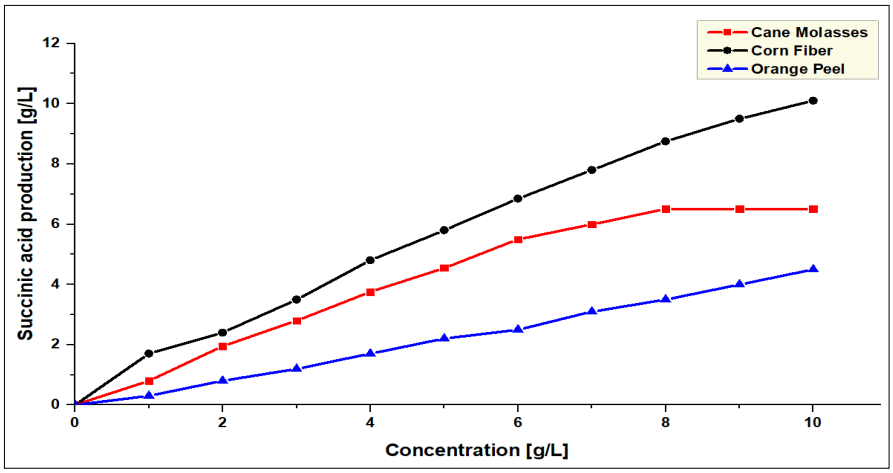

Fig 4: Succinic acid production after cultivation for corn fiber, cane molasses and orange peel at 37°C for 48 h.

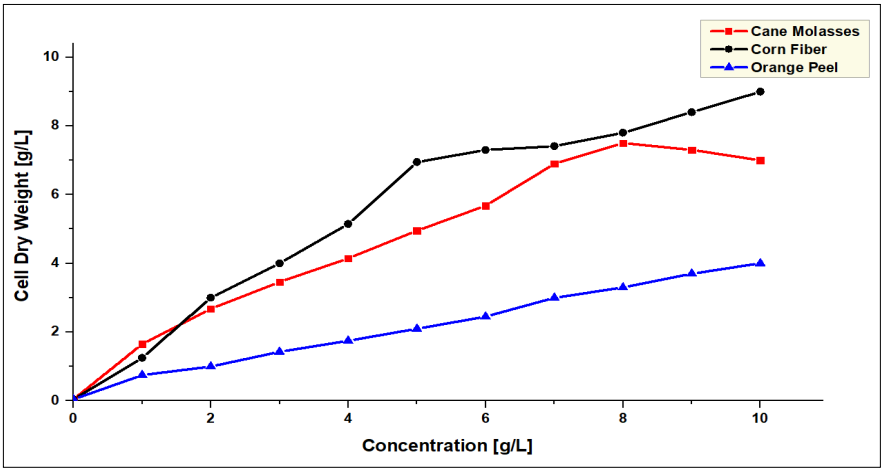

Fig 5: Dry cell weight after cultivation for corn fiber, cane molasses and orange peel at 37°C for 48 h.

consuming the culture content. However, this is not the case for the orange peel and the cane molasses. The sugar depletion follows a linear trend for increasing the concentration for all samples.

The findings support the study hypothesis. The corn fiber has successfully attained all the criteria required to obtain SA in the flask experiments. No consideration could limit the ultimate SA production by the S02 at the corn fiber

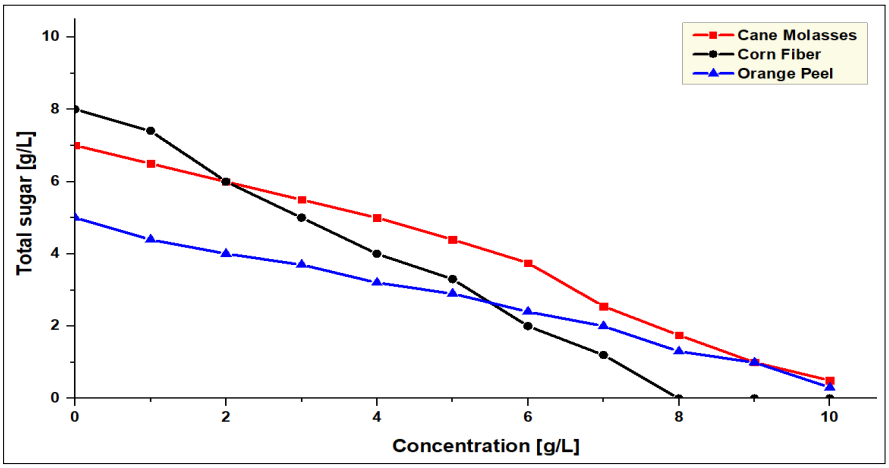

Fig 6: Total sugar after cultivation for corn fiber, cane molasses and orange peel at 37°C for 48 h.

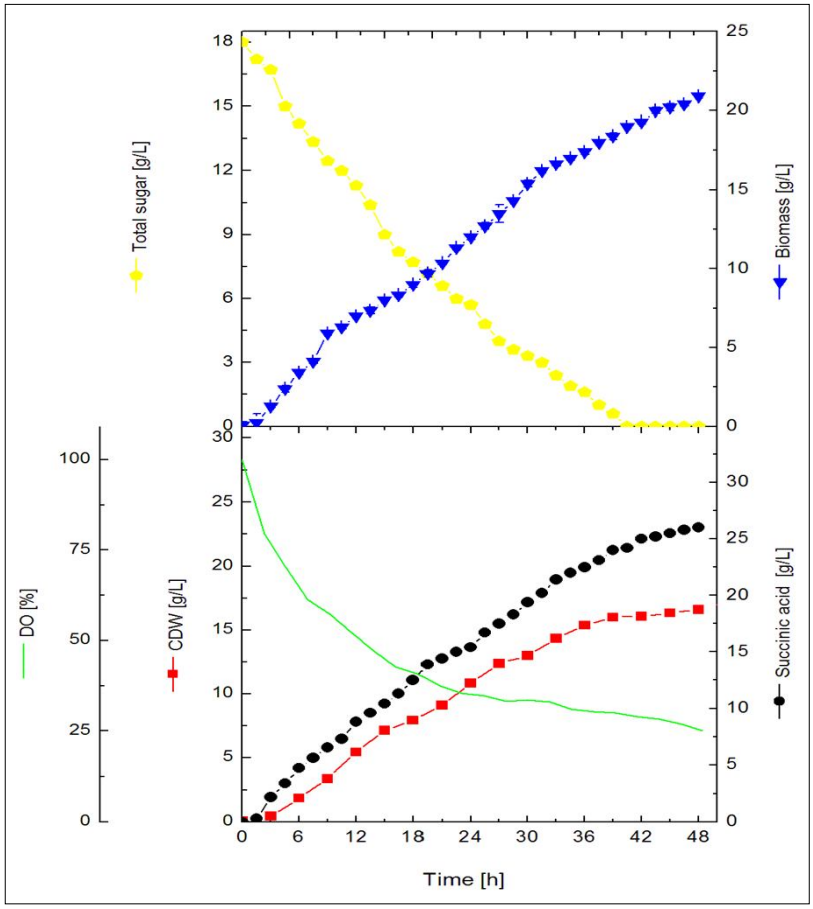

Fig 7: SA production, cell dry weight, biomass, total sugar and dissolved oxygen changes in Bioreactor 4-L cultivation.

medium. Thus, it will be continued to the 4-L bioreactor examination to predict the industrialization benefits.

Succinic acid production and kinetics of bioprocess parameter in 4-litter bioreactor

Corn fiber was selected to continue the scale-up examination for the large-scale fermentation process. 10 g/L of Corn fiber was utilized to isolate *Actinobacillus succinogenes*. in the 4-L Bioreactor. The medium used in the process is composed of Corn fiber 10.0, 10.0 g yeast extract, 8.0 g $NaHCO_3$, 8.5 g $NaH_2PO_4 \cdot H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH4)_2SO_4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$. The bioreactor ran under controlled pH conditions, where the initial pH sat at 7.0. The cultivation process was taken after 48 h; as had been practiced in the flask experiments.

It was noticed that the pH reduced to 6.5 after 3 h of cultivation. From the observation, the cells reached the stationary phase only after 3h. The cells entered the exponential phase with a growth rate of 0.175 [g/L.h]. The maximum growth rate and specific SA production are 1.024 [g/L] and1.175 [g/g], respectively.

During the process, the SA increased over time, as illustrated in Fig 7. SA production is 5.75, 11.30, 15.50, 22.00 g/L at 6, 12 and 24h. This indicates that the Bioreactor was able to give a similar result to the shake flask only after 12 hours. It is a quarter of the time needed previously. The maximal SA production value is 25.00 g/L. Besides, the cell dry weight and biomass, as seen in (Fig 7), were escalated during the fermentation process and reached a maximum value of 19.00 g/L and 20.50 g/L, respectively.

Apparently, both values are 50% higher than reported for the shake flask culture. The total sugar in batch bioreactor 4-L cultivation is reducing gradually from 18.00 g/L to null. Presumably, the sugar diminishes has occurred approximately between 36 h to 48 h. The quantity of dissolved oxygen (DO) decreased gradually from 100% to 25.00% at the end of fermentation. All the findings at the bioreactor stage could be explained by the effects of corn fiber (sugar complexity) and the production of many acids during the *Actinobacillus succinogenes* fermentation pathway (Alsaheb *et al*., 2020; Raje Nimbalkar *et al*., 2022).

CONCLUSION

The current biotechnology tends to utilize fermentation optimization process to choose appropriate Bacteria and medium to produce SA. The current study explored the succinic bacteria in different to enhance the understanding of the relationship between fermentation and raw carbon source. Encapsulating the findings can be as follow:

1-There is clear evidence that the *Actinobacillus succinogenes* is the best succinate bacteria to provide commercial SA. The 10 g glucose presence is sufficient to improve all succinate bacteria performance. The highest SA production was 12.5 g/L.

2-The comparison between the corn fiber, cane molasses and orange peel indicates potential replacement for the traditional carbon source. This is proved by the highest SA provided by corn fiber/ *Actinobacillus succinogenes* fermentation.

3-At the 4-L Bioreactor, the 10 g corn fiber provided 25 g/L of SA. The exponential growth rate of 0.175 [g/L.h] and specific growth rate of 1.024 g/L is a unique characteristics of the *Actinobacillus succinogenes* in the selected medium. Although the comparison proved the corn fiber applicability, further investigation on different medium composition/ conditions is needed.

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Conflict of interest: None.

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