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Succinic acid Production from Bovine Rumen – Isolation and Optimization

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Abstract: In this study, a novel succinic acid producing bacterium was isolated from cow bovine rumen. Two bacteria's are found to give better results for the production of succinic acid from the isolated colonies. Different synthetic carbon sources such as glucose, sucrose, fructose, dextrose, starch and cellulose and different crude carbon sources such as rice straw, sorghum stalk, baggase and sugar mill waste were studied. The fermentation process was carried out under CO_2 atmosphere with N_2 gas. The effect of pH, temperature and initial dextrose concentration were studied and optimized. The optimum fermentation conditions for the newly isolated strain were found to be: pH – 6.5, Temperature – 30°C, Initial dextrose concentration – 10 g/L. At these optimized condition, the maximum production of succinic acid was found to be 15g/l.

Keywords: Succinic acid, Bovine rumen, Optimization, Rice straw, Sorghum stalk.

1. INTRODUCTION

Succinic acid is a dicarboxylic acid and otherwise known as butanedioic acid. It is a common metabolite formed by plants, animals and microorganisms. It is an intermediate compound in the tricarboxylic acid cycle (TCA). It is also one of the fermentation products of energy metabolism [(1)]. It has been synthesized from petrochemical based maleic acid, but its fermentation production is drawing much attention in response to the current need to develop sustainable process using renewable resources[(2)]. This is an important point, as succinic acid can be produced from renewable, environmentally sound carbohydrates rather than relying on limited petrochemical hydrocarbons. It is synthesized by carbon-di-oxide fixation based carboxylation of C_3 metabolism. This unique carbon-di-oxide fixation makes fermentative succinic acid production even more attractive.

As the importance of succinic acid for use as a biodegradable polymer has increased, the biological production by fermentation has been focused on the alternative to the petrochemical based process. Newly developed facultative anaerobic bacteria *Actinobacillus succinogens*[(3)] and *Mannheimia succinicproducens*[(4)] are considered as the effective succinic acid producers because they can endure high glucose osmotic pressure and produce significant amounts of succinic acid with a high productivity.

The rumen is the first division of the stomach of a ruminant animal. More than 200 kinds of bacteria inhabit the bovine rumen. A number of functionally important rumen bacteria produce succinic acid during fermentation of carbohydrate, although succinic acid is seldom detected in measurable amounts in ruminal because it is rapidly converted to propionic acid. Several anaerobic and facultative bacteria that produce succinic acid from carbohydrates have been isolated from rumen and other sources: *Ruminococcus albus*[(5)], *cellulolytic Prevotella ruminicola*[(6)], *Bacteroides amylophilus*[(7)] *and Bacteroides fragilis* [(8)]. (Lee PC et

al: 2002) studied the production of succinic acid using bovine serum. In these studies, isolation of succinic acid producing strains was found and the process parameters for the maximum production of succinic acid using dextrose were found. This is the first report on the isolation of the bacterium from the cow bovine rumen.

2. MATERIALS AND METHODS

2.1. ORGANISM AND GROWTH CONDITIONS

Bovine rumen is taken from the blood serum of cow. Seed culture were prepared by growing cells at 39°C in a sealed anaerobic flask containing (MH) medium with CO_2 head space.

The isolation of a novel succinic acid producing bacterium, from bovine rumen was carried out. Anaerobic cultivation technique was used for the growth of organisms and preparation of media. The isolation medium contains (g/L): dextrose – 200; polypeptone -5; yeast extract – 5; NaCl- 2; Bactogar-12; sodium bicarbonate – 0.4; cysteine HCl – 0.25. Culture media were gassed with oxygen-free CO_2 and autoclaved for 15 minutes at 121°C.

2.2. ENRICHMENT AND ISOLATION

Ruminal contents were taken from a cow and were inoculated into an enrichment medium containing the ionoporic antibiotics lasalocid and monensin which inhibit rumen microorganisms that produce acetic acid and hydrogen, thereby favoring enrichment of those bacteria producing propionic acid. Subcultures were carried out after 18 h of incubation at 37°C. The culture was diluted to 10^{-6} in Phosphate buffered saline (abbreviated **PBS**) buffer. The diluted cultures (0.1ml) were spread onto screening agar plates and were incubated in an anaerobic jar under CO_2 atmosphere at 37°C. After 24-48 h, isolated colonies were picked with a sterilized 22-gauge needle and injected into vials containing enrichment medium plus lasalocid and monensin. Primary isolates were cultured for 24 h and their fermentation products were analyzed by HPLC. Isolates producing succinic acid were preserved for further analysis.

2.3. GROWTH CONDITIONS AND PHENOTYPIC ANALYSIS

Cells were grown anaerobically in sealed anaerobic bottles containing 100 ml of MH medium plus 10 g/L of glucose under CO_2 atmosphere. MH medium contains (g/L): polypeptone - 10, 5g yeast extract, 3 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.2g $CaCl_2 2H_2O$. 0.2 g $MgCl_2 6H_2O$ and 10 g $MgCO_3$ $Na_2S.9H_2O$ was added to a final concentration of 2 mg/L to ensure anaerobic conditions. For flask cultures, exponentially growing cells, washed anaerobically with MH medium, were used to inoculate sealed anaerobic medium bottles containing 100 ml of MH medium plus 10 g/L of glucose or other carbon sources. MH medium with lower amounts of polypeptone (0, 2 and 5 g/L) and yeast extract (0, 1 and 2 g/L) was also examined to see the effect of complex nitrogen sources on cell growth. Utilization of various carbon sources was examined by monitoring their concentrations during cultivation. Batch cultures were carried out at 39°C in a jar fermentor containing 1 1 MH medium plus 20 g/L glucose $Na_2S.9H_2O$ was again added to a final concentration of 1 mg/L. The pH was controlled at 6.5 using 5 N NaOH. Foaming was controlled by the addition of Antifoam 289. During aerobic cultivation, air was sparged and dissolved oxygen concentration was maintained at over 40% of air saturation by increase of agitation speed up to 1,000 rpm. CO_2 and N_2 gases were sparged during anaerobic cultivation and gas sparging rates and agitation speed were controlled at 0.25 vvm and 200 rpm, respectively. CO_2 and N_2 gases were scrubbed free of oxygen by passing them through a gas purifier. The sensivity of *M.Succiniciproducens* MBEL55E to various antibiotics was examined by counting colony-forming units (cfu) on agar plates containing various concentrations of these antibiotics.

2.4. ANALYTICAL PROCEDURES

The concentrations of fermentation products and carbon compounds were determined by HPLC equipped with an ion exchange column using 0.012 N H_2SO_4 as mobile phase. Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer. Dry cell weight (DCW) was calculated from a curve relating the OD_{660} of 1.0 was equivalent to 400 ± 20 mg DCW 1^{-1} was calculated from a curve relating the OD_{660} to DCW. An OD_{660} of 0.1 was equivalent to 400 ± 20 mg DCW1^{-1}. The yields of fermentation products were defined as grams of product formed form 1 g of glucose and were expressed as a percentage.

2.5. ESTIMATION OF CELL MASS

The estimation of growth is carried out by spectrophotometric method. The optical density of all cultures is measured using Elico-SLV 164, Double beam UV-VIS spectrophotometer at 660 nm with blanks of the appropriate growth medium. Suspension with an OD above 1.0 is diluted with appropriate growth medium. Curves relating OD to dry weight are constructed by harvesting cultures at room temperature, washing with the appropriate growth medium. Curves relating OD to dry weight are constructed by harvesting cultures at room temperature, washing with distilled water and

resuspending the cells in distilled waster to about 10 mg of dry weight per ml. Portions (5ml) are dried at 100°C and weighted. The dry weight of the cells is determined. The strain produces an extra cellular slime and in turn produces turbid solutions. In such cases, the optical density is read against a culture supernatant blank, diluting the blank in the same ration as the culture.

2.6. ESTIMATION OF CALCIUM SUCCINATE

The precipated calcium succinate was filtered at 39°C by vacuum filtration. Broths from IL and 2L fermentations were filtered using Whatman No.1 filter paper (Whatman Inc., Clifton Heights, N.J.) in a 17 cm dia. Ceramic Buchner funnel. The filtrate was collected in a 2L Pyrex vacuum flask. The filter cake was washed with the minimum volume required to remove all the filtrate. For larger fermentations a 20-ounce cotton twill filter cloth in a 12-inch diameter Buchner funnel was used for the filtration. The filtrate was then heated to 80°C, seeded with calcium succinate and mixed for 25 minutes allowing equilibrium to be established. The hot slurry was then filtered. The calcium succinate filter cake was washed with enough 80°C water to remove all filtrate.

3. RESULTS AND DISCUSSION

3.1. ISOLATION OF THE STRAIN

The strain used in this study was isolated from a cow bovine rumen which is enriched with a medium containing the ionophoric antibiotics Lasalocid and Monensin which inhibit rumen micro organisms that produce acetic acid and hydrogen, there by favoring enrichment of those bacteria producing propionic acid. Seven strains of strictly anaerobic succinic acid producing bacterium were isolated in pure culture from a bovine rumen contents. The growth conditions were maintained at temperature - 30°C and pH - 6.5.

The bacterium produced various fermentation products from a wide range of carbon substrates under anaerobic conditions. Large amount of succinic acid was produced under anaerobic conditions in the presence of CO_2. Under anaerobic conditions, phosphoenolpyruvate (PEP) can be directed to C_4 branch (succinic acid) and/or C_2, C_3 branch (ethanol, lactic and acetic acids) pathway by PEP carboxykinase and pyruvate kinase, respectively. PEP carboxykinase is a CO_2 fixing enzyme that converts PEP to oxaloacetate, which is further converted to succinic acid as a final compound via several reactions. Pyruvate kinase converts PEP to pyruvate, which is subsequently converted to several end-products including acetic, and formic acids. Therefore, PEP carboxulation catalyzed by PEP carboxykinase is a reaction for succinic acid production and the availability of CO_2 controls the partition of PEP to various metabolites such as succinic, lactic and acetic acids. This seems to be why overall cellular activities and succinic acid production were significantly repressed under N_2.

3.2. SCREENING OF THE STRAIN

The utilization of carbon sources by the bacterium was also examined. The bacterium could utilize glucose, sucrose, dextrose, starch, fructose and cellulose as a synthetic carbon source and as a crude carbon source rice straw, sorghum stalk, baggase and sugar mill waste was used at 30°C with continuous supply of CO_2. Accordingly fig 3.1 shows, succinic acid yield on dextrose was higher (i.e) 15 g/L from Ia strain, 10 g/L from Ic strain and 10.8 g/L from IIIc strain respectively and fig 3.2 shows sorghum stalk was higher on all the strains.

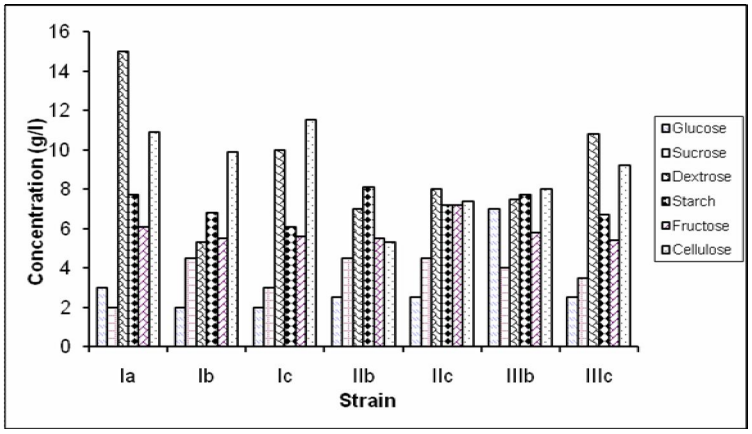

Fig. 3.1 Production of succinic acid from different isolates using various synthetic carbon sources

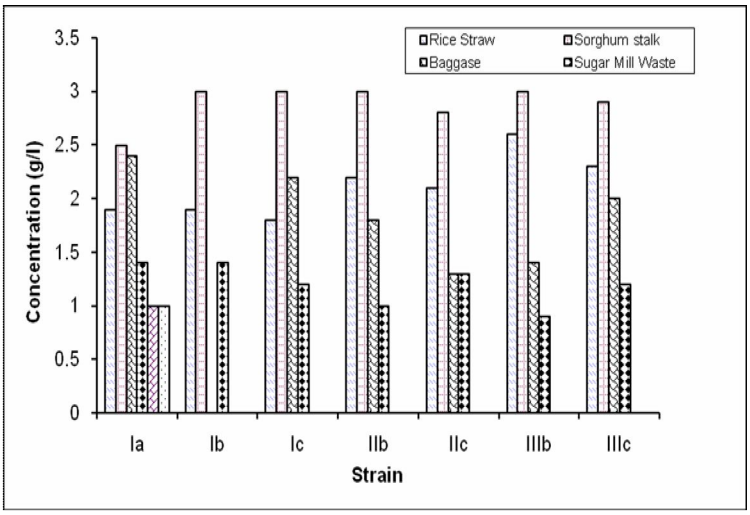

Fig. 3.2 Production of Succinic acid from different isolates using different crude carbon sources

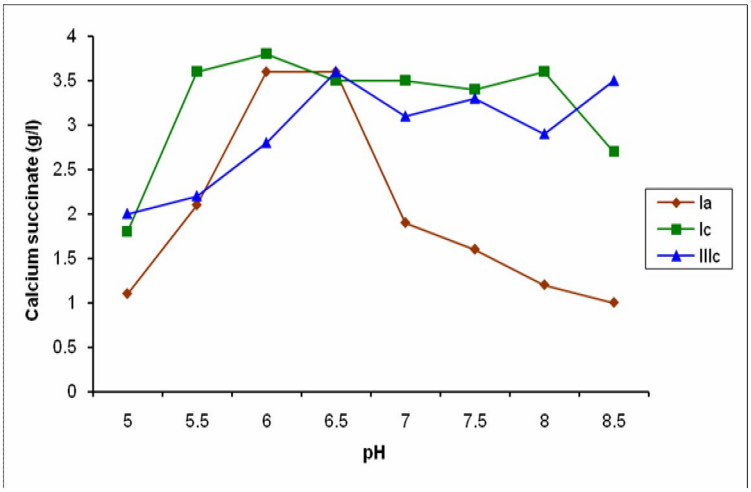

Fig.3. 3. Effect of initial pH on the production of succinic acid using selected strain

3.3. EFFECT OF INITIAL PH

The effect of initial pH on the production of calcium succinate using new isolated strain was investigated by conducting the fermentation for 48 hours. The results were shown in Fig. 3.3. The fermentors were gassed with 100% CO_2. When the pH was maintained at 5.5, cell growth was poor. As the pH was increased from 6.0 to 7.5, cells grow better and the dextrose consumption rate increased. When the pH was maintained at 8.0, cell growth was severely impaired. Initial pH 6.5 was found to be an optimum initial pH which results the highest yield of calcium succinate the ratio of end-products formed was not much affected by changing the culture pH, growth rate, on the other hand, was not significantly affected. Thus the results show that the fermentation should be conducted at the pH 6.5. This is the optimum pH for cell growth. The highest succinate yield based on dextrose consumption at optimum fermentation pH 3.6g/l.

3.4. EFFECT OF TEMPERATURE

The effect of fermentation temperature on the performance of the newly isolated strain was investigated. Table 3.1 summarizes comparative results obtained when fermentation were run at four different temperature values, temperature 30°C, 32°C, 35°C, 36°C. The samples were analyzed for the production of Calcium succinate. At the end of the fermentation Calcium succinate concentration was higher at the temperature 30°C. The initial concentration of calcium succinate was increased then the other concentration of temperature. The fig 3.4 shows that the fermentation temperature of 30°C was found to be an optimum temperature with resulted the highest succinate yield based on dextrose consumption is 3.6 g/l.

Table 3.1. Effect of temperature on the production of Succinic acid using selected strain

Strain	Calcium succinate (g/l)			
	Temperature			
	30°C	32°C	35°C	36°C
Ia	3.6	2.2	1.7	3.2
Ic	3.5	2.2	1.7	3.5
IIIc	3.6	2.5	2.2	2.8

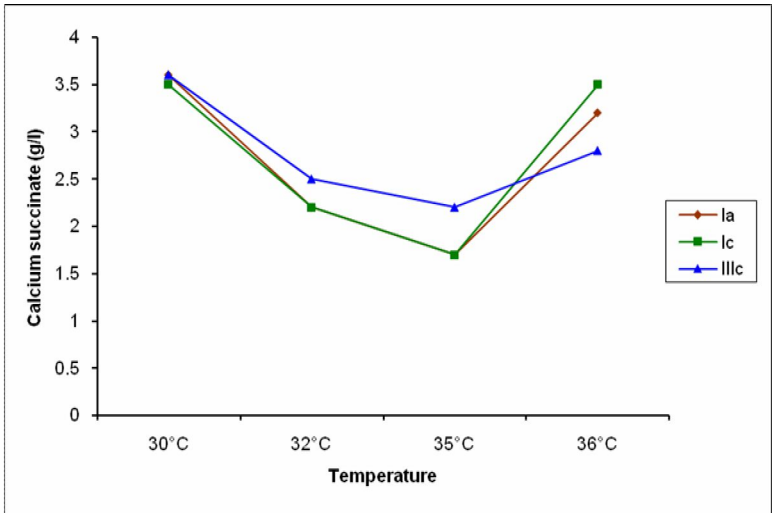

Fig. 3.4. Effect of temperature on the production of succinic acid using selected strain

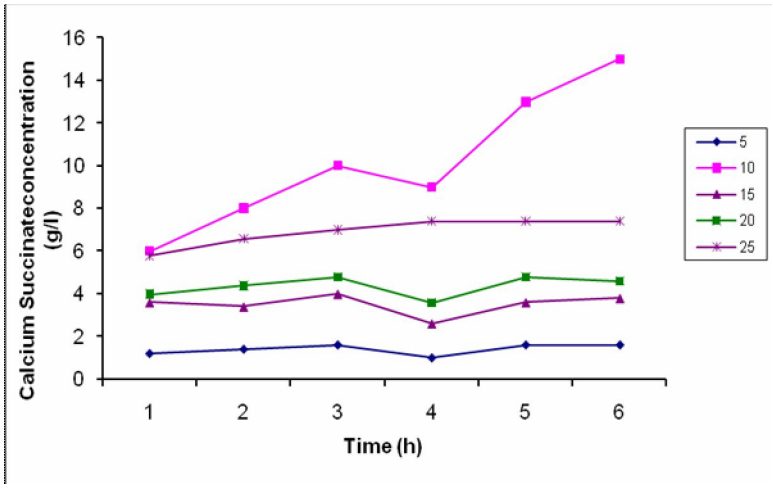

Fig. 3.5. Effect of initial dextrose concentration on the production of Calcium succinate.

3.5. EFFECT OF INITIAL DEXTROSE CONCENTRATION

Metabolic engineering of a succinic acid producer should allow production of succinic acid to a high concentration with high productivity and high yield without byproducts formation. Of course, this strain development process needs to be integrated with the fermentation process development which also considers the best available carbon source, calcium succinate, optimal cell mass and other culture condition. Taken together future of fermentative production of succinic acid is bright considering the increasingly acceptable raw material cost, large potential market size, the additional of CO_2 fixation and advances in the development of strategies for strain improvement, fermentation and purification. Fig 3.5 shows that, 10g/l of dextrose gives the highest succinate yield of 15g/L.

4. CONCLUSION

Production of succinic acid using new strain was investigated and the factors affecting the production were optimized. Ia strain may be suggested as a candidate strain for the production of succinic acid because of its high succinic acid productivity. The initial pH of the culture medium significantly affects the production. A suitable substrate concentration level is essential to get high production; however, excessively low concentrations of substrate level affect the growth of organism resulting in reduced production. Temperature is another important factor and it also affects the growth of the microorganism. The optimum values there found to be time 48h, initial pH-6.5, Temperature 30°C and dextrose 10g/1. At these optimized condition, the maximum succinic acid production was found 15g/1. From the results, it can be concluded that Ia may therefore be considered as a promising candidate for the strain and process development which is necessary for cost efficient succinic acid production. Still there is considerable task of lowering the costs of succinic acid recovery technology. Decreasing the number of unit operations and developing lower-cost liquid extraction procedures appear to be promising ways of improving the overall process economics.

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