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Research Article

ORGANIC ACID PRODUCTION BY *CORYNEBACTERIUM VITAERUMINIS*

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ABSTRACT
A new facultative anaerobic bacterial strain producing lactic acid and succinic acid was isolated from bovine rumen fluid. 16S rDNA analysis of the isolated strain showed closest match to *Corynebacterium vitaeruminis* with 99.13 % similarity score. Batch fermentation of *Corynebacterium vitaeruminis* MTCC 5488 was studied in a 7 l fermenter under optimum culture conditions and the isolated strain exhibited a maximum lactic acid and succinic acid concentrations of 15.3 g/l and 2.2 g/l respectively in 14 h of fermentation with a maximum cell absorbance of 8.5 at 600 nm. Since high concentration of carbon source (glucose) inhibits the acid production, fed-batch strategy was designed. Based on the carbon consumption rate data in batch process, glucose solution (370 g/l) was fed continuously to the reactor from 8 h (the time, when the culture enters the log phase) till the end of fermentation to maintain the glucose concentration in the range of 5-8 g/l in the fermentation medium. By continuous feeding of carbon source (glucose), lactic acid and succinic acid concentrations of 38.5 g/l and 8.4 g/l respectively were obtained. The concentrations of lactic acid and succinic acid obtained by the continuous feeding of glucose were 2.6 and 4 times respectively higher than that obtained in batch process.

Key Words: *Fermentation, Lactic Acid, Succinic Acid and Fed-Batch Fermentation*

INTRODUCTION
Organic acids constitute a key group among building block chemicals that can be produced by microbial processes and have long history of use as raw materials in food, cosmetic, chemical and pharmaceutical industries. In recent times, lactic acid and succinic acid have received greatest interest for use as monomers for the production of biodegradable polymers (e.g. Polylactic acid and Polybutylene succinate). The esters of lactic and succinic acid (Ethyl lactate and Diethyl succinate) have also become the focus of attention as environmental friendly green solvents. Lactic acid a C_3 monocarboxylic acid is also used for the production of other organic chemicals, including acrylic acid, propylene glycol etc (Datta *et al.*, 1995; Sauer *et al*., 2008). Succinic acid, a C_4-dicarboxylic acid (butanedioic acid) is predicted to be one of the future platform chemicals because a huge variety of bulk chemicals such as 1, 4-butanediol, tetrahydrofuran, γ-butyrolactone, biodegradable polymers etc. can be produced from it by chemical conversion (Bechthold *et al*., 2008). Microbial fermentation provides a simple and environmental friendly process for the production of these organic acids. Many microorganisms have been isolated and studied for the production of lactic acid and succinic acid from different raw materials such as saccharides, molasses, whey, starchy & cellulosic materials (Song and Lee, 2006; Wee *et al.*, 2006; Shanmugan and Ingram, 2008). Lactic acid is mainly produced by *Lactobacillus* species with high yield and productivity (Carra *et al.*, 2002). For succinic acid, three naturally occurring bacteria (*Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens* and *Mannheimia succiniciproducens)* and one genetically engineered *E.coli* have been reported to produce it as a major fermentation product along with acetate, ethanol, formate and lactate as co-products at various levels (Lee *et al.*, 2000; Kim *et al.*, 2004; Scholten and Dagele, 2008). However, most microorganisms currently available are anaerobic that require zero oxygen concentration to survive. The need of strictly anaerobic conditions requires additional expensive equipments for the production of these organic acids adding to the cost of their production. Therefore, there is a need for a microorganism that can tolerate oxygen and is

capable of producing succinic acid and lactic acid at a high yield. There is also a need for a process that would allow for microbial production of succinic acid and lactic acid in a simple and efficient manner. However, as far as we know till date, this is the first report on production of lactic acid and succinic acid under facultative anaerobic conditions using *Corynebacterium vitaeruminis* MTCC 5488 culture.

Present study involves the isolation of a new bacterium from rumen fluid, identification and batch fermentation studies. Based on batch data, fed batch fermentation was designed to maintain a low sugar concentration during the fermentation process so that cells can grow faster and organic acid production phase can be prolonged due to elimination of the substrate inhibition.

MATERIALS AND METHODS

Media components were obtained from HiMedia Laboratories, India and Becton Dickinson and Company USA. Genomic DNA Isolation kit was obtained from Zymo Research USA. PCR reagents and Primers were obtained from Sigma Aldrich India. Vector pTZ57R was obtained from Fermentas USA. All other chemicals were of analytical grade and obtained from Sisco Research Laboratories, India.

Isolation and selection of organic acids producing bacteria

To isolate a lactic acid and succinic acid producing microbial strain, nutrient broth containing 1% glucose was inoculated with 10% bovine rumen fluid. This culture was incubated under facultative anaerobic conditions with carbon dioxide atmosphere at 37^{o} C for 72h. In our study, facultative anaerobic condition was created by supplying carbon dioxide without removal of existing oxygen from the bioreactor. After 72 h of growth, the culture was transferred to fresh sterilized nutrient broth with 1% glucose and incubated for 48 h at 37^{o}C. The final grown culture was diluted to 10^{-4} in phosphate buffer (0.1 M) and 0.1 ml of this diluted sample was spread on nutrient agar plates. The plates were incubated in an anaerobic jar with carbon dioxide atmosphere at a temperature of 37^{o}C. Single, well isolated colonies were picked up and transferred to test tube containing 5 ml sterilized tryptic soy broth and incubated in an anaerobic jar with carbon dioxide atmosphere for 48 h at 37^{o}C. After 48 h of incubation, the acid concentrations were analyzed by high performance liquid chromatography (HPLC, Aminex, HPX – 87H column). A strain producing more than 1 g/l of succinic acid and lactic acid was selected for further studies.

Identification of the selected bacteria producing organic acid

The isolated microbial strain producing organic acids was identified using 16S rDNA (ribosomal DNA) gene sequencing. Genomic DNA was isolated from the selected strain using Zymo Research® kit and used as template in the Polymerase Chain Reaction (PCR) to amplify the 16S rDNA fragment. This Polymerase Chain Reaction (PCR) was carried out with primers 27F (5'-AGAGTTTGATCMTGGCTCAG–3') and 1492R (5'- TACGGYTACCTTGTTACGACTT – 3'), the primer numbers corresponding to that of the *E. coli* 16S rDNA gene. The PCR fragments so obtained were then cloned into pTZ57R vector (Fermentas®) and sequenced using M13 forward and reverse primers. The sequence similarity of the 16S rDNA to other known strains was analyzed.

Batch fermentation of Corynebacterium vitaeruminis MTCC 5488

7 l laboratory fermenter (Scigenics India Pvt Ltd.) with working volume of 5 l was used to carry out batch fermentation. Inoculum was grown in tryptic soy broth media for 20 h at 37^{o}C under static conditions and was transferred (10% v/v) to the 7 l fermenter containing glucose 20 g/l, Yeast Extract 5 g/l, Peptone 10 g/l, K_2HPO_4 3 g/l, NaCl 1 g/l, $CaCl_2.2H_2O$ 0.02 g/l, $MgCl_2.7H_2O$ 0.02g/l. Fermentations were carried out for 16 h at temperature 37 $\pm$ 0.5 ^{o}C and agitation speed of 300 rpm. The pH of the fermentation medium was maintained at 6.8 $\pm$ 0.25 using 5 N NaOH. Carbon dioxide gas was continuously supplied at the rate of 0.2 – 0.25 vvm to maintain facultative anaerobic conditions throughout the fermentation. Samples were withdrawn at regular time interval during fermentation and analyzed for cell biomass, residual glucose, lactic acid and succinic acid.

Fed-batch fermentation of Corynebacterium vitaeruminis MTCC 5488

The Fed-batch fermentation was also carried out in 7 l reactor. Inoculum was grown in tryptic soy broth media for 20 h at 37^{o}C under static conditions and was transferred (10 % v/v) to the 7 l fermenter containing glucose 20 g/l, Yeast Extract 5 g/l, Peptone 10 g/l, K_2HPO_4 3 g/l, NaCl 1 g/l, $CaCl_2.2H_2O$ 0.02 g/l, $MgCl_2.7H_2O$ 0.02g/l. Fermentations were carried out at temperature 37 $\pm$ 0.5 ^{o}C and agitation speed of 300 rpm. The pH of the fermentation medium was maintained at 6.8 $\pm$ 0.25 using 5 N NaOH. Carbon dioxide gas was continuously supplied at the rate of 0.2 – 0.25 vvm to maintain facultative anaerobic conditions throughout the fermentation. A concentrated glucose solution (370 g/l) was fed at the rate of 0.5 ml/min to the reactor continuously from 8 h (the time, when the culture enters the log phase) to 27 h (the time, when the culture enters the stationary phase) to maintain the concentration of glucose (5 – 8 g/l) in the fermentation medium. After 27 h of fermentation the feeding rate of glucose solution (370 g/l) was reduced to 0.1 ml/min till the end of fermentation. Samples were withdrawn at regular time interval during fermentation and analyzed for cell biomass, residual glucose, lactic acid and succinic acid.

Analytical Methods

After the incubation period, the samples were centrifuged (10,000 rpm) using Beckman Coulter Microfuge 22R Centrifuge for 10 min at 4^{o}C. The cell pellets were washed two times with distilled water to remove all media components and the cell growth was measured by taking absorbance at 600 nm wavelength using a photometer (Eppendorf). The concentrations of organic acids (lactic acid and succinic acid) and residual sugars were quantified using high performance liquid chromatography (HPLC, Aminex, HPX – 87H column) under the following conditions: mobile phase - 0.008 N H_2SO_4, Flow rate - 0.6 ml/min, Temperature - 35^{o}C, Detector - refractive index (Liu *et al*., 2008).

RESULTS AND DISCUSSION

Isolation and Identification

The novel organic acid producing bacterial strain was isolated from rumen fluid of buffalo. The isolated strain has coccobacillus cell morphology with non-spore forming and facultative anaerobic growth characteristics. The sequence similarity of the 16S rDNA to other known strains. The strain showed closest similarity to *Corynebacterium vitaeruminis* strain with 99.13 % similarity score. This strain was deposited with accession number 5488 at the Microbial Type Culture Collection (MTCC) in IMTECH, Chandigarh, India (Shukla *et al*., 2009).

Batch fermentation of Corynebacterium vitaeruminis MTCC 5488

The batch kinetic profiles of the growth of *Corynebacterium vitaeruminis* MTCC 5488, with respect to biomass absorbance, residual substrate and products (Lactic acid and succinic acid) are shown in Figure 1. The entire carbon source (glucose) was consumed in 14 h of fermentation and the specific glucose consumption rate was found to be 2.8 g/l.h in the log period of the culture growth. At the end of log phase the maximum cell absorbance was found to be 8.5 (at 600 nm). Culture started synthesizing lactic acid as a main product and succinic acid as a co product during the log period of growth and their maximum concentrations at the end of fermentation were 15.3 g/l and 2.2 g/l respectively. This was significantly higher than the concentrations reported for lactic acid and succinic acid in the literature for batch cultivation of *Corynebacterium glutamicum* and *Bacteroides fragilis* (Okino *et al*., 2005; Isar *et al*., 2006). Once the culture reached to stationary phase the rate of lactic acid production decreased and rate of succinic acid production increased. The maximum yield and productivity of lactic acid at the end of fermentation were 70% and 1.1 g/l.h respectively. For succinic acid the yield and productivity were 10 % and 0.16 g/l.h respectively.

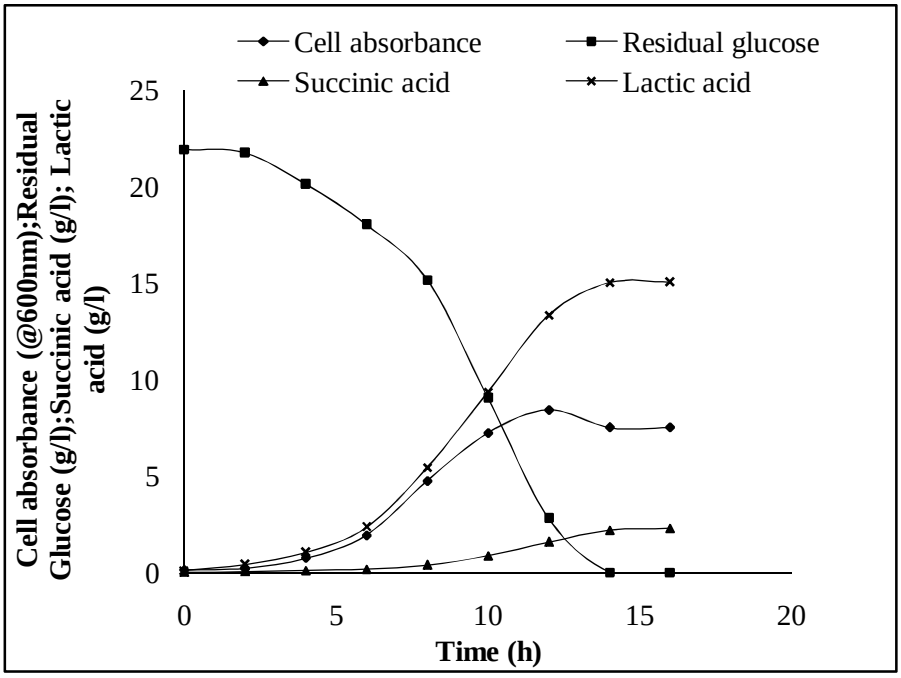

Figure 1: Batch fermentation profile of *Corynebacterium vitaeruminis* MTCC 5488

Fed-batch fermentation of Corynebacterium vitaeruminis MTCC 5488

The growth of *Corynebacterium vitaeruminis* MTCC 5488 and organic acid production strongly depends upon the initial concentration of carbon source. It has been reported in the literature that cell growth and organic acids production rates decrease with increase in glucose concentrations in the fermentation medium (Ding and Tan, 2006; Lin *et al*., 2008). Based on the glucose consumption rate data in batch process the glucose nutrient feeding strategy was designed. The glucose solution (370 g/l) was fed at the rate of 0.5 ml/min to the reactor continuously from 8 h to 27 h to maintain the concentration of glucose (5 – 8 g/l) in the medium. After 27 h of fermentation, the glucose solution (370 g/l) was fed at the rate of 0.1 ml/min till the end of fermentation. The fed-batch kinetic profiles of the growth of *Corynebacterium vitaeruminis* MTCC 5488 with respect to biomass absorbance, residual substrate and products (Lactic acid and succinic acid) are shown in Figure 2. Continuous feeding of glucose increased the biomass absorbance from 2.2 to 12.1 (at 600 nm) as the culture enters the logarithmic phase of growth which is 1.5 times higher than the batch culture. At the end of fermentation the maximum concentrations of lactic acid and succinic acid were 38.3 g/l and 8.4 g/l respectively. The yields of lactic acid and succinic acid were 79% and 17% respectively which are higher than that obtained in the batch fermentation. Although the productivity of lactic acid (1.1 g/l.h) did not change in fed-batch, the productivity of succinic acid (0.24 g/l.h) was found to be 1.5 times higher than that obtained in the batch process, because the culture entered into the stationary phase. It has been found that cells of stationary phase can produce more amount of succinic acid as compared to cells of log phase (Shanmugam and Ingram, 2008). In the present study, production of byproducts such as acetic acid and propionic acid could not be detected due to very low concentrations, eliminating the need for extensive purification.

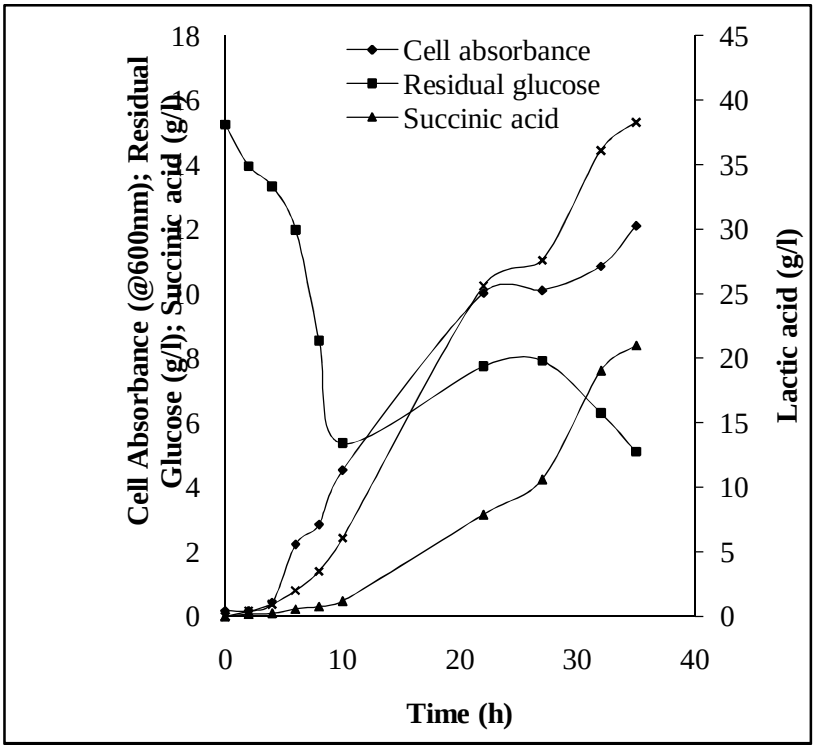

Figure 2: Fed-Batch fermentation profile of *Corynebacterium vitaeruminis* MTCC 5488

In conclusion, a new bacteria *Corynebacterium vitaeruminis* MTCC 5488 was isolated and identified. The isolated microbial strain *Corynebacterium vitaeruminis* MTCC 5488 produces two organic acids mainly lactic acid and succinic acid as major fermentative products. Moreover, as the isolated microbial strain is tolerant to oxygen, it allows for the production of succinic acid and lactic acid under facultative anaerobic conditions. This eliminates the problem of process instability which usually occurs due to the presence of oxygen in the conventional fermentation process of producing succinic acid and lactic acid using obligate anaerobes. This method was shown to be very effective and easily controllable for production of organic acids using newly isolated strain and also useful to reduce the recovery cost of the product.

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