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Microbial production of succinic acid using crude and purified glycerol from a *Crotalaria juncea* based biorefinery

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ABSTRACT

Microbial conversion of crude and purified glycerol obtained in the process of biorefining *Crotalaria juncea* is carried out to produce succinic acid using *Escherichia coli*. Batch tests are performed for nine different substrate concentrations of commercial, purified and crude glycerol, in order to observe cell growth and substrate utilization rate. Inhibitory (Halden-Andrew, Aiba-Edward, Tessier type and Andrews) as well as non-inhibitory (Monod, Moser and Tessier) models are fitted to the relationship between specific growth rate and substrate concentration obtained from the growth curves. Considering the inhibition effect, Aiba-Edward model ranked 1 out of 7 in case of two samples and Haldane-Andrew model ranked 1 in case of one sample. Aiba-Edward model gave the best fitment for a large range of concentrations of all the three types of glycerol, crude, purified and laboratory grade. Maximum production of succinic acid is obtained from commercial glycerol at pH 7 and 37.5 °C.

1. Introduction

Bio-refinery are currently the focus of many researches as they can be used as a substitute of petroleum refinery for converting agricultural waste materials, through appropriate microbiological or chemical treatment, into either gaseous or liquid fuels or value added biochemical. Besides environmental benefits, a bio-refinery is also capable of generating economic benefits.

The dramatic growth of biodiesel industry all over the world and the shift towards the use of waste materials as feedstock, has created environmental and economic concerns by generating glycerol by-products [9,31]. Technical grade crude glycerol (raw glycerol) produced from the reaction is usually of 80% purity (approximately). Disposal of surplus glycerol is, therefore, becoming an increasing challenge and potential innovative uses need to be found out. Different useful chemicals can be produced from glycerol by fermentation (some of them using technical grade glycerol) [12,22,26]. Due to its reduced nature of carbon atoms, fuels and reduced chemicals can be produced from glycerol at higher yields than those obtained from common sugars such as glucose or xylose. Using glycerol as a feed stock in the fermentation process, there is a significant increase in the product yield of chemicals, such as succinate, ethanol, and propanediols, whose production is limited from these sugars.

Succinic acid ($C_4H_6O_4$), also known as amber acid or butanedioic acid, a dicarboxylic acid is mostly produced by the chemical route from *n*-butane through maleic anhydride [11,23]. Microbial

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production has been mainly restricted to the food and pharmaceutical industries. The biotechnological production of succinic acid at the industrial scale, for wider end-user companies, is related to its market price. Succinic acid can be produced by different kinds of anaerobic and facultative bacteria as a fermentation end-product. However, only a few species can produce it as the major end-product with high yield, such as *Anaerobiospirullum succiniciproducens, Actinobacillus succinogenes and Mannheimia succiniciproducens* [15,17,18,25,30,33]. *Escherichia coli* produce succinic acid as a minor fermentation product (typically 7.8% of total) under anaerobic conditions [14]. Several metabolic engineering strategies have been used for the enhanced production of succinic acid by *E. coli* with some good results and production yields [6,13,16,29,32].

A variety of mathematical microbial growth kinetic models have been developed, proposed and used by many researchers to predict the amount of biomass production at a particular time, substrate utilization and bacterial growth [5,24]. These models are capable of predicting the reduction of chemicals to certain concentration due to utilization by microbes.

In the present research work, waste glycerol produced by trans-esterification of *Crotalaria juncea* oil, was purified using several physico-chemical steps. *E. coli* was used to produce succinic acid using commercial, purified and crude glycerol obtained from Sunn-hemp seeds as the carbon source. A number of batch fermentations were conducted for glycerol concentrations ranging from 1 to 30 g/100 ml in order to evaluate cell growth. Seven growth models namely, Monod, Moser, Tessier, Halden-Andrew, Aiba-Edward, Tessier type and Andrews were used to determine the model-specific growth kinetic parameters: specific growth rate (μ), substrate saturation constant (K_S), and substrate inhibition constant ($K_{I,S}$). Finally, the effects of process parameters were optimized by Response Surface Methodology (RSM) using a Face Centered Central Composite Design (FCCCD).

2. Materials and methods

2.1. Preparation of glycerol for the fermentation substrate [20]

In this research work, Sunn-hemp seeds were collected from Central Research Institute for Jute and Allied Fibers (CRIJAF), Kolkata, India. The raw seeds were cleaned and dried and then crushed using a domestic kitchen mill. Oil was extracted from 107 g crushed seeds on adding 500 ml of isopropanol into a standard Soxhlet extractor [7]. *Trans*-esterification of Sunn-hemp oil was carried out with 20 g oil in a 500 ml three-necked round-bottom flask, equipped with a mechanical stirrer and a water-cooled condenser. The reaction was carried out with an oil-methanol molar ratio of 1:11 at 4 h and 60 °C temperature in presence of 2 wt % KOH. After completion of the reaction, the mixture was taken into a separating funnel for separating the phases [19].

Crude glycerol, obtained from the bottom layer, was purified through several physico-chemical steps, namely: acidification, neutralization, solvent extraction, adsorption and finally pressure filtration through a membrane. Initially, excess alcohol from trans-esterification process was removed by evaporation, using a rotary evaporator. Then glycerol was acidified using dilute sulphuric acid or phosphoric acid in order to split the soap. The charred substances produced were filtered off. The samples were then decanted to recover the crude fatty acids. The aqueous glycerine solutions were neutralized by 50% sodium hydroxide. The salt, crystallizing out, was removed by decantation. In order to purify and concentrate the solutions further, they were solvent extracted and filtered to remove the residual salt. Finally, they were evaporated at 79 °C to obtain the crude glycerine. Decolourisation was done on addition of activated carbon into the remaining glycerol. Finally, it was purified by vacuum filtration using cellulose acetate membrane filter (**Make**: Sartorius Stedim Biotech S.A.; Pore size: 0.2 μm). The pressure was maintained at 750 mm Hg (vacuum gauge) by a vacuum pump (**Make**: Tarsons; **Model**: Rockyvac 300).

2.2. Preparation of microorganism and culture

In this study, *E. coli* (ATCC 8739) was acquired from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The bacteria was cultivated by transferring 2 ml of a stock culture to 100 ml of liquid medium into a 250-ml Erlenmeyer flask, in which the cells were aerobically incubated at 37 °C and 120 rpm in a shaker incubator (**Make**: Scigenics Biotech; **Model**: 400 LJ31L) overnight. The liquid medium contained beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g and distilled water 1.0L and was sterilised at 121 °C for 15 min.

2.3. Experiments on growth kinetics

Batch experiments were carried out to investigate the effect of different types/grades of glycerol on the growth of *Escherichia coli*. Nine different concentrations (1, 2, 3, 5, 10, 15, 20, 25 and 30 g/100 ml) of commercial, purified and crude glycerol, obtained as a by-product of the trans-esterification route, were used as the only carbon source for the growth of this culture. Inocula were developed after transferring 2 ml of fresh cultures into a 250 ml Erlenmeyer flask which contained liquid medium: 100 ml mineral salt medium (per liter: 3.5 g of KH_2PO_4; 5.0 g of K_2HPO_4; 3.5 g of $(NH_4)_2HPO_4$, 0.25 g of $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, 0.5 mg of thiamine, and 1 ml of trace metal stock) with yeast extract 3.0 g L^{-1}, peptone 4.0 g L^{-1} and glycerol (as mentioned above). The trace metal stock was prepared in 0.1 M HCl (per liter: 1.6 g of $FeCl_3$/0.2 g of $CoCl_2.6H_2O$/0.1 g of $CuCl_2$/0.2 g of $ZnCl_2.4H_2O$/0.2 g of $NaMoO_4$/0.05 g of H_3BO_3). pH of the media was maintained by adding 2.4 M K_2CO_3 and 1.2 M KOH. The cultures were incubated at 120 rpm and 37 °C in a shaker incubator for 72 h. Cell growth was monitored using optical density at 600 nm (OD_{600}) and stopped when bacteria had reached the lag phase and no further glycerol consumption was detected. Table 1 shows the details of the experiments for the batch fermentation to study cell growth for various glycerol concentrations.

2.4. Optimization of process parameters for succinic acid production

Different physico-chemical parameters, such as type of glycerol [commercial or lab grade glycerol (code≡ 1), purified (code≡ 2) and crude glycerol (code≡ 3)], pH of fermentation medium (6.5,

Table 1
Details of the experiments for the batch study.

Expt. #	Glycerol used	Fermentation time	Experimental result									
			Substrate concentrations in g/100 ml	1	2	3	5	10	15	20	25	30
1	Commercial (lab grade)	72 h	Specific growth rate μ, h^{-1}	0.025	0.035	0.038	0.05	0.057	0.045	0.041	0.03	0.029
2	Purified			0.018	0.022	0.032	0.039	0.044	0.04	0.036	0.031	0.024
3	Crude			0.003	0.007	0.015	0.021	0.017	0.016	0.014	0.009	0.006