Biotechnology Reports 10 (2016) 84–93

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

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

Suvra Sadhukhan[a], Raffaella Villa[b], Ujjaini Sarkar[a,*]

[a] Department of Chemical Engineering, Jadavpur University, Kolkata 700032, India
[b] School of Energy, Environment and Agrifood, Cranfield University, Beds MK43 0AL, United Kingdom

ARTICLE INFO

Article history:
Received 12 November 2015
Received in revised form 8 February 2016
Accepted 31 March 2016
Available online 2 April 2016

Chemical compounds studied in this article:
Isopropanol (PubChem CID: 3776)
Methanol (PubChem CID: 887)
Phosphoric acid (PubChem CID: 1004)
Sodium chloride (PubChem CID: 5234)
Potassium dihydrogen phosphate (PubChem CID: 516951)
Dipotassium hydrogen phosphate (PubChem CID: 24450)
Diammonium phosphate (PubChem CID: 24540)
Magnesium sulfate (PubChem CID: 24083)
Ferric chloride (PubCem CID: 24380)
Copper chloride (PubCem CID: 24014)

Keywords:
Biorefinery
Glycerol
Growth kinetics
Succinic acid
Inhibition

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

* Corresponding author.
E-mail addresses: r.villa@cranfield.ac.uk (R. Villa), usarkar@chemical.jdvu.ac.in, abhigyan@hotmail.com, abhi_nandan47@rediffmail.com (U. Sarkar).

http://dx.doi.org/10.1016/j.btre.2016.03.008

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

7 and 7.5) and incubation temperature (35, 37.5 and 40 °C) were varied in order to improve process yields. Glycerol concentration and yeast extract were always added at 10 g/100 ml and 3.0 g L^{-1}, respectively. Response Surface Methodology (RSM) was applied using face centered central composite design (FCCCD) as the experimental design, in order to optimize the production rate of succinic acid with the statistical software, Design Expert version 9.0.3.1. (Stat-Ease Inc., USA). A set of twenty experiments were carried out for 8 factorial points, 6 axial points ($\alpha = 1$) and 6 centre points. The experimental data for each of the runs were analyzed and a second-order quadratic polynomial Eq. (1) was obtained.

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i X_i + \sum_{i=1}^{N} \beta_{ii} X_i^2 + \sum_{i=1}^{N} \sum_{j>1}^{N} \beta_{ij} X_i X_j \quad (1)$$

It described that response was correlated with three input factors of the process (type of glycerol, pH of the media and incubation temperature), where Y is the response (Yield of succinic acid), X_i, X_j are the coded variables, β_0 is the intercept, β_i is the linear, β_{ii} is the quadratic and β_{ij} is the interaction coefficients. N is the number of factors studied in the experiment. The model was justified by the coefficients of determination (R^2) and analysis of variance (ANOVA) with the contour plots for the independent variables developed from the experimental data.

2.5. Analytical techniques

Bacterial growths were monitored by measuring the absorbance of cell solutions at 600 nm (OD_{600}) using an UV–vis spectrophotometer (**Make**: PerkinElmer, **Model**: Precisely Lamda 25 UV/Visible). Then the cells were separated by centrifugation at 10000 rpm for 10 min and dry weight measurement was carried out after washing the cells with distilled water followed by drying at 80 °C for 24 h. The specific growth rate μ, h^{-1}, refers to a cellular concentration at an instant and was calculated for each concentration of glycerol during the exponential growth period.

The supernatant collected after centrifugation was analyzed to measure the substrate utilization using a UV–vis Spectrophotometer based colorimetric method. This was a two-step reaction process. First, glycerol present in the sample was treated with sodium per-iodate and consequently formaldehyde was formed. Then this formaldehyde would react with acetyle acetone and a yellow complex, 3,5-diacetyl-1,4-dihydrolutidine, was formed. Then the intensity of colour of the solution was measured by absorption spectra at 410 nm. From the absorption intensity data, concentration of the solution was calculated using the standard curve, prepared beforehand.

The succinic acid produced was estimated on a High-Performance Liquid Chromatography (HPLC) system (**Make**: Waters, **Model**: Series 200) equipped with a 190–400 nm wavelength UV detector, with a C_{18} column using 1% acetonitrile and 20 mM K_2HPO_4 as the mobile phase, a quaternary gradient system pump with a pressure range of 0–6200 psi and a column oven, which has an operating range of 5 °C above ambient to 100 °C. In our study, temperature of the column was maintained at 35 °C and the pressure at 81 bar. UV detector at 210 nm wavelength was used for the detection of succinic acid. Standard curves were prepared by plotting peak area versus known concentrations of succinic acid samples to determine the unknown concentrations of solutions.

2.6. Development of growth kinetic models

The kinetics of microbial growth (μ) and utilization of substrate concentration (S) are correlated using various mathematical models. In a microbial process, inhibition is a part in which some

Table 2
Statistical analysis of growth kinetic parameters obtained for various models.

Sample	Model	Values of kinetic parameters and fitting constant								
		μ_{max} (h^{-1})	Ks (g/100 ml)	$K_{I,S}$(g/100 ml)	n	Correlation coefficient (R^2)	Residual μ (residual μ = predicted μ −observed μ)/observe μ	Variance (σ^2)	Standard deviation (σ)	Root mean square error
Commercial	Monod	0.043	0.409			0.902	−0.282 to+ 0.449	0.063	0.252	0.244
	Moser	0.042	0.702		2.573	0.567	−0.272 to +0.431	0.055	0.234	0.227
	Tessier	0.042	1.076			0.761	−0.267 to +0.441	0.055	0.234	0.225
	Haldane-Andrew	0.156	6.721	7.513		0.859	−0.205 to +0.132	0.011	0.106	0.1
	Aiba-Edward	0.118	4.31	22.47		0.907	−0.15 to +0.115	0.007	0.086	0.081
	Tessier-type	0.081	3.044	27.93		0.895	−0.21 to +0.115	0.01	0.099	0.094
	Andrews	0.206	7.015	7.014		0.836	−0.127 to +0.172	0.01	0.101	0.096
Purified	Monod	0.037	0.779			0.93	−0.218 to +0.507	0.054	0.232	0.224
	Moser	0.036	1.258		2.028	0.797	−0.2 to +0.481	0.048	0.22	0.21
	Tessier	0.036	1.54			0.859	−0.19 to +0.487	0.044	0.209	0.2
	Haldane-Andrew	0.131	8.331	8.367		0.914	−0.228 to +0.126	0.01	0.101	0.096
	Aiba-Edward	0.104	5.755	24.176		0.936	−0.178 to +0.123	0.006	0.08	0.076
	Tessier-type	0.066	3.583	31.764		0.933	−0.222 to +0.099	0.008	0.09	0.086
	Andrews	0.167	8.439	8.44		0.894	−0.0123 to + 0.19	0.011	0.104	0.099
Crude	Monod	0.014	0.835			0.934	−0.421 to +1.579	0.517	0.719	0.744
	Moser	0.018	44.323		4.943	0.978	−0.868 to +0.125	0.139	0.373	0.365
	Tessier	0.014	1.613			0.871	−0.372 to +1.3	0.374	0.612	0.637
	Haldane-Andrew	0.327	42.897	0.807		0.997	−0.164 to +0.376	0.036	0.189	0.178
	Aiba-Edward	0.22	34.66	10.758		0.965	−0.169 to +0.876	0.11	0.332	0.338
	Tessier-type	0.075	6.066	12.622		0.963	−0.168 to +0.883	0.111	0.334	0.34
	Andrews	0.066	7.226	7.226		0.932	−0.46 to +7.878	7.084	2.662	2.665

of the substrate or the product tend to inhibit the growth of the microorganisms, due to which the cell growth rate ceases. The reason for termination of growth may be either exhaustion of an essential nutrient or accumulation of toxic products. If an inhibitory product is produced that accumulates in the medium, the growth rate will slow down, depending on inhibitor production rate and at a certain level of inhibitor concentration, growth will stop. During the growth and decline phases of batch culture, the specific growth rate of the cells depends on the concentration of nutrients of the medium. Often, a single substrate exerts a dominant influence on the rate of growth; this component is known as growth-limiting substrate. The growth limiting substrate is often the carbon or nitrogen source [1,4,21]. A set of empirically derived rate laws are generated to describe the behavior of a given system. The growth models are divided into two: those that incorporate limiting substrate-inhibition kinetics and those that contained only growth kinetic parameters. These models are based on spatial homogeneity, being ensured by a well-mixed environment (e.g. shaker incubator). Glycerol in the media is treated as the only carbon source. The fluid volume and pH are assumed to remain constant throughout the operation.

2.6.1. Monod model (Model 1)

Monod model, the most simple and fundamental model of growth kinetics is generally used to correlate the growth rate to the concentration of a single growth-limiting substrate with the parameters μ_{max} and K_s. At low substrate concentration, a proportional relationship between specific growth rate and initial substrate concentration is described by this model, Eq. (2).

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{2}$$

where μ = specific growth rate, μ_{max} = maximum specific growth rate, S = substrate concentration, K_s = substrate saturation constant (i.e. substrate concentration at half μ_{max}). Monod made the assumption implicit in Eq. (2) that, μ is tightly coupled to S, i.e. a change in S during growth of a culture results in a corresponding change in, μ. However, the Monod model often fails to account for substrate inhibition of growth at higher substrate concentrations. There are some general formulae in the literature which, by modifying the Monod equation, take inhibition into account. The models of Moser, Tessier, Haldane, Andrews, Aiba and Edward are employed in order to overcome this drawback.

2.6.2. Moser model (Model 2)

A number of structured and unstructured kinetic expressions are generated to overcome the limitations of Monod model in order to explain the characteristics of a growth curve for the microorganisms. A modified Monod equation with power function of substrate concentration is described by Moser model, Eq. (3).

$$\mu = \frac{\mu_{max} S^n}{K_S + S^n} \tag{3}$$

Value of the power determines the degree of inhibition without explaining critical substrate concentration or inhibition constant. When n = 1, Moser equation describes a Monod model.

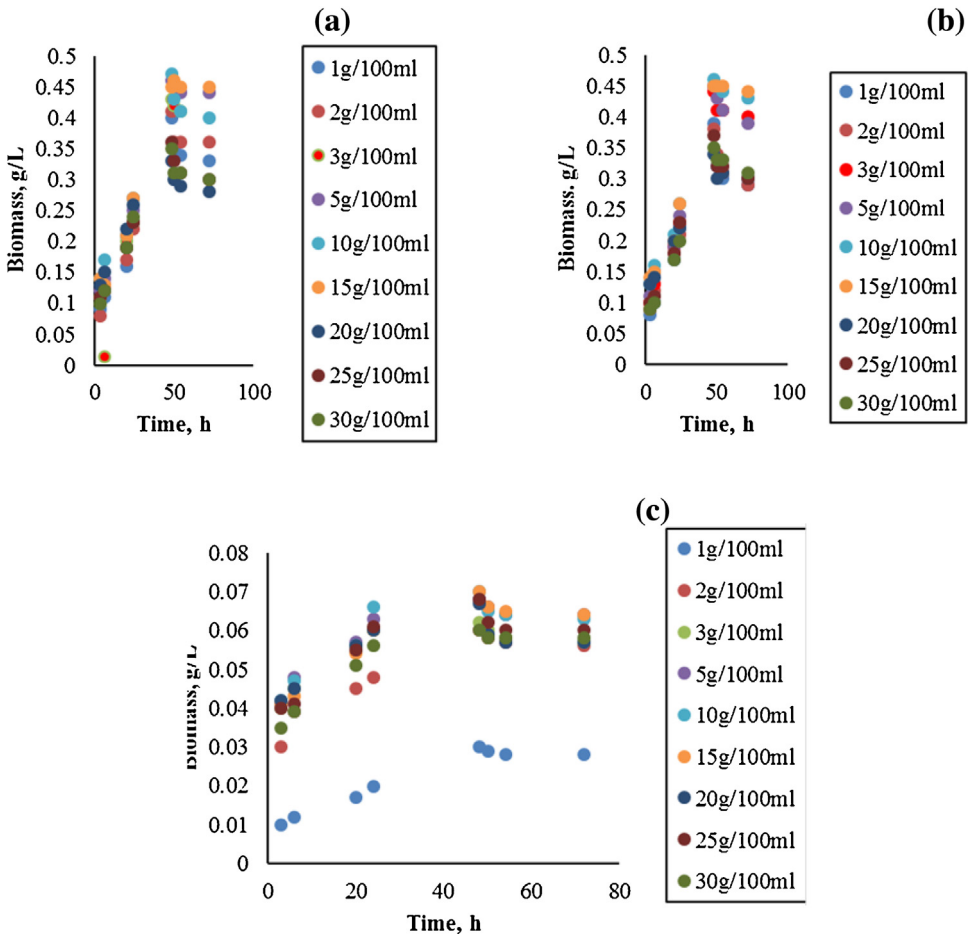

Fig. 1. Evolution of the concentrations of biomass with respect to time for different initial glycerol concentrations: (a) Commercial, (b) Purified, (c) Crude.

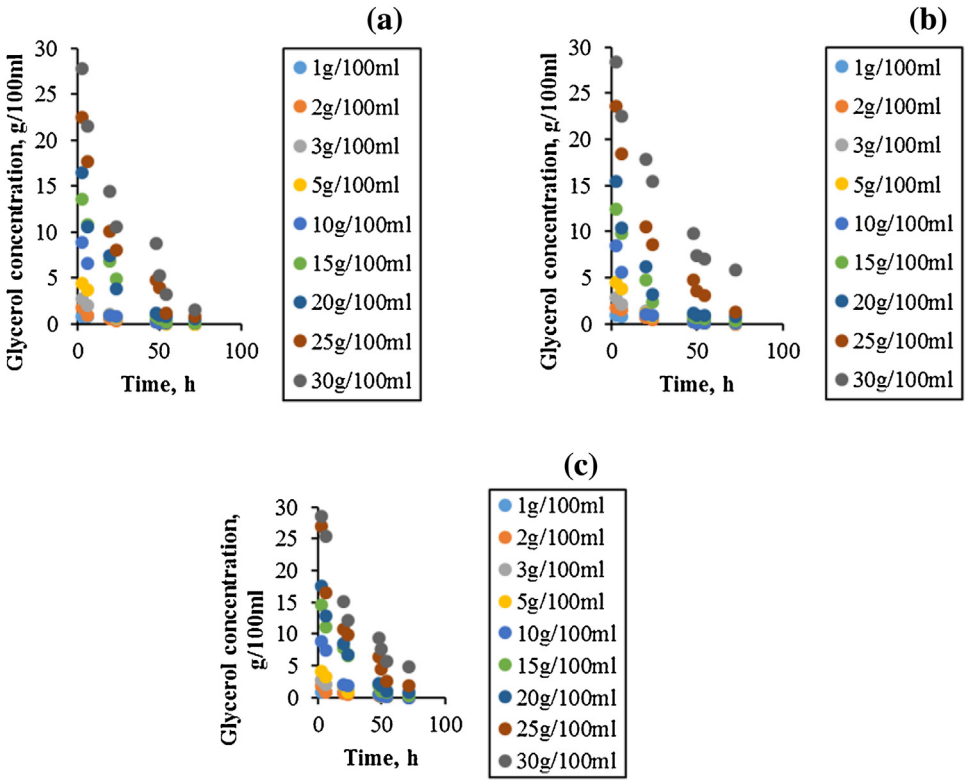

Fig. 2. Evolution of the utilization of glycerol with respect to time for different initial glycerol concentrations: (a) Commercial, (b) Purified, (c) Crude.

2.6.3. Tessier model (Model 3)

Another unstructured, non-segregated model, Tessier model, Eq. (4) is based on an exponential function and assumes one limiting substrate.

$$\mu = \mu_{max}(1 - e^{-\frac{S}{K_S}}) \quad (4)$$

2.6.4. Haldane–Andrews model (Model 4)

Substrate inhibition, generated at high substrate concentrations, is primarily caused by more than one substrate molecule binding to an active site or different sub-sites within the substrate molecules [27]. Haldane proposed the first and most popular model for substrate inhibition kinetics. Haldane-Andrews model, Eq. (5) is an extension of the Monod model which explains a substrate inhibition by introducing an inhibition parameter $K_{I,S}$ (substrate inhibition constant).

$$\mu = \frac{\mu_{max} S}{S + K_S + (S^2 / K_{I,S})} \quad (5)$$

Due to its mathematical simplicity, this growth model is accepted to predict the growth kinetics of inhibitory substrates. In general, Haldane's growth kinetics model is used on the premise that this has less number of parameters.

2.6.5. Aiba-Edward model (Model 5)

A modified version of Monod equation was proposed by Aiba and was later adapted by Edward [8]. This is a widely used model to analyze product inhibition. Aiba model was generated to describe inhibitory kinetics of the product in alcohol fermentation, where specific growth rate decreases with increasing product concentration [2]. Aiba-Edward model Eq. (6) cannot explain the critical value of inhibitory substrate/product concentration.

$$\mu = \mu_{max} \frac{S}{S + K_S} e^{\frac{-S}{K_{I,S}}} \quad (6)$$

2.6.6. Tessier-type (Model 6) 8

Tessier proposed another model Eq. (7) to explain the inhibitory effect of substrate at high concentrations for the growth of microorganisms.

$$\mu = \mu_{max}(e^{\frac{-S}{K_{I,S}}} - e^{\frac{-S}{K_S}}) \quad (7)$$

2.6.7. Andrews model (Model 7)

Andrew's model Eq. (8) is most widely used among the substrate inhibition models, which explains inhibitory effects of substrate at higher concentrations. At very large inhibition constant, it reduces to Monod's equation [3].

$$\mu = \frac{\mu_{max}}{(1 + \frac{K_S}{S})(1 + \frac{S}{K_{I,S}})} \quad (8)$$

2.7. Parameter estimation method

In the present study, the parameters in each of the seven growth-kinetic models [Eqs. (2)–(8)], consisting of a set of mathematical equations, were fitted by linear and nonlinear least squares methods using MATLAB (2009b) software to evaluate the best-fit values of rate constants. In each of these growth models, appropriate initial guesses of the parameters were made, and then the ordinary differential equations were solved to determine the calculated values of particular growth parameters. Table 2 describes the results of parameter estimation along with the

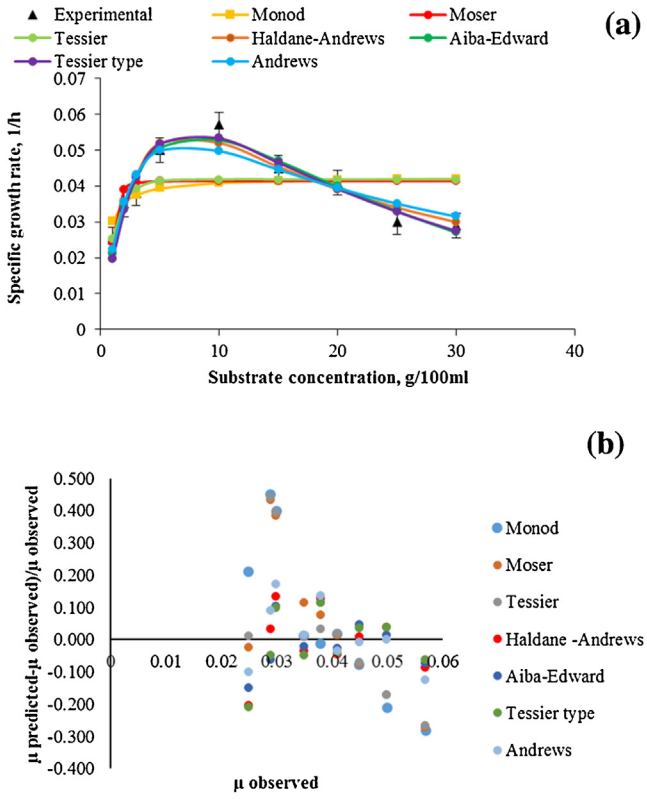

Fig. 3. (a) Comparison of different growth kinetic models for commercial glycerol. (b) Plot of residual growths for the different kinetic models for commercial glycerol.

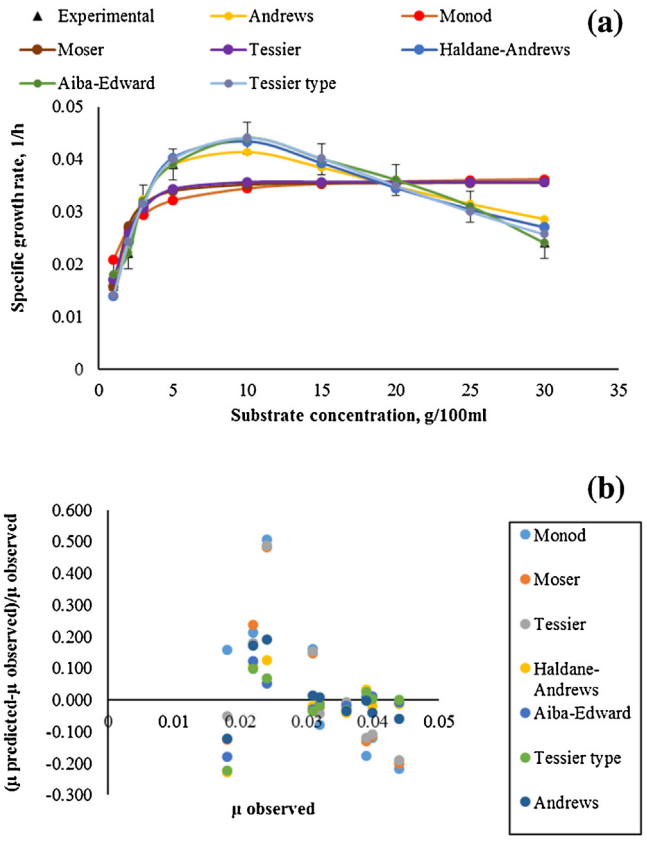

Fig. 4. (a) Comparison of different growth kinetic models for purified glycerol. (b) Plot of residual growths for the different kinetic models for purified glycerol.

statistical values of each parameter for each of the models for the three types of samples.

3. Results

3.1. Effect of glycerol concentration on growth of coli

The influence of three types of glycerol namely, crude, purified and laboratory grade, on the generation of biomass are given in Fig. 1a–c, respectively. Cell grows at maximum with 10 g/100 ml of glycerol concentration.

The increase in glycerol concentration from 15 to 30 g/100 ml produces a decrease in biomass growth, probably as a result of substrate inhibition. Cells show a longer lag phase with 30 g/100 ml glycerol concentration. Commercial (laboratory grade) and purified glycerol show a similar trend for biomass formation. Higher glycerol concentration produces less amount of biomass. Glycerol consumption rates are described in Fig. 2a–c, for three types of glycerol. The growth of *E. coli* gradually slowed down with time, indicating that *E. coli* is no longer able to produce succinic acid due to substrate inhibition [see Supplementary material A].

3.2. Cell growth kinetics

The experimental results of μ and S for commercial and pure glycerol are plotted in Figs. 3a and 4a , together with fitted data by assuming that substrate concentrations higher than 10 g/100 ml become inhibitory. Using commercial glycerol, Aiba-Edward model gives the best fit with a correlation coefficient ($R^2 = 0.907$) with lowest variance ($\sigma^2 = 0.007$) [shown in Fig. 3a]. Smaller variance of a model can predict a better fit of the data as compared to a model with larger variance. Fig. 4a similarly shows that this model gives the best fit with highest correlation coefficient ($R^2 = 0.936$) and lowest variance ($\sigma^2 = 0.006$) by using pure glycerol as the substrate. In both cases, *E. coli* showed the highest specific growth rate and substrate saturation constant at a low substrate concentration for the Andrew model. In case of crude glycerol (Fig. 5a), Haldane-Andrew model gives the best fitment ($R^2 = 0.997$, $\sigma^2 = 0.036$). The substrate concentration increases with observed specific growth rates up to 5 g/100 ml, after which inhibition occurs and the specific growth rate tends to decrease rather sharply. From this point onward, Haldane–Andrew model can fit the observed data well, while Moser model is not applied for a higher concentration (20–30 g/100 ml). Aiba-Edward model again gives best fitment for all concentrations (1–30 g/100 ml) with $R^2 = 0.965$, $\sigma^2 = 0.11$. For judging the appropriateness of any model, a residual plot is also used together with R^2 value as the latter is not always a key factor to represent goodness of fit for an accurate prediction. The residual plots defined as: residual growth = (predicted growth − observed growth)/observed growth, with respect to the seven models, are tested. In Figs. 3b, 4b and 5b, residual growths are plotted against the observed growth. In case of commercial glycerol, the residuals are randomly distributed in the range of −0.15 to +0.115 (see Fig. 3b), indicating that model 5 represents the observed data in a better way, while Model 1 has a similar regression trend with the residuals ranging from −0.282+ to 0.449. By showing the similar random nature of their distribution of residuals, other growth models predict appropriateness of the model being used. In Fig. 4b, no appreciable departure from the model assumptions are observed with the residuals ranging from −0.178 to +0.123 for

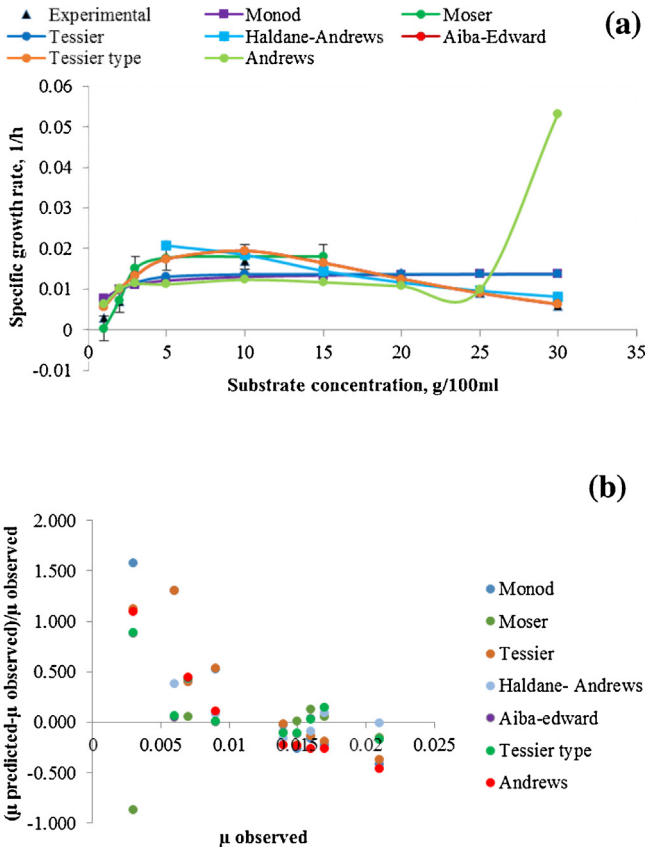

Fig. 5. (a) Comparison of different growth kinetic models for crude glycerol. (b) Plot of residual growths for the different kinetic models for crude glycerol.

model 5. Model 5 performed best with a comparatively low standard deviation ($\sigma = 0.08$) with a root mean square error of 0.076, while Model 6 is ranked second with a standard deviation (σ) of 0.09 and root mean square error of 0.086. For crude glycerol, Fig. 5b does not show any prominent departure from the model assumptions with the residuals ranging from −0.164 to +0.376. Model 4 is ranked 1 (in one case) with a low standard deviation ($\sigma = 0.189$) and root mean square error of 0.178.

3.3. Optimization of succinic acid poduction: statistical analysis for design of experiments

Twenty experiments are performed to obtain maximum production of succinic acid.

Table 3 shows that maximum production of succinic acid of 30.76 g L^{-1} is obtained from commercial glycerol at pH 7 and at 37.5 °C temperature, followed by 23.31 g L^{-1} and 1.36 g L^{-1} with purified and crude glycerol obtained after trans-esterification of *juncea* oil. A summary of production of succinic acid by various research groups all over the world, using microbial fermentation, is enlisted in Table 4.

Accuracy of the model is determined after choosing three process parameters, namely type of glycerol (A), pH of the media (B) and incubation temperature (C) for the production of succinic acid. Table 3 gives the arrangement of experiments, based on face centered central composite design (FCCCD). Here, the values of responses are analyzed by ANOVA following a quadratic polynomial Eq. (9) for the yield of succinic acid (Y) with the coded variables.

$$Y = 22.32 - 12.72A - 1.41B - 0.014C + 2.7AB$$
$$-0.16AC - 0.47BC - 4.86A^2 + 1.86B^2 - 6.31C^2 \quad (9)$$

Statistical significance of the model is established after obtaining results of the ANOVA, which gives a P-value < 0.0001, corresponding to the F value of 61.07 with a high coefficient of determination ($R^2 = 0.98$), adjusted coefficient of determination (*adjusted* $R^2 = 0.97$), predicted coefficient of determination (*Predicted* $R^2 = 0.89$) and a low value of the coefficient of variation (CV = 11.05%). Optimization of the process is carried out with the Design Expert Software which shows that choosing pH of the media as 6.7 at the temperature 38 °C, purified glycerol gives best yield as 32.5 g L^{-1}, with a desirability of 1.

4. Discussion

In this study, crude glycerol obtained from trans-esterification of *C. juncea* oil is purified through physico-chemical treatments and used as the feedstock for the production of succinic acid. The inhibitory effect of glycerol on the growth of *E. coli is* demonstrated in this study. Crude glycerol in particular, produces a very slow growth rate, and it takes more time for *E. coli* to reach the exponential phase. This is probably due to the presence of inhibitory compounds such as methanol or residual salt from

Table 3
Experimental data and results of FCCCD for production of succinic acid.

Run	Type of glycerol (A)	pH of the media (B)	Incubation temperature (°C) (C)	Yield of succinic acid(gL^{-1})	
				Experimental	Predicted
1	2	7	37.5	23.31	22.32
2	2	7	40	15.44	15.99
3	1	7	37.5	30.76	30.18
4	2	7	37.5	23.3	22.32
5	2	7	37.5	23	22.32
6	1	7.5	35	21.46	21.94
7	2	7	37.5	23.28	22.32
8	3	6.5	40	0.48	−0.70
9	1	7.5	40	20.58	21.31
10	2	7	37.5	23.29	22.32
11	1	6.5	35	29.84	29.22
12	3	7	37.5	1.36	4.73
13	3	6.5	35	0.14	−1.29
14	1	6.5	40	30.48	30.46
15	2	6.5	37.5	22.35	25.59
16	2	7.5	37.5	23.21	22.76
17	3	7.5	40	1	0.92
18	2	7	35	13.78	16.02
19	2	7	37.5	23.3	22.32
20	3	7.5	35	2.9	2.22

the trans-esterification reaction or plant component residues from the oil extraction step. As reported by [10] cell growth and glycerol fermentation at pH 7.5 were reduced by low glycerol concentrations (2 g/L) and the presence of high levels of phosphate and potassium. In our case, glycerol concentrations were higher than the reported 2 g/L but the levels of residual potassium (KOH was used for the trans-esterification), could have been high enough to produce inhibition. These results are further confirmed by the lack of inhibition in our systems using post-trans-esterification purified glycerol as the carbon source. Indeed, in this case, the residual salts from the trans-esterification process had been removed during the purification steps (acidification and filtration steps).

In addition, similar to this work, other studies have shown that the growth rate of *E. coli* for succinic acid production during glycerol fermentation can be very slow [32]. Kinetic parameters for the growth rates quantify this effect. Seven growth models are tested to fit the observed data of μ–S, to describe the specific growth rate of *E. coli* (see Table 2). Inhibition constant is highest ($K_{I,S}$ = 27.93, 31.764 and 12.622 g/100 ml for commercial, purified and crude glycerol, respectively) for Tessier-type model, implying that this cell can grow well at a higher substrate concentration of purified glycerol as compared to the other two types of glycerol.

For commercial (laboratory grade) glycerol, the estimated growth parameter is higher, $\mu_{max} = 0.043\ h^{-1}$ for Monod model than other two growth only models (Moser, Tessier). Similar results are found for pure glycerol, which showed the highest μ_{max} (0.037 h-1) for Monod model. But substrate saturation constant, K_s (1.076 and 1.54 g/100 ml) is higher for Tessier model in both substrates (commercial and purified glycerol). Higher estimation of μ_{max} is attributed to comparatively low K_S. By increasing K_S, μ_{max} decreases and vice versa. But for the models with substrate inhibition (Models 4–7), totally different results are observed, where increase in K_S leads to an increase in μ_{max}.

It is evident that, the substrate inhibition kinetic model for fermentation of glycerol fits the growth data more accurately, which is in agreement with works previously reported [5,28].

For crude glycerol, Ks is highest (44.323 g/100 ml) for Moser model and μ_{max} ($0.327\ h^{-1}$) is highest for the Haldane-Andrew model. It could be concluded that Model 5 has the best performance with a rank of 1 out of 7 (in two cases) based on the estimate of minimum variance and standard deviation (σ = 0.086) with a root mean square error of 0.081.

In this study, RSM is used to optimize the process parameters to obtain maximum production of succinic acid. Fig. 6a represents yield of succinic acid due to the combined effect of the process variables: 'type of glycerol' vs pH of the media at constant incubation temperature (C = 37.5 °C).

The data reported in this figure reveals that, with changing type of glycerol from crude to commercial (3–1), succinic acid production increased. If the inhibitory effect of the residual potassium in the crude glycerol could be mitigated without an expensive purification sequence, the crude glycerol, currently producing only $1.36\ g\ L^{-1}$, could become a prospective feedstock for succinic acid production. Indeed, the surface plot suggests that pH mitigation (increases from 6.5 to 6.7–6.8.) would improve these yields by increasing for more than a factor of 10.

The data in Fig. 6b represents the response surface plot of the effect of incubation temperature (35, 37.5 and 40) and media pH

Table 4
Comparison of different studies that produce fermentative succinic acid from glucose and glycerol.

C-source	Microorganism	Incubation condition	Yield (gL^{-1})	Reference
Glucose	*Escherichia coli* W3110	pH 6.7 Glu conc.5.5%	33.8	[5]
Glucose	*Actinobacillus succinogenes* ZT-130 (ATCC 55617)	pH 7.0 Glu conc.2%	24.2	[11]
Glycerol	*Actinobacillus succinogenes* (ATCC 55618)	pH 6.4 Gly conc.3%	29.3	[27]
Glycerol	*Escherichia coli* (ATCC 8739)	pH 7.0 Gly conc. 5%	38	[31]
Crude glycerol obtained from Sunn-hemp seed	*Escherichia coli* (ATCC 8739)	pH 6.7 Gly conc. 10%	1.36	This study
Purified glycerol obtained from Sunn-hemp seed	*Escherichia coli* (ATCC 8739)	pH 6.7 Gly conc. 10%	23.31	This study
Commercial glycerol	*Escherichia coli* (ATCC 8739)	pH 6.7 Gly conc. 10%	30.76	This study

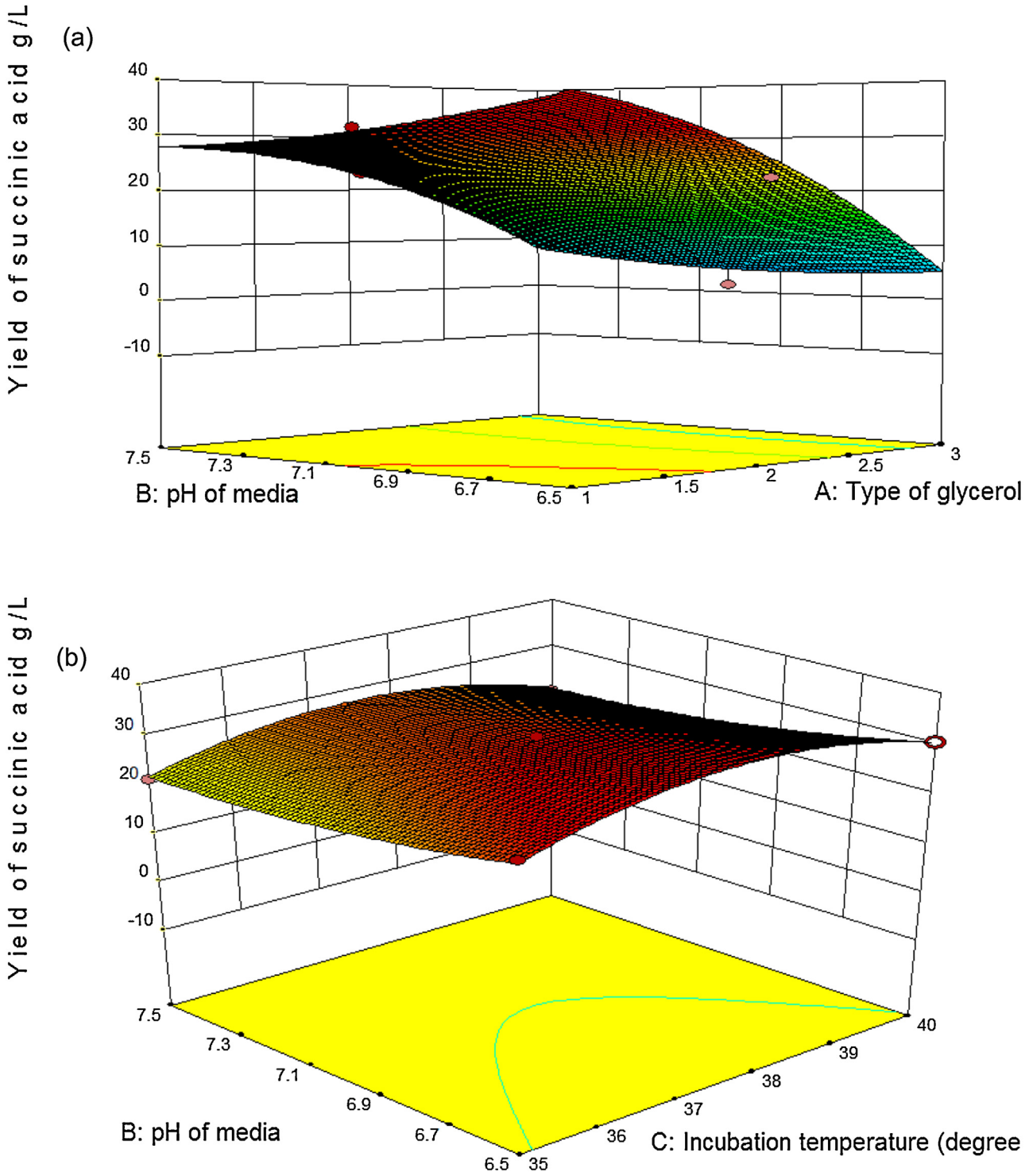

Fig. 6. (a) Response surface plot for combined effect of type of glycerol (1 = commercial, 2 = purified, 3 = crude) and pH (6.5, 7 and 7.5) at 37.5 °C incubation temperature. b. Response surface plot for combined effect of pH (6.5, 7 and 7.5) of the media and incubation temperature (35, 37.5 and 40) for commercial glycerol.

(6.5, 7 and 7.5) on succinic acid production for commercial glycerol (type of glycerol = 1). Here, succinic acid production increases upto a pH (of the media) of 7 with an incubation temperature of 37.5 °C.

5. Conclusion

In this study, we proved the feasibility of using crude glycerol obtained from trans-esterification of *C. juncea* oil for the production of succinic acid. Crude glycerol was purified through a sequence of physico-chemical treatments and used as an additional feedstock, other than crude and commercially pure glycerol. Seven different kinetic models were fitted with the experimental data for design and optimization of batch fermentation processes. The models with substrate inhibition, and in particular the Aiba-Edward model, showed to be the best fit for our systems. However, it is clear that, if crude glycerol was to be used for the fermentation process, it would be necessary to remove the main impurities. In particular, residual potassium, present in the

system due to excess KOH, proved to be harmful to *E. coli*. Finally, as not much difference in kinetic parameters, for both purified and commercial glycerol, was detected, the first one could be used as a replacement of commercial glycerol in case of industrial applications.

Acknowledgment

The first author is particularly grateful to CSIR, India for providing her with a fellowship of SRF (File No.: 09/096(787)/2013-EMR-I dated 21.03.2013 and UGC sponsored Major Research Project entitled 'Biorefining Crotalaria Juncea (Sunn Hemp): Prospects of Utilizing Waste Glycerol for the Production of Succinic Acid' [File No.: 43-153/2014 (SR) dated August, 2015] for providing with the consumables and chemicals.

References

[1] T. Abuhamed, E. Bayraktar, T. Mehmetoglu, U. Mehmetoglu, Kinetics model for growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation, Process Biochem. 39 (2004) 983–988.
[2] S. Aiba, M. Shoda, M. Nagatani, Kinetics of product inhibition in alcohol fermentation, Biotechnol. Bioeng. 10 (1968) 845–864.
[3] J.F. Andrews, A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates, Biotechnol. Bioeng. 10 (1968) 707–723.
[4] M.S.M. Annuar, I.K.P. Tan, S. Ibrahim, K.B. Ramachandran, A kinetic model for growth and biosynthesis of medium-chain-length poly-(3-hydroxyalkanoates) in *Pseudomonas putida*, Brazil. J. Chem. Eng. 25 (2008) 217–228.
[5] R.I. Corona-Gonzalez, A. Bories, V. Gonzalez-Alvarez, C. Pelayo-Ortiz, Kinetic study of succinic acid production by *Actinobacillus succinogenes* ZT-130, Process Biochem. 43 (2008) 1047–1053.
[6] C. Du, S.K.C. Lin, A. Koutinas, R. Wang, C. Webb, Succinic acid production from wheat using a biorefining strategy, Appl. Microbiol. Biotechnol. 76 (2007) 1263–1270.
[7] R. Dutta, U. Sarkar, A. Mukherjee, Extraction of oil from *Crotalaria Juncea* seeds in a modified Soxhlet apparatus: physical and chemical characterization of a prospective bio-fuel, Fuel 116 (2014) 794–802.
[8] V.H. Edwards, The influence of high substrate concentrations on microbial kinetics, Biotechnol. Bioeng. 12 (1970) 679–712.
[9] M. Fan, J. Huang, J. Yang, P. Zhang, Biodiesel production by transesterification catalyzed by an efficient choline ionic liquid catalyst, Appl. Energy 108 (2013) 333–339.
[10] R. Gonzalez, A. Murarka, Y. Dharmadi, S.S. Yazdani, A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*, Metab. Eng. 10 (5) (2008) 234–245.
[11] J. Isar, L. Agarwal, S. Saran, R.K. Saxena, A statistical method for enhancing the production of succinic acid from *Escherichia coli* under anaerobic conditions, Bioresour. Technol. 97 (2006) 1443–1448.
[12] S. Khanna, S. Jaiswal, A. Goyal, V.S. Moholkar, Ultrasound enhanced bioconversion of glycerol by *Clostridium pasteurianum:* a mechanistic investigation, Chem. Eng. J. 200 (2012) 416–425.
[13] R. Liu, L. Liang, M. Jiang, J. Ma, K. Chen, H. Jia, P. Wei, P. Ouyang, Effects of redox potential control on succinic acid production by engineered *Escherichia coli* under anaerobic conditions, Process Biochem. 49 (2014) 740–744.
[14] O.M. Neijssel, M.J.T. De Mattos, D.W. Tempest, Growth yield and energy distribution, in: F.C. Neidhardt, others (Eds.), *Escherichia Coli* and *Salmonella*: Cellular and Molecular Biology, American Society for Microbiology, Washington, DC, 1996, pp. 1683–1692.
[15] S. Okino, R. Noburyu, M. Suda, T. Jojima, M. Inui, H. Yukawa, An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain, Appl. Microbiol. Biotechnol. 81 (2008) 459–464.
[16] A. Orjuela, A.J. Yanez, A. Santhanakrishnan, C.T. Lira, D.J. Miller, Kinetics of mixed succinic acid/acetic acid esterification with Amberlyst 70 ion exchange resin as catalyst, Chem. Eng. J. 188 (2012) 98–107.
[17] J.M. Otero, L. Olsson, J. Nielsen, Metabolic engineering of *Saccharomyces cerevisiae* microbial cell factories for succinic acid production, J. Biotechnol. 131 (2007) S205.
[18] C. Roca, G. Albuquerque, M. Reis, Evolutionary engineering of *Actinobacillus succinogenes* for improved succinic acid production on glycerol, J. Biotechnol. 150 (2010) 373.
[19] S. Sadhukhan, U. Sarkar, Production of biodiesel from *Crotalaria juncea* (Sunn-hemp) oil using catalytic trans-esterification: process optimisation using a Factorial and Box-behnken design, Waste Biomass Valorization (2015), doi: http://dx.doi.org/10.1007/s12649-015-9454-4.
[20] S. Sadhukhan, U. Sarkar, Production of purified glycerol using sequential desalination and extraction of crude glycerol obtained during trans-esterification of Crotalaria juncea oil, Energy Convers. Manage. 118 (2016) 450–458.
[21] P. Saravanan, K. Pakshirajan, P. Saha, Batch growth kinetics of an indigenous mixed microbial culture utilizing m-cresol as the sole carbon source, J. Hazard. Mater. 162 (2009) 476–481.
[22] N. Sdrula, A study using classical or membrane separation in the biodiesel process, Desalination 250 (2010) 1070–1072.
[23] H. Song, Y.S. Huh, S.Y. Lee, W.H. Hong, Y.K. Hong, Recovery of succinic acid produced by fermentation of a metabolically engineered *Mannheimia succiniciproducens* strain, J. Biotechnol. 132 (2007) 445–452.
[24] H. Song, S.H. Jang, J.M. Park, S.Y. Lee, Modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens*, Biochem. Eng. J. 40 (2008) 107–115.
[25] H. Song, S.Y. Lee, Production of succinic acid by bacterial fermentation, Enzyme Microbial. Technol. 39 (2006) 352–361.
[26] H.W. Tan, A.R.A. Aziz, M.K. Aroua, Glycerol production and its applications as a raw material: a review, Renew. Sustain. Energy Rev. 27 (2013) 118–127.
[27] Y. Tan, Z.X. Wang, K.C. Marshall, Modeling substrate inhibition of microbial growth, Biotechnol. Bioeng. 52 (1996) 602–608.
[28] A. Vlysidis, M. Binns, C. Webb, C. Theodoropoulos, Glycerol utilisation for the production of chemicals: conversion to succinic acid, a combined experimental and computational study, Biochem. Eng. J. 58 (2011) 1–11.
[29] Y.-L. Xi, K. Chen -q, J. Li, X. Fang -j, X. Zheng -y, S. Sui -s, M. Jiang, P. Wei, Optimization of culture conditions in CO_2 fixation for succinic acid production using *Actinobacillus succinogenes*, J. Ind. Microbiol. Biotechnol. 38 (2011) 1605–1612.
[30] J. Yu, Z. Li, Q. Ye, Y. Yang, S. Chen, Development of succinic acid production from corncob hydrolysate by *Actinobacillus succinogenes*, J. Ind. Microbiol. Biotechnol. 37 (2010) 1033–1040.
[31] J. Zhang, S. Chen, R. Yang, Y. Yan, Biodiesel production from vegetable oil using heterogenous acid and alkali catalyst, Fuel 89 (2010) 2939–2944.
[32] X. Zhang, K.T. Shanmugam, L.O. Ingram, Fermentation of glycerol to succinate by metabolically engineered strains of *Escherichia coli*, Appl. Environ. Microbiol. 76 (2010) 2397–2401.
[33] P. Zheng, K. Zhang, Q. Yan, Y. Xu, Z. Sun, Enhanced succinic acid production by *Actinobacillus succinogenes* after genome shuffling, J. Ind. Microbiol. Biotechnol. 40 (2013) 831–840.