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Original Research

Mathematical modelling of bioethanol production from algal starch hydrolysate by *Saccharomyces cerevisiae*

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Abstract: Bioethanol is an excellent alternative for petrol and has long-term economic advantages over non-renewable liquid biofuels. Bioethanol can be produced from different biomass materials, and it is categorized into three generations by biomass. First generation bioethanol directly competes with food items while second generation bioethanol requires more land area and fertilizers. Bioethanol produced from algae comes under third generation bioethanol and has many advantages over first and second generation bioethanol. Algae have higher growth rates than plants and require less land area also they do not need additional fertilizers for their growth. Microalgae can fix atmospheric CO_2 from the environment and assimilate it into lipid and carbohydrates which can be used as a substrate for biofuel production. In this work, microalgae *Chlorella* sp. was cultivated at 28°C and light intensity of 25µmol m^{-2} s^{-1} and used as a source of starch for bioethanol production. Chlorella starch was hydrolyzed into fermentable sugars by amylases produced from *Aspergillus niger* NCIM 616 for bioethanol production by yeast *Saccharomyces cerevisiae* NCIM 3494. Mathematical modelling gave the insight to predict the experimental profiles of bioethanol production from algal biomass. Microbial growth, reducing sugar consumption, and bioethanol production were described quantitatively by using Logistic, Pirt, and Luedeking-Piret equations respectively. The equations used in modelling of the fermentation kinetics matched very well with the experimental profiles, thus concluding that ethanol production from algal starch hydrolysate was growth-associated under the evaluated conditions.

Key words: Bioethanol; Algae; *Chlorella sp.*; Modelling.

Introduction

Today world is facing a new threat from global warming because of excessive use of conventional resources like coal, natural gas and crude oils and deforestation (1). There is a need of the hour to increase the use of renewable resources like wind, solar, hydro and bioenergy in place of nonrenewable energy resources to reduce the effects of global warming (1-3). But only bioenergy is focused here on the sustainable development. Generation of algal biomass is one of the approaches that can be used for CO2 sequestration and production of bioethanol. Bioethanol is an excellent alternative for petrol and has long-term economic advantages over non-renewable liquid biofuels. Bioethanol is produced from different biomass materials, and it is categorized into three generations by biomass (4). First generation bioethanol directly competes with food stuff while second generation bioethanol requires more land area and fertilizers. Bioethanol produced from algae comes under third generation bioethanol and has many advantages over first and second generation bioethanol. Algae both micro and macro, have higher growth rates than plants and require less land area also they do not need additional fertilizers for their growth (5). Microalgae can fix atmospheric CO2 from the environment and assimilate it into lipid and carbohydrates which can be used for biofuel production (6). *Chlorella* sp. usually contains 12-17% starch on a dry weight basis, but this can be enhanced by growing microalgae in stressed conditions. Dragone et al. used nutrient limitation of mainly nitrogen and iron as a strategy for increasing starch accumulation in microalgae (7). Cycloheximide is a proteosynthesis inhibitor which inhibits protein synthesis in algae while increases the starch content (7-8). Starch obtained from microalgae is used as a substrate for production of bioethanol. Amylases hydrolyze starch into glucose that can be utilized by Saccharomyces cerevisiae to produce bioethanol (8). Mathematical modelling is used to predict the behaviour of many biochemical engineering processes. For this purpose, many unstructured models have been developed. These models validate quantitatively experimental data and also to support fermenter reactor design and operations (9).

In this work, Microalgae *Chlorella* sp. was grown in suitable environmental conditions and then treated with cycloheximide to obtain higher starch content. Amylases hydrolyzed microalgal starch into glucose which is further utilized for the bioethanol production by *Saccharomyces cerevisiae*. The experimental data of yeast growth, reducing sugar consumption, and bioethanol production were described quantitatively by using Logistic, Pirt, and Luedeking-Piret equations respectively.

Materials and Methods

Isolation and cultivation of microalgae

Microalgae, *Chlorella sp.* was isolated from the Ganga River at Varanasi. A small amount of algal sample was spread onto BBM agar plates and cultured under

irradiance of 60 µmol m^{-2} s^{-1}, light: dark period of 16:8 and at a temperature of 28°C. Single colonies were picked up and transferred to BBM media. *Chlorella sp.* was confirmed by morphological analysis. The algal strain has been maintained in BBM agar slants for further use (10). The growth analysis of *Chlorella sp.* was done in BBM media for 32 days. 10 ml of algal culture was inoculated into 200 ml of BBM media. The culture broth was grown at 28°C and light intensity of 60 µmol $m^{-2}s^{-1}$. The culture samples were taken out periodically, and microalgal growth was measured at a wavelength of 680 nm. The alga growth was also measured on the basis of dry cell weight. The biomass cultivation of microalga was done in 10 litres photobioreactor having 5 litres of working volume.

Effect of cycloheximide treatment

A concentration of 1mg/ L of cycloheximide was added in algal culture broth at the early stationary phase of the growth cycle. The addition of cycloheximide inhibits the synthesis of protein in eukaryotic cells by binding with the ribosome. The addition of cycloheximide increases the starch content within the microalgae (8). The effect of cycloheximide is shown in figure 3.

Preparation of substrate

Algal biomass was harvested by centrifugation at 10,000 rpm for 15 min. The centrifuged algal cell mass was collected and dried in an oven at 80°C. Dried microalgae biomass was crushed into Mortar and pestle and sonicated for 15 minutes which causes breakage of algal cells. This operation caused the starch granules to come out and which was further hydrolyzed by adding crude amylase having the activity of 40 Units/ ml. The crude amylase was produced by *Aspergillus niger* NCIM 616 and the procedure described by Gupta *et al.* (11). The reducing sugars obtained from enzymatic hydrolysis were used as fermentation media for ethanol production. The fermentation media was autoclaved at 15 psi for 15 min to sterilize the media. The initial pH of the medium was adjusted to 5.5 using 0.1 N NaOH and 1 N HCl (12). The composition of fermentation media is given in Table 1.

Yeast strain

For the fermentation of reducing sugars *S. cerevisiae* NCIM 3494, was obtained from NCIM (National Chemical Laboratories Pune) India. Yeast culture was grown on MGYP media (3.0 g/L malt, 10.0 g/L glucose, 3.0 g/L yeast extract, 5.0 g/L peptone) having pH of 6.0 at 30°C and 150 rpm for 48 h. 5% (v/v) of fresh yeast culture broth was used as inoculums for ethanol fermentation.

Fermentation of algal starch

Fermentation of algal starch was done in two step process. In the first step, all the starch is hydrolyzed into simple sugars by amylase enzymes and in second the step, the reducing sugars were utilized by yeast *Saccharomyces cerevisiae* for production of ethanol**.** The fermentation media, as composition given in Table 1, was prepared in 250 mL of Erlenmeyer flask and autoclaved. After cooling, 10 mL of crude amylase was added aseptically in sterilized fermentation media. The enzymatic hydrolysis was performed at 55°C and 100 rpm for 1 hour. Before fermentation cellular debris was removed by centrifugation at 2000 rpm for 5 minutes. The pH was adjusted 5.5 using 1 N NaOH and 1 N HCl. 5% (v/v) of yeast inoculum was added into fermentation media hydrolyzed sugars as the main substrate. The ethanol fermentation was done at 30°C for 64 h under anaerobic condition. In fermentation broth, starch, glucose, ethanol and cell mass were analysed at regular intervals. Glucose obtained from starch hydrolysis was measured by DNS method. The residual starch concentration during fermentation was measured using anthrone reagent method (13). The estimation of ethanol during fermentation was done by using gas chromatography method. The growth kinetics of *S. cerevisiae* was calculated by measuring the weight of dry cell mass. The concentration profiles of glucose (*G*), starch (*S*) and ethanol (*E*) and yeast cell mass (*X*) during fermentation were shown in fig. 5.

Table 1. Composition of fermentation media.

Fermentation media	Amount
Glucose (derived from *Chlorella* starch)	50 g
KH_2PO_4	0.19 g
$(NH_4)_2SO_4$	0.95 g
$MgSO_4.7H_2O$	0.095 g
Yeast extract	0.19 g
Distilled water	190 mL
pH	5.5

Estimation of bioethanol

The ethanol concentrations in the fermentation broth were determined using gas chromatograph (Nucon GC-5765, India) equipped with a flame ion detector (FID) and Porapak-Q column (2 m length and 0.125 inch ID). The injector, detector and oven temperatures were set to 190, 250 and 165°C, respectively. Nitrogen gas was used as a carrier gas with a flow rate of 30 mL per minute while Hydrogen was used as a fuel gas and compressed Oxygen helped in burning. The standard curve for ethanol concentration was plotted by injecting different concentrations of standard ethanol solution ranging from 0.1 to 10.0 % (v/v) ethanol in water. A fixed percentage 5% (v/v) of n-Propanol is added as internal standard in each ethanol samples. 1.0 µL of sample was injected into the gas chromatograph and the ethanol retention time was determined as 2.65 min (14).

Data modelling

The relationships between the cell growth and substrate consumption, and cell growth and product synthesis have been given by Jiménez-Islas *et al.* using logistic, Pirt, and Luedeking-Piret equations (9). The integration of the logistic equation is given in equation 1 which describes the relationship of cell biomass with time. The relationship between substrate consumption and cell growth is given by equation 2 which is obtai-

ned by dividing the Pirt equation by the logistic equation followed by integration. Equation 3 describes the relation between product synthesis and cell growth and obtained by dividing the Luedeking-Piret equations by the logistic equation followed by integration(9, 15).

$$X(t) = \frac{X_{max}}{1+\left(\frac{X_{max}}{X_0}-1\right)e^{-\mu t}} \quad (1)$$

$$S(X) = S_0 - \frac{1}{Y_{X/S}}(X - X_0) - \frac{m X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \quad (2)$$

$$E(X) = E + \alpha(X - X_0) + \frac{\beta X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right) \quad (3)$$

The experimental data of fermentation processes were compared with model data of equations 1-3. The simulation program was designed in such a way that it can minimize normalized error (the sum of difference square of model data and experimental data) in solver function. The different kinetic parameters for microbial growth, sugar consumption, and ethanol production were evaluated by fitting the experimental data with equations 1-3. The lists of different kinetic parameters are shown in Table 2.

The experimental data with model data were analysed by using the regression curve fitting with statistical significance (p=0.05).

Results

Isolated microalgae

The isolated *Chlorella* strain was confirmed by morphological analysis under a compound light microscope. The photograph of isolated microalgae, *Chlorella sp.,* is shown in figure 1(a), and large cultivation of microalgae shown in figure 1(b) was done in a photobioreactor with periodical mixing.

Kinetics of microalgae

The *Chlorella sp.* was grown in BBM medium and determined its growth curve, which is shown in figure 2. The specific growth rate of *Chlorella sp.* ($\mu = 0.147$ day $^{-1}$) was obtained during exponential phase and doubling time (t_d) was 4.714 day.

Effect of Cycloheximide

At the later stage of the exponential phase of the growth curve, 1mg/l of Cycloheximide was added, and its effect on starch content is shown in figure 3. Microalgae without Cycloheximide treatment contain 19.3% (w/w) of starch on dry weight basis while the other batch of microalgae after the Cycloheximide treatment contains 38.2% (w/w) of starch (dry weight).

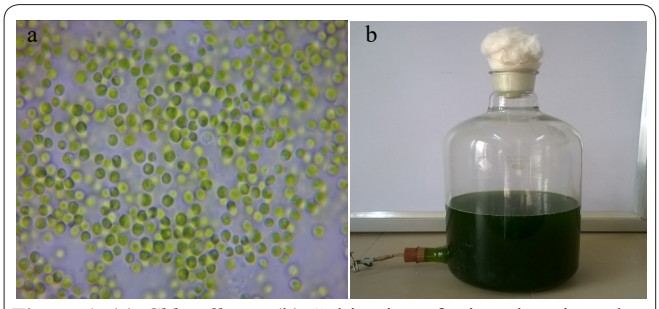

Figure 1. (a) *Chlorella sp.* (b) Cultivation of microalgae in a photobioreactor.

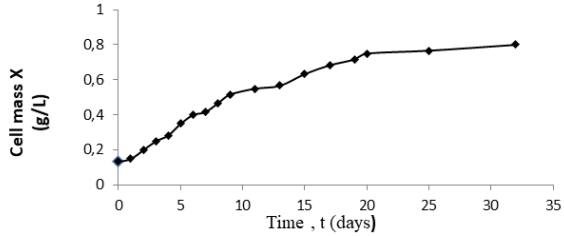

Figure 2. Growth curve of microalga *Chlorella sp.*

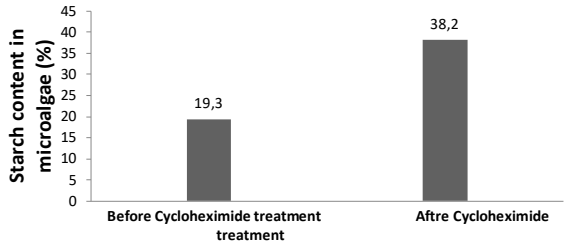

Figure 3. Effect of Cycloheximide treatment on algal starch content.

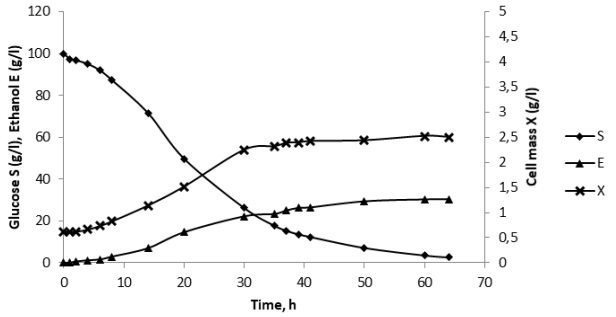

Figure 4. The ethanol (E), yeast cell mass (X) and reducing sugar (S) concentrations during ethanol fermentation.

Ethanol fermentation

The course profiles of yeast cell mass generation, reducing sugar consumption and ethanol production during the batch fermentation of hydrolyzed microalgal starch by *Saccharomyces cerevisiae* are shown in figure 4. The presented figure shows that highest biomass was produced after 40 h of fermentation and which was approximate constant during the rest of the fermentation. This biomass concentration was 2.5 g/L which was produced at 30°C and pH of 5.5.

The reducing sugar consumption is also shown in figure 4. The initial sugar concentration was 100 g/L which was consumed by *Saccharomyces cerevisiae* in cell mass growth and ethanol production. The result shows that till 8 hrs of fermentation, the sugar consumption was slow and after that, it increases rapidly till 40 hrs. After 40 hrs, the rate of sugar consumption as well as the rate of ethanol production slows down till the end of fermentation. The rate of ethanol production was slow in the beginning but it increases rapidly till the 40 hrs. The maximum ethanol concentration which was achieved after the fermentation was 30.2 g/ L.

The experimental profiles of biomass generation, reducing sugar consumption and ethanol production were compared with model data given by a logistic, Pirt, and Luedeking-Piret equations respectively (figure 6 and 7). The different unknown parameters used in model equations were evaluated using solver function. The list of

Table 2. The value of different kinetic parameters.

Kinetic parameters	Units	Values
X_{max}	gL^{-1}	2.6363
μ	h^{-1}	0.083641
$Y^{max}_{X/S}$	gg^{-1}	0.021485
m	$gg^{-1}h^{-1}$	0.074548
α	gg^{-1}	11.18044
β	$gg^{-1}h^{-1}$	0.096109
$Y_{P/S}$	gg^{-1}	0.302

Table 3. Coefficient of determination (R^2) for different models.

Predicted models for	Models	Coefficient of Determination (R^2)
Glucose	$S(X) = S_0 - \frac{1}{Yx_{/s}}(X - X_0) - \frac{m.X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right)$	0.997605
Cell mass	$X(t) = \frac{X_{max}}{1 + \left(\frac{X_{max}}{X_0} - 1\right)e^{-\mu t}}$	0.89504
Ethanol	$E(X) = E + \alpha(X - X_0) + \frac{\beta.X_{max}}{\mu}\ln\left(\frac{X_{max}-X_0}{X_{max}-X}\right)$	0.999874

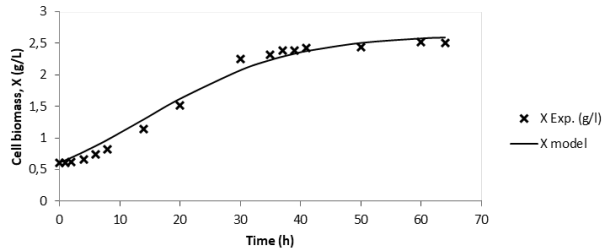

Figure 5. The experimental and predicted profiles of yeast cell mass generation. Line (---) shows the model data while cross (**x**) shows the experimental data.

these kinetic parameters is given in table 2.

The result shown in figure 5 compares the experimental data of biomass generation with model data generated by the logistic equation.

The kinetic models give the higher correlation coefficient values (R^2- values) with experimental data. That shows the good prediction of experimental behaviour. The values of coefficient of determinations for respective models are given in table 3.

Discussion

The microalgae have been used as substrate for ethanol production because of its high carbohydrate content (6, 16). The starch content of microalgae varies depending on the species. The starch content can be enhanced by applying the nutrient limitation strategy or by some chemical treatment (8, 17). The growth rate of isolated microalgae was found slower than the reported strain by Brányiková *et al.* because of the limited availability of light and other culture conditions (8). Therefore the starch content of microalgae could not be reached up to the reported value i.e. 60% (w/w).

The mathematical models are used to mimic the chemical, physical and biochemical processes. In our study, a mathematical model has been proposed for ethanol production from microalgal starch hydrolysate. We

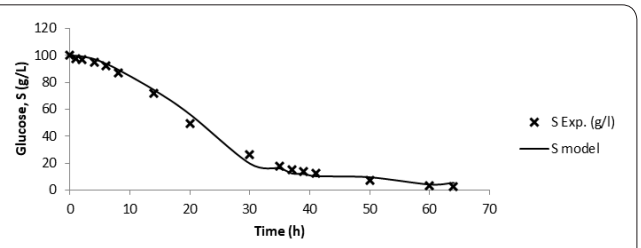

Figure 6. The experimental and predicted profiles of reducing sugar consumption. Line (---) shows the model data while cross (**x**) shows the experimental data.

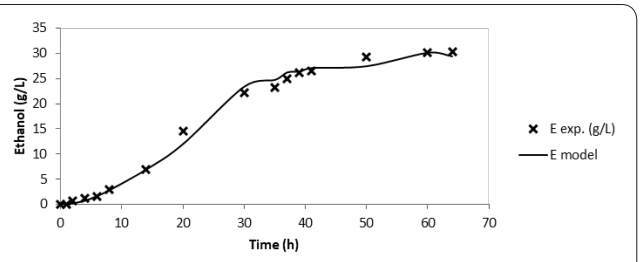

Figure 7. The experimental and predicted profiles of ethanol production. Line (---) shows the model data while cross (**x**) shows the experimental data.

found that all the model equations fitted very well with the experimental data. A mathematical model of ethanol production from starch in immobilized recombinant amylase-producing yeast culture was suggested by Kobayashi *et al* for estimating the dynamic behaviour of yeast cell growth, starch depletion, glucose accumulation/consumption, and ethanol production in batch fermentation by immobilized yeast (18). Similar profiles of starch depletion and cell growth were observed in our study except for the glucose consumption profile because the starch was completely hydrolyzed before the fermentation.

In conclusion, the role of microalgae as a suitable source for ethanol production was explored, and the behaviour of fermentation processes was predicted using mathematical modelling. The indigenous microalga,

Chlorella sp. was used in this study as a source of starch for ethanol production. Starch obtained from microalgae was hydrolyzed by amylase enzymes produced from *Aspergillus niger.* The hydrolyzed sugars were further used for the production of ethanol by yeast *Saccharomyces cerevisiae* in optimum conditions. Mathematical modelling was used to predict the experimental behaviour of glucose consumption, ethanol production, and yeast biomass generation from hydrolyzed microalgal starch. It was found that both glucose consumption and ethanol production profiles are given by models fitted very well with experimental profiles using different kinetic parameters and provided R^2- values 0.997 and 0.999 for glucose consumption and ethanol production respectively. Whereas, the logistic equation used for biomass generation showed fit with experimental profile and provided R^2-value just 0.895.

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References

1. Singh A, Nigam PS, Murphy JD. Renewable fuels from algae: An answer to debatable land based fuels. Bioresour. Technol. 2011; 102: 10-16.
2. Popp J, Lakner Z, Harangi-Rákos M, Fári M. The effect of bioenergy expansion: Food, energy, and environment. Renewable and Sustainable Energy Reviews 2014; 32: 559-578.
3. Kundu S, Singh S, Ojha S, Kundu K. Role of biorefining and biomass utilization in environmental control. Proc. International Science Index, London United Kingdom Jan 19-20, 2015, 13 (01) Part IV 2015: 886-889.
4. Dragone G, Fernandes BD, Vicente AA, Teixeira JA. Third generation biofuels from microalgae. Current research, technology and education topics in applied microbiology and microbial biotechnology 2010; 2: 1355-1366.
5. John RP, Anisha G, Nampoothiri KM, Pandey A. Micro and macroalgal biomass: A renewable source for bioethanol. Bioresour. Technol. 2011; 102: 186-193.
6. Harun R, Danquah MK, Forde GM. Microalgal biomass as a fermentation feedstock for bioethanol production. J. Chem. Technol. Biotechnol. 2010; 85: 199-203.
7. Douskova I, Doucha J, Machat J, Novak P, Umysova D, Vitova M, Zachleder V. Microalgae as a means for converting flue gas co2 into biomass with high content of starch. Proceedings of the International Conference: Bioenergy: Challenges and Opportunities, 6th/9th April 2008.
8. Brányiková I, Maršálková B, Doucha J, Brányik T, Bišová K, Zachleder V, Vítová M. Microalgae—novel highly efficient starch producers. Biotechnol. Bioeng. 2011; 108: 766-776.
9. Jiménez-Islas D, Páez-Lerma J, Soto-Cruz NO, Gracida J. Modelling of ethanol production from red beet juice by saccharomyces cerevisiae under thermal and acid stress conditions. Food Technol. Biotechnol. 2014; 52: 93.
10. Jang J-S, Cho Y, Jeong G-T, Kim S-K. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (ssf) from seaweed, saccharina japonica. Bioprocess Biosyst. Eng. 2012; 35: 11-18.
11. Gupta A, Gupta V, Modi D, Yadava L. Production and characterization of α-amylase from aspergillus niger. Biotechnology 2008; 7: 551-556.
12. Asada C, Doi K, Sasaki C, Nakamura Y. Efficient extraction of starch from microalgae using ultrasonic homogenizer and its conversion into ethanol by simultaneous saccharification and fermentation. 2012.
13. Fernandes B, Dragone G, Abreu AP, Geada P, Teixeira J, Vicente A. Starch determination in chlorella vulgaris—a comparison between acid and enzymatic methods. J. Appl. Phycol. 2012; 24: 1203-1208.
14. Stackler B, Christensen E. Quantitative determination of ethanol in wine by gas chromatography. Am. J. Enol. Vitic. 1974; 25: 202-207.
15. Soto-Cruz O, Favela-Torres E, Saucedo-Castañeda G. Modeling of growth, lactate consumption, and volatile fatty acid production by megasphaera elsdenii cultivated in minimal and complex media. Biotechnol. Prog. 2002; 18: 193-200.
16. Li K, Liu S, Liu X. An overview of algae bioethanol production. Int. J. Energ. Res. 2014; 38: 965-977.
17. Dragone G, Fernandes BD, Abreu AP, Vicente AA, Teixeira JA. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl. Energy 2011; 88: 3331-3335.
18. Kobayashi F, Nakamura Y. Mathematical model of direct ethanol production from starch in immobilized recombinant yeast culture. Biochem. Eng. J. 2004; 21: 93-101.