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Bioethanol production from micro-algae, *Schizocytrium* sp., using hydrothermal treatment and biological conversion

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Abstract−Hydrothermal fractionation for micro-algae, *Schizocytrium* sp., was investigated to separate sugars, lipids, and proteins. This fractionation process produced protein-rich solid cake and liquid hydrolysates, which contained oligomeric sugars and lipids. Oligomeric sugars and lipids were easily separated by liquid-liquid separation. Sugars in the separated hydrolyzate were determined to be mainly D-glucose and L-galactose. Fractionation conditions were optimized by response surface methodology (RSM). Optimal conditions were found to be 115.5 °C of reaction temperature, 46.7 min of reaction time, and 25% (w/w) of solid loading. The model predicted that maximum oligomeric sugar yield (based on untreated micro-algae weight), which can be recovered by hydrothermal fractionation at the optimum conditions, was 19.4 wt% (based on the total biomass weight). Experimental results were in agreement with the model prediction of 16.6 wt%. Production of bioethanol using micro-algae-induced glucan and *E. coli* KO11 was tested with SSF (simultaneous saccharification and fermentation), which resulted in 11.8 g-ethanol/*l* was produced from 25.7 g/*l* of glucose; i.e. the theoretical maximum ethanol yield based on glucan in hydrolyzate was 89.8%.

Key words: Biofuel, Simultaneous Saccharification and Fermentation (SSF), Fractionation, KO11, Hot Water, *Schizochytrium* sp.

INTRODUCTION

Energy consumption throughout the world has drastically increased during the past several decades. Over 80% of our energy demands are met by the combustion of fossil fuels such as oil, coal and natural gas [1]. These fossil resources are finite, which will finally lead to the predictable depletion of fossil resources in the near future. It will also cause an accelerated increase of the carbon dioxide in the atmosphere, which is one of the major green house gases.

Bioethanol produced from various renewable feedstocks such as lignocellulosic biomass is currently considered as a clean and renewable energy, which is an ideal substitute for gasoline. Recent research and development efforts have been focused on the commercialization of ethanol production and development of abundant and inexpensive renewable resources other than lignocellulosic biomass such as crop residues and biomass waste [2-10], municipal solid wastes [11-14], municipal sludge [15], and dairy and cattle manures [16]. In the past, micro-algae have been primarily considered as a feedstock to produce biodiesel [17-20] and to produce high-value product such as docosahexaenoic acid (DHA) due to their high lipid content [21]. It was also reported that micro-algae have high a potential to produce bioethanol because they contain polysaccharides, i.e., glucan and galactan [22,23].

Microalgae are unicellular algae which normally grow in suspension within a body of water. Micro-algae have many advantages as biofuel feedstocks; they have high growth rates, they can produce up to 300 times more oil per acre than conventional crops, such as rapeseed, palms, soybeans, or Jatropha, and they have higher photosynthetic efficiency than other biomasses [24,25]. Matsumoto et al. also reported micro-algae have the potential to be a better substrate than lignocellulosic biomass [26].

Micro-algae consist of three major components: lipids/natural oils, proteins and carbohydrates. The oil content of microalgae is typically 20-50 wt% depending upon the species. It was observed that nitrogen-deficient conditions resulted in higher carbohydrate production and reduction of protein [21,27].

If micro-algae are easily fractionated to sugar and lipid component for bioethanol or biodiesel production, and sugar or lipid production is optimized, the utilization of micro-algae as a feedstock for biofuel production can be maximized. Unfortunately, the previous method for lipid extraction from micro-algae contains several toxic chemicals and processing steps [28].

In this study, hydrothermal treatment was used to fractionate *Schizochytrium* sp., which is currently being used for commercial DHA production [29]. The hydrothermal pretreatment was carried out to easily fractionate the micro-algae to the sugar and lipid phase. A feasibility study of bioethanol fermentation using hot water fractionated micro-algae was also performed. The production of sugar for bioethanol fermentation from micro-algae was optimized by a response surface methodology (RSM) based on the 2^3 factorial central composite design (CCD).

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Table 1. Composition of *Schizocytrium* sp. used in this study

Composition	Value
Carbohydrate (wt%)	17.3
Glucan	10.8
Galactan	6.5
Lipid (wt%)	25.1
DHA	8.2

MATERIALS AND METHODS

1. Feedstock

Schizochytrium sp. was purchased in the form of a dried orange powder from NOVUS (DHA Gold, St. Charles, MO, USA). The compositional analysis of *Schizochytrium* sp. is presented in Table 1.

2. Enzyme and Microorganisms

Spezyme Xtra (Product Code A05347, Genencor International Inc., Rochester, NY, USA), and Distillase L400 (Product Code A060515, Genencor International Inc., Rochester, NY, USA) were used for enzymatic saccharification of *Schizochytrium* sp. The activity of Spezyme Xtra was 14,000 α-amylase units (AAU)/g (one AAU unit of α-amylase activity is defined as the amount of enzyme required to hydrolyze 10 mg of starch per minute under specified conditions) and the activity of Distillase L400 was 350 glucoamylase units (GAU)/g (one GAU unit is defined as the amount of enzyme that will liberate one gram of reducing sugars, which is calculated as the amount glucose per hour from soluble starch substrate under the conditions of the assay).

E. coli KO11 was used for the ethanol fermentation. The strain was grown on LB medium containing 40 mg/L chloramphenicol. The cells were cultured in a shaking incubator at 37 °C and 150 rpm.

3. Experimental Setup and Operation for Hydrothermal Fractionation

The reaction was performed in temperature-programmable GC oven (Hewlett Packard 5890A, Agilent Technologies, Santa Clara, CA, USA). The reactor (10 cm^3 of internal volume) was constructed out of 240 mm of stainless tubing with an ID of 23 mm. The reactor

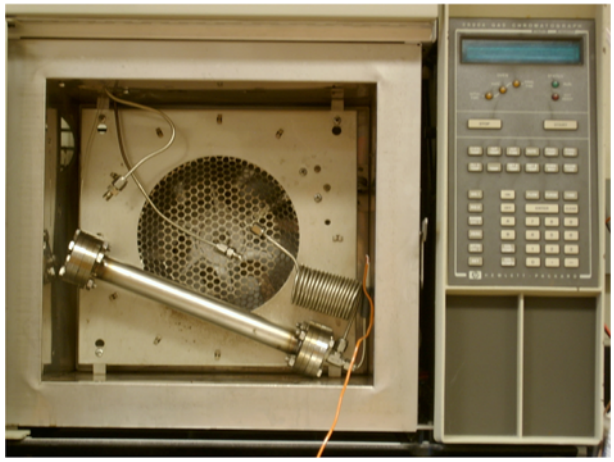

Fig. 1. Laboratory hydrothermal treatment reactor set up.

Table 2. Experimental design (conditions and responses) for hydrothermal fractionation

Run	Temp. (°C), X_1		Time (min), X_2		Solid loading (%), X_3		Total oligomeric sugar content (wt%), Y_1
1	−1.00	90	−1.00	30	−1.00	10	16.33
2	+1.00	150	−1.00	30	−1.00	10	16.71
3	−1.00	90	+1.00	60	−1.00	10	16.5
4	+1.00	150	+1.00	60	−1.00	10	13.66
5	−1.00	90	−1.00	30	+1.00	20	17.60
6	+1.00	150	−1.00	30	+1.00	20	15.79
7	−1.00	90	+1.00	60	+1.00	20	17.33
8	+1.00	150	+1.00	60	+1.00	20	13.61
9	−2.00	60	0.00	45	0.00	15	16.10
10	+2.00	180	0.00	45	0.00	15	5.08
11	0.00	120	−2.00	15	0.00	15	16.20
12	0.00	120	+2.00	75	0.00	15	17.72
13	0.00	120	0.00	45	−2.00	5	16.24
14	0.00	120	0.00	45	+2.00	25	19.89
15	0.00	120	0.00	45	0.00	15	16.85
16	0.00	120	0.00	45	0.00	15	16.23
17	0.00	120	0.00	45	0.00	15	18.85
18	0.00	120	0.00	45	0.00	15	17.03
19	0.00	120	0.00	45	0.00	15	21.62
20	0.00	120	0.00	45	0.00	15	16.77

For statistical calculations, the relationship between the coded values and actual values are described as the following equation: $x_i=(X_i-X_0)/\Delta X_i$, i=1, 2, 3, …..k

where; x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_0 is the real value of an independent variable at the center point and ΔX_i is the step change of variable

setup is shown in Fig. 1, and the reaction conditions are summarized in Table 2.

For the hydrothermal fractionation experiment, the biomass sample was packed into the reactor according to the solid loading ratio. The total packed volume was 80% of total reactor volume. The oven was preheated for 15 min to reach the target temperature before each experiment.

4. Response Surface Methodology

To produce oligomeric sugar from micro-algae, hydrothermal fractionation conditions were optimized by RSM based on the 2^3 factorial central composite design. Twenty experiments were conducted with three variables, and each variable varied at five levels (α=2) for oligomeric sugar content. Oligomeric sugar content was the response (dependent) variable. The reduced cubic polynomial model was fitted for the oligomeric sugar content (Y), giving the following Eq. (1):

$$Y=\alpha_0+\alpha_1X_1+\alpha_2X_2+\alpha_3X_3+\alpha_{11}X_1^2+\alpha_{22}X_2^2+\alpha_{33}X_3^2+\alpha_{12}X_1X_2 +\alpha_{13}X_1X_3+\alpha_{23}X_2X_3+\alpha'_{12}X_1^2X_2+\alpha'_{13}X_1^2X_3+\alpha''_{12}X_1X_2^2 \quad (1)$$

where X_1, X_2, and X_3 represent coded levels of the independent variables; α_0 is intercept terms; α_1, α_2, and α_3 are linear terms; α_{11}, α_{22}, and α_{33} are quadric terms; α_{12}, α_{13}, α_{23}, α'_{12}, α'_{13}, and α''_{12} are interaction terms. The statistical analysis of the data was performed using "Design Expert" software (version 7.1.1, Stat-Ease, Inc., Minneapolis, USA). The series of experiments designed and conducted are shown in Table 2.

5. Partial Simultaneous Saccharification and Fermentation

A 250 mL Erlenmeyer flask was used as the bioreactor. It was shaken in the incubator shaker at 37 °C at 150 rpm under anaerobic condition. Hydrothermal fractionated liquid phase of micro-algae was introduced in a reactor of 100 ml of working volume of liquid. Raw micro-algae in which hydrothermal fractionation was not performed were put through the same procedure as the control. The loading of amylase enzyme (Spezyme Xtra) was approximately 13,000 AAU/g-glucan and, approximately 660 GAU/g-glucan of glucoamylase (Distillase L400). Enzymatic saccharification was performed for 24 h and then *E. coli* KO11 was inoculated in the bioreactor for the bioethanol production. The ethanol yield in partial SSF test was calculated as follows:

Theoretical maximum ethanol yield (%)
=(Ethanol produced (g) in reactor×100)
/Initial sugar (g) in reactor×0.511

Note: Sugar is interpreted as glucose in the SSCF work.

6. Analytical Method

Sugars and ethanol were determined by HPLC using a Bio-Rad Aminex HPX-87P and 87H columns and a refractive index detector. The total fat and fatty acid content were determined by the method developed by Du et al. [28].

RESULTS AND DISCUSSION

1. Composition Analysis of *Schizochytrium* sp.

Table 1 shows the initial compositions of *Schizochytrium* sp. Carbohydrate components, which can be potentially converted to ethanol by ethanol fermenting organisms, were determined by two methods: enzymatic saccharification by amylase enzyme, and phenol sulfuric acid method [30]. The phenol sulfuric acid method was used to obtain a more accurate estimate of carbohydrate component. In both of the methods, glucan and galactan were determined to be from the carbohydrate of *Schizochytrium* sp., of which, 10.8 wt% of glucan and 0.6 wt% of galactan were determined by enzymatic saccharification. However, in case of phenol sulfuric acid methods, 7.2 wt% of glucan and 6.5 wt% of galactan were determined. The difference of glucan content of the two methods was found to be from the decomposition of sugar component by sulfuric acid. In the case of galactan content, it was determined that the enzyme used in the hydrolysis step did not contain galactanase activity. It should be noted that although HPLC can analyze galactose, HPLC does not identify L- and D- form of galactose. However, *E. coli* KO11 can consume only D-galactose sugar; therefore, the L-galactose content in the hydrolysate can be estimated by fermentation of hydrolysate using *E. coli* KO11. The total carbohydrate content of *Schizochytrium* sp. was approximately 17.3 wt% which included 10.8 wt% of glucan and 6.5 wt% of galactan. Our result indicated that the galactan produced in our hydrothermal fractionation was assumed to be an L (levo)-form galactose polymer since the L-galactose cannot be used for ethanol fermentation by *E. coli* KO11. The carbohydrate composition found in this study was similar to reported values of Darley et al. [31]. They mentioned that the sugars of *Schizochytrium aggregatum* are glucose and L-form galactose with the principal sugar being L-form galactose. In addition to the carbohydrate composition, approximately 25.1 wt% of *Schizochytrium* sp. is comprised of lipid component, which can be used to produce biodiesel. Also, among the fatty acid, DHA content was found to be 8.2%. The strategy in this study was to easily fractionate the sugar and lipid components in the micro-algae by hydrothermal fractionation for bioethanol, biodiesel, or high-value product production. Since the current predominant method uses several toxic chemicals and complicated processing steps to separate lipid from micro-algae, the hydrothermal fractionation suggested in this study can result in a simple conversion scheme that is expected to be environmentally friendly and economically viable.

2. Statistical Analysis for Hydrothermal Fractionation

Response surface methodology (RSM) based on the 2^3 factorial central composite design (CCD) was used to optimize the conditions of hydrothermal fractionation for bioethanol production from *Schizocytrium* sp. A central composite factorial design experiment was performed to examine the combined effects of the three independent variables on oligomeric sugar content (Table 2). The CCD was originally introduced by Box and Willson [32]. Several researches on CCD are available in the literature [33-37]. Mathematical models, which represent a reduced cubic polynomial, are given by Eq. (2), where variables take their coded values.

The reduced cubic polynomial model for ethanol production (Y) is as follows:

$$Y=17.9-2.76X_1+0.38X_2+0.91X_3-1.82X_1^2-0.23X_2^2+0.05X_3^2 -0.64X_1X_2-0.38X_1X_3+0.05X_2X_3-1.05X_1^2X_2 -0.77X_1^2X_3+1.76X_1X_3^2 \quad (2)$$

Where, Y represents the responses or, oligomeric sugar content (%); X_1-X_3 are independent variables (X_1: Temperature, X_2: Reaction time, and X_3: Solid loading).

Table 3. Analysis of variance for reduced cubic model

Source	SS[a]	DF[b]	MS[c]	*F*-value	Prob (*P*)>*F*
[R^2=0.8925, CV=10.59%]					
Model	173.14	12	14.43	4.84	<0.0226
Residual (error)	20.86	7	2.98		
Lack of fit	0.23	2	0.16	0.03	0.9725
Pure error	3.67	5	0.73		
Total	194	19			

[a]SS, sum of squares
[b]DF, degrees of freedom
[c]MS, mean squares

The statistical significance of the respective model equation was checked using *F*-test analysis of variance (ANOVA) (Table 3). The fitness of the model was also expressed by the coefficient of determination, R^2, which was found to be 0.8925 on the oligomeric sugar content. This value indicates 89.25% of the response variability in oligomeric sugar content. The closer the R^2 is to 1, the stronger the model and the better it predicts the response [38]. The lower the value of the coefficient of the variation (CV) (10.59 wt% for oligomeric sugar content) is, the greater the precision and reliability of the experiments carried out. The probability *p*-value for models of less than 0.0226 also indicated that the models were highly significant, and insignificant *p*-value of lack of fit for models indicated that experimental data obtained are in good agreement with the model. If the *p*-value for lack of fit for model is significant ($p<0.05$), then a more complicated model would be required to fit the data [39].

3. Optimization of Hydrothermal Fractionation

The traditional 'one-factor at a time' optimization technique is comparatively simple, and the individual effects of bioprocess factors can be graphically depicted without the need for the statistical analysis. Unfortunately, this method often fails to seek the region of optimum response since the joint effects of factors on the response are not considered in the technique. A combination of factors generating a certain optimum response can be identified though factorial design and the use of response surface methodology (RSM) [40,41].

The 3-D response surface plots graphically represent the regression equation. By using the response surface plot, the interaction between two variables and their optimum levels can be easily understood and located. Plots showing interaction between reaction time and temperature, solid loading and temperature, solid loading and reaction time are depicted in Fig. 2.

The maximum sugar content was observed with a relatively high temperature in the interaction between reaction time and temperature (Fig. 2(a)). On the other hand, when reaction time was fixed at 45 min, optimum solid loading and temperature were high around the center point (Fig. 2(b)). As seen in Fig. 2(c), the maximum ethanol production was achieved with high solid loading and long reaction time in the interaction between solid loading and reaction time. Optimal values of the test variables in coded units were numerically solved as follows: $X_1=-0.15$, $X_2=0.11$, $X_3=2.00$ corresponding to $Y_1=19.4\%$. Actual values were obtained by putting the respective values of X_i in equation of Table 2: temperature, 115.5 °C; reaction time, 46.7 min, and solid loading, 25%. To confirm the predicted

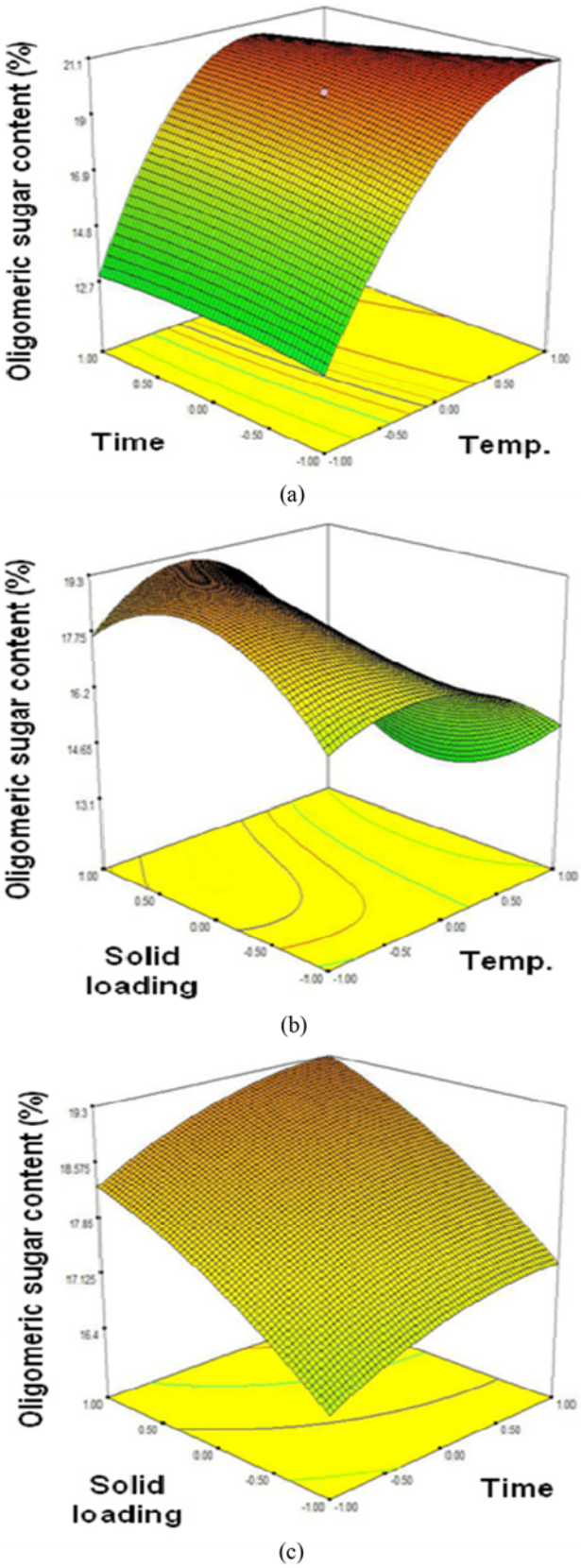

Fig. 2. Response surface plots showing the effect of temperature, reaction time, and solid loading and their combined effect on the oligomeric sugar content. (a) Reaction time and temperature, (b) Solid loading and temperature, and (c) Solid loading and time.

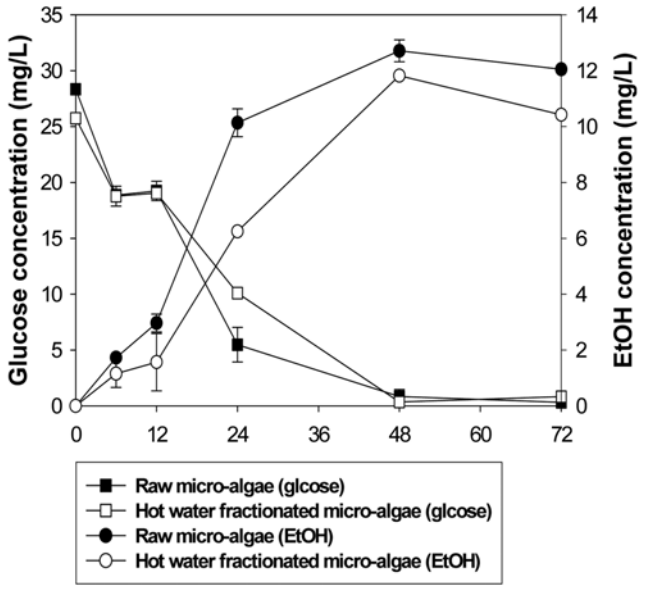

Fig. 3. SSF of micro-algae induced sugars.

optimization conditions, experiments were performed using conditions determined above. Similarly, experimental results of sugar content were 16.6 wt%, which was in close agreement with the model prediction. Time course of ethanol production using hot-water fractionated liquid phase of micro-algae in the optimum conditions is shown in Fig. 3. This fermentability test showed that 11.8 g/*l* of ethanol was produced from 25.7 g/*l* of glucose by *E. coli* KO11 with the maximum bioethanol production yield being 89.8 wt%. So if 1,000 kg of micro-algae (*Schizocytrium* sp.) containing about 10.8 wt% glucan and about 6.5 wt% galactan were and fermented by the procedure above to produce ethanol, 55.1 kg ethanol could be produced.

Additional fermentation test was conducted to confirm the effect of the addition of the lipids to the fermentation broth (data not shown). The lipids separated from the hydrolyzate were used to test an inhibitory effect on microbial activity in an SSF reactor. The SSF test result showed that the addition of lipid had no negative effect on ethanol fermentation using KO11 strain.

CONCLUSION

Hydrothermal treatment can easily fractionate micro-algae to sugars, lipids, and protein phase with no additional chemical extraction process. Statistical optimization of hydrothermal fractionation of micro-algae for ethanol production has been successfully carried out using RSM based on the 2^3 factorial CCD. The optimal conditions for sugar content were determined as follows: reaction temperature of 115.5 °C, reaction time of 46.7 min, and solid loading of 25% (w/w).

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REFERENCES

1. D. Das and T. N. Veziroğlu, *Int. J. Hydrog. Energy*, **26**, 13 (2001).
2. C. Asada, Y. Nakamura and F. Kobayashi, *Bioprocess Eng.*, **10**, 346 (2005).
3. Z. Kádár, Z. Szengyel and K. Réczey, *Ind. Crop. Prod.*, **20**, 103 (2004).
4. S. Kim and B. E. Dale, *Biomass Bioenerg.*, **26**, 361 (2004).
5. G Lissens, H. Klinke, W. Verstraete, B. Ahring and A. B. Thomsen, *J. Chem. Technol. Biotechnol.*, **79**, 889 (2004).
6. Z. Fan, C. South, K. Lyford, J. Munsie, P. van Walsum and L. R. Lynd, *Bioprocess Biosyst. Eng.*, **26**, 93 (2003).
7. Y. Nakamura and T. Sawada, *Biotechnol. Bioprocess Eng.*, **8**, 205 (2003).
8. T. H. Kim, J. S. Kim, C. Sunwoo and Y. Y. Lee, *Bioresour. Technol.*, **90**, 39 (2003).
9. T. H. Kim and Y. Y. Lee, *Appl. Biochem. Biotechnol.*, **137-140**(1-12), 81 (2007).
10. T. H. Kim., Y. Y. Lee, C. Sunwoo and J. S. Kim *Appl. Biochem. Biotechnol.*, **133**(1), 41 (2006).
11. G. Mtui and Y. Nakamura, *Biodegradation*, **16**, 493 (2005).
12. N. Lark, Y. Xia, C. G. Qin, C. S. Gong and G. T. Tsao, *Biomass Bioenerg.*, **12**, 135 (1997).
13. M. Green and G. Shelef, *Chem. Eng. J.*, **40**, B25 (1989).
14. M. Green, S. Kimchie, A. I. Malester, B. Rugg and G. Shelef, *Biol. Wastes*, **26**, 285 (1988).
15. S. W. Cheung and B. C. Anderson, *Bioresour. Technol.*, **59**, 81 (1997).
16. Z. Wen, W. Liao and S. Chen, *Bioresour. Technol.*, **91**, 31 (2004).
17. S. Sawayama, S. Inoue, Y. Dote and S. Y. Yokoyama, *Energy Convers. Manage.*, **36**, 729 (1995).
18. T. G Dunahay, E. E. Jarvis, S. S. Dais and P. G Roessler, *Appl. Biochem. Biotechnol.*, **57-58**, 223 (1996).
19. A. Banerjee, R. Sharma, Y. Chisti and U. C. Banerjee, *Crit. Rev. Biotechnol.*, **22**, 245 (2002).
20. M. Gavrilescu and Y. Chisti, *Biotechnol. Adv.*, **23**, 471 (2005).
21. B. H. Um and Y. S. Kim, *J. Ind. Eng. Chem.*, **15**, 1 (2009).
22. S. Kim, Y. Jeon, W. Kim, H. Back, P. Park, H. Byun and S. Bai, *J. Fish. Sci. Technol.*, **4**, 75 (2001).
23. R. Harun, M. K. Danquah and G. M. Forde, *J. Chem. Technol. Biotechnol.*, **85**(2), 199 (2010).
24. A. B. Ross, J. M. Jones, M. L. Kubacki and T. Bridgeman, *Bioresour. Technol.*, **99**, 6494 (2008).
25. A. Widjaja, C. C. Chien and Y. H. Ju, *J. Taiwan Inst. Chem. Eng.*, **40**, 13 (2009).
26. M. Matsumoto, H. Yokouchi, N. Suzuki, H. Ohata and T. Matsunaga, *Appl. Biochem. Biotechnol.*, **105-108**, 247 (2003).
27. J. Sheehan, T. Dunahay, J. Benemann and P. Roessler, A Look Back at the U.S. Department of Energy's Aquatic Species Program-Biodiesel from Algae, Technical Report for NREL (1998).
28. M. Du, D. U. Ahn and J. L. Sell, *Poultry Sci.*, **79**, 1749 (2000).
29. L. Sijtsma and M. E. de Swaaf, *Appl. Microbiol. Biotechnol.*, **64**, 146 (2004).
30. N. Dubois, K. A. Gillies, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
31. W. M. Darley, D. Porter and M. S. Fuller, *Ach. Mikrobiol.*, **90**, 89 (1973).

32. G. E. P. Box and K. B. Wilson, *J. R. Stat. Soc.*, *(Ser B)*, **13**, 1 (1951).
33. S. F. G. Oskouie, F. Tabandeh, B. Yakhchali and F. Eftekhar, *Biochem. Eng. J.*, **39**, 37 (2008).
34. C. Tari, H. Genckal and F. Tokatli, *Process Biochem.*, **41**, 659 (2006).
35. V. V. R. Bandaru, S. R. Somalanka, D. R. Menduc, N. R. Madicherla and A. Chityala, *Enzyme. Microb. Technol.*, **38**, 209 (2006).
36. S. Sharma, A. Malik and S. Satya, *J. Hazard. Mater.*, **164**, 1198 (2009).
37. B. H. Um and T. R. Hanley, *Korean J. Chem. Eng.*, **25**(5), 1094 (2008).
38. K. Chauhan, U. Trivedi and K. C. Patel, *J. Microbiol. Biotechnol.*, **16**, 1410 (2006).
39. R. M. Banik, A. Santhiagu and S. Upadhyay, *Bioresour. Technol.*, **98**, 792 (2007).
40. G. E. P. Box, W. G. Hunter and J. S. Hunter, *Statistics for experimenters: an introduction to design, data analysis and model building*, John Wiley and Sons Inc., New York, 653 (1978).
41. M. L. Cazetta, M. A. P. C. Celligoi, J. B. Buzato and I. S. Scarmino, *Bioresour. Technol.*, **98**, 2824 (2007).