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Optimization of enzymatic hydrolysis conditions for saccharification of carbohydrates in algal biomass: An integral walk for bioethanol production

Poonam Sharma, Nivedita Sharma and Nisha Sharma

Abstract
Fermentation of carbohydrates to produce bioethanol is one of the pathways to produce biofuels from microalgae. In the present work, enzymatic pre-treatment was performed on microalgal species to disrupt and break down complex carbohydrates into simple sugars, as a prerequisite step to produce bioethanol. A mixture of different in house enzymes (cellulase: amylase: xylanase: pectinase) was used for enzymatic hydrolysis of untreated and pretreated algal biomass and different process parameters were optimized by using one factor at a time approach. The process parameters optimized for enhanced hydrolysis were enzymatic ratio, enzymatic dosage, temperature and incubation time. The best optimized conditions obtained were enzymatic ratio 5:3:1:1 (cellulase: amylase: xylanase: pectinase) @ dose of 12.5 ml/g of biomass, temperature 45 ˚C and incubation period 48 h yielding 140.72 mg/g and 195.84 mg/g of reducing sugars for untreated and pretreated biomass respectively. The results obtained proved the effectiveness of enzymatic hydrolysis to enhance complex carbohydrates break down into simple sugars in bioethanol production process from micro algal biomass.

Keywords: Fermentation, algae, enzymes, optimization, bioethanol

1. Introduction
The fast growth of the world population and rapid development of a number of emerging economies have led to increase in global energy consumption (Harun *et al*., 2010) [12]. However, the use of fossil fuels is associated with environmental pollution, the greenhouse effect, and climate change (Ho *et al*., 2011; Sivakumar *et al*., 2010) [13, 26], and thus many countries are now increasing their efforts with regard to developing renewable energy sources, which are both more economic and environmentally friendly (Mussatto *et al*., 2010) [19]. In this aspect biofuel is a sustainable option. Agricultural lands cannot be compromised for biofuel production due to the requirement of food for the increasing population. Certain species of algae can produce ethanol during anaerobic fermentation and thus serve as a direct source for bioethanol production. Indeed, algae offer a number of potential advantages compared to higher plants. Microalgae have shown to be more efficient than terrestrial plants in converting sunlight to biochemical energy being its production tenfold higher (Stephenson *et al*., 2011, Pedersen *et al*., 1996) [28, 20]. Microalgae consume CO_2, reducing greenhouse gas emissions and their growth is not dependent on arable land availability (Rittman, 2008, Stephens *et al*., 2012) [22]. The carbohydrates in green algae mainly come from starch in chloroplasts and cellulose/polysaccharides on cell walls (Domozych *et al*., 2012; Richmond, 2004) [4, 21], which are not readily fermentable for ethanol production by microorganisms. Therefore, prior to ethanol fermentation, the polysaccharides of microalgae should be hydrolyzed to fermentable sugars (Hahn-Ha gerdal *et al*., 2007) [9]. In general, chemical (acid and alkaline) or enzymatic hydrolysis are common methods used for this purpose. While acid hydrolysis is faster, easier and cheaper than other types of hydrolysis, but acidic conditions may lead to decomposition of the sugars into unwanted compounds that inhibit the fermentation process (Girio *et al*., 2010; Moxley and Zhang, 2007) [7, 18]. In contrast, enzymatic hydrolysis is an environmentally benign process and can obtain higher glucose yields without producing inhibitory products. Despite of the many cell disrupting methods tested in literature for microalgal cell wall disruption, a standard pre-treatment has not been identified to treat most of microalgal species. Furthermore, data in literature concerning biomass pretreatments are not comparable, because quite different microalgae strains, conditions and techniques are used, making it difficult to compare these results between microalgae. Optimization of enzymatic hydrolysis process is one of the most

important stages in the development of an efficient and cost effective saccharification strategy. The process efficiency depends on several parameters such as enzyme, substrate loading, pH, temperature and incubation time. The optimal enzymatic process conditions vary also depending on the composition of carbohydrates between the green, brown and red algae. Optimization of multifactorial system by conventional techniques is generally done with one-factor at a time. In this context, the effect of enzymatic pretreatment on algae *Rhizoclonium* sp. was studied and different factors for enzymatic pretreatment were optimized by using one factor at a time approach (OFAT) for enhancing hydrolysis of algal biomass.

2. Materials and Methods

2.1. Biomass collection and Preparation

The fresh water green algae were collected in sterile polythene bags from different districts of Himachal Pradesh, India. The algae samples were washed thoroughly with tap water to remove salts, epiphytes and debris and dried to a constant weight at temperature of 50 ºC. After drying, the samples were powdered using grinder and stored in plastic containers at room temperature.

2.2. Identification of the algal biomass: Identification done by applying two step approach: Detailed studies were made by examining specimens under microscope. Algal identification was done by referring the literature of Anand (1998) [2] and Bellinger and Sigee (2010) [3].

2.3 Pretreatment for algal biomass: Microwave irradiation i.e. 150, 300, 450 W for different time intervals of 30 and 60 sec.

2.4. Enzymatic hydrolysis of algal biomass

2.4.1 Enzymes used: Enzymatic mixture of different in house hydrolytic enzymes (cellulase: amylase: xylanase: pectinase) was employed for biomass hydrolysis.

i) α-amylase (Vyas, 2015) [30]
ii) Xylanase (Sharma, 2013) [24]
iii) Cellulase (Sharma, 2013) [24]
iv) Pectinase (Handa, 2016) [10]

2.4.2 Hydrolysis: To each 1 g untreated and pretreated algae powder in 100 ml Erlenmeyer flasks added 10 ml phosphate buffer and autoclaved. Then enzymatic mixture of different in house enzymes in different ratio was added to each flask under sterile conditions. The flasks were incubated at 45 ºC for 72 h to undergo enzymatic hydrolysis. After 72 h, biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars (Miller, 1959) [17].

2.5 Optimization of enzymatic ratio

The different enzymatic ratios for enzymatic hydrolysis were selected on the basis of composition of algal biomass i.e. 4:3:1.5:1.5, 4:4:1:1, 3:5:1:1, 6:2:1:1, 7:1:1:1, 5:3:1:1 (cellulase: amylase: xylanase: pectinase) for optimization.

2.6 Optimization of Enzyme dose

The best selected enzymatic ratio of 5:3:1:1 (cellulase: amylase: xylanase: pectinase) with varying enzyme dose @ 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 ml/g was studied.

2.7 Optimization of hydrolysis temperature

Different temperatures i.e. 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC and 60 ºC for 72 h were used for enzymatic saccharification.

2.8 Optimization of hydrolysis period

The hydrolysis period was varied from 12 h, 24 h, 36 h, 48 h, 60 h and 72 h for enzymatic hydrolysis.

3. Results and Discussion

Mixture of different partially purified in house enzymes was used for the enzymatic hydrolysis of untreated algal biomass. Cellulase, amylase, xylanase and pectinase were used for the hydrolysis of untreated algal biomass for its conversion into simple sugars. Different enzymatic ratios were selected on the basis of biochemical composition of algal biomass to evaluate their effect on the saccharification of untreated *Rhizoclonium* sp. biomass.

3.1 Optimization of different enzymatic ratio: The saccharification of the algae *Rhizoclonium* sp. Within house hydrolytic enzymes was optimized with respect to enzymatic ratio, enzyme dosage, temperature and incubation period by using one factor at a time (OFAT) approach. The composition of hydrolytic enzymes may significantly affect the efficiency of enzymatic hydrolysis of microalgae biomass for obtaining the fermentable sugars, due to the specific structures and proportions of cellulose and starch. Different enzymatic ratio of (cellulase: amylase: xylanase: pectinase) i.e. 4:3:1.5:1.5, 4:4:1:1, 3:5:1:1, 6:2:1:1, 7:1:1:1 and 5:3:1:1 (v/w) resulted into a reducing sugar yield of 63.36, 69.12, 54.72, 89.6, 52.84, 122.8 mg/g biomass, respectively (Table 1). The enzymatic ratio of 5:3:1:1 was observed for maximum sugars and was employed for hydrolysis of biomass in subsequent optimization studies. Same results were obtained from untreated algal biomass with enzymatic ratio of 5:3:1:1. resulted into highest reducing sugar yield 63.28 mg/g. Coupling of microwave pretreatment with enzymatic hydrolysis caused an appreciable increase in production of reducing sugars because enzyme action was rapid and caused greater break down of already fragmented and complex carbohydrate chains for release of maximum sugars. The enzymatic ratio of 3:5:1:1 was proven least effective for production of reducing sugars as up to 31.36 and 54.72 mg/g only were produced from untreated and pretreated biomass. The minimum amount of sugars produced by this ratio was due to variation in cell wall composition to ratio of different enzymes in enzymatic mixture. Harun *et al.* (2014) [12] studied the enzymatic hydrolysis of microalgal biomass *Chlorococcum infusionum* by using the enzymes cellulase from *Trichoderma reesei* (ATCC 26921) and cellobiase from *Aspergillus niger* (Novozyme 188). Rodrigues *et al.* (2015) [23] studied the enzymatic hydrolysis of untreated *Chlorella homosphaera* biomass by using mixture of different hydrolytic enzymes. High rates of enzymatic hydrolysis were achieved for untreated *C. homosphaera* biomass with enzymes containing endoglucanase and β-glucosidase activities.

3.2 Effect of enzyme dosage: After optimization of enzymatic ratio, enzyme dosage@ 2.5 ml/g to 20 ml/g resulted in reducing sugar yield of maximum 112.3 mg/g and 183.6 mg/g @12.5 ml/g for untreated and pretreated biomass respectively. The reducing sugar yields were increased from

dose @ 2.5 ml/g to 12.5 ml/g of biomass and ranging between 43.2 to 183.6 mg for pretreated biomass. For untreated biomass reducing sugars ranged from 28 mg/g to 112.3 mg/g of biomass. In case of both untreated and pretreated biomass a decline in reducing sugars was noticed after enzyme dose of 12.5 ml/g till 20 ml/g with highest sugars in pretreated biomass (Table 2). A lower enzymatic dose leads to reduced release of reducing sugars as less amount of enzymes were available for action on substrate. With increase in enzymatic dose an appreciable increase was observed in amount of sugars, as higher enzymatic dose provides suitable conditions for hydrolysis of biomass (Karki *et al*., 2011) [14]. High enzyme loading and prolonged hydrolysis provides complete cellulose conversion. An increase in the enzyme dosage beyond optimal level i.e. 15 ml/g to 20 ml/g did not improve sugar yield because of increase in rate of transglycosylation reactions and hydrodynamic instability thus counteracting the rate of hydrolysis (Griggs *et al*., 2014) [8]. Trivedi *et al*. (2013) [29] studied the enzymatic hydrolysis of macrophytic green alga *Ulva fasciata* with the help of commercial cellulase enzymes. Different enzyme dosage of 1%, 2% and 5% (w/v) resulted into a reducing sugar range of 48.65 ± 4.3, 159.22 ± 11.9 to 132.69 ± 8.2 mg/g biomass, respectively. The enzyme dosage of 2% (w/v) was found optimum and was employed for hydrolysis of biomass. Kassim *et al.* (2014) [15] optimized the enzymatic saccharification of untreated and pretreated microalgal (*Tetraselmis suecica*) biomass by degradation enzyme produced from *Trichoderma longibrachiatum*. Pre-treated microalgal biomass similar to our findings produced higher reducing sugar as compared to untreated microalgal biomass.

3.3 Effect of temperature: Different temperatures were used for optimization with one factor at a time approach. The reducing sugar yield were estimated to increase linearly with increase in temperature from 25 ºC to 45 ºC and ranged between 82.08 mg/g and 184.68 mg/g for pretreated biomass. The same pattern was observed for untreated samples as their reducing sugar varied from 55.46 mg and 116.24 mg/g. Further increase in temperature to 60 ºC showed a decline in reducing sugar yield to 109.20 mg/g for pretreated and 75.67 mg/g for untreated algal biomass. Due to unavailability of sufficient energy required for action of enzymes on biomass, the process of hydrolysis was slow at 30 ºC. At higher temperature of 45 ºC, there was an increase in degree of hydrolysis due to breakage of peptide bonds exposed to heat treatment. The rate of enzymatic reaction increases with increase in temperature up to a threshold limit beyond which rate of reaction decreases. This is attributed due to the thermal inactivation of enzymes as most of enzymes work at specific temperature and pH. The reduced rate of reaction leads to poor performance of enzymes and low rate of hydrolysis of substrate. The reduction in rate of hydrolysis may also be due to other factors such as decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme deactivation. An increase in the temperature affects the kinetic energy of enzymatic reactions, which in turn increases the frequency of collision between the substrate and the active sites of an enzyme. Such a thermal agitation may lead to denaturation of enzymes, thereby reducing the availability of active sites (Shuler, 2002) [25]. Eldalatony *et al.* (2015) [5] carried out the hydrolysis of *Chlamydomonas mexicana* at different temperatures ranging from 20 to 60 ºC with the optimum [E]: [S] ratio (1:5) and pH 5. The total reducing sugars for 24 h ascended with an increase in temperature from 20 to 50 ºC and then descended with a further increase in temperature to 60 ºC. The optimum enzymatic hydrolysis temperature was 50 ºC, reflecting the highest total reducing sugar of 280.5 mg/g after 24 h of hydrolysis. Kim *et al.* (2014) [16] reported that over-dehydration of total reducing sugar was incurred on exposure to high temperature, resulting in the formation of by-products such as 5-hydroxymethylfurfural, levulinic acid, formic acid.

3.4 Effect of incubation period: The optimization of incubation period resulted in an optimum reducing sugar yield of 195.84 mg/g for pretreated biomass when incubation period was adjusted to 48 h and the same declined with increase in incubation period to 81.43 mg/g to 96 h. The same results were obtained for untreated biomass resulting into maximum reducing sugars of 140.71 mg/g to 48 h and further increase in incubation period resulted in decline of reducing sugar to 56.51 mg/g for 96 h. The reducing sugar yield for the untreated sample was appreciably lower than that of the pretreated biomass. Thus enzyme ratio (Cellulase: Amylase: Xylanase: Pectinase) of 5:3:1:1, enzyme dosage of 12.5 ml/g of biomass, temperature at 45 ºC, incubation period for 48 h was found optimum for hydrolysis of both untreated algal biomass. Hydrolysis time is one of the most critical factors among different physical factors affecting the yield of sugar. A short duration of time such as 12h generally produces less amount of sugars as the enzymes need a sufficient stretch of time for hydrolysis of biomass effectively. The longer duration of time as 48 h allows the increased enzymatic hydrolysis due to increased cleavage of peptides bonds for extraction of maximum sugars, resulting in increase in solubility of substrate, more stability of enzymes and the reaction equilibrium towards more product formation. The drastic decline in enzymatic hydrolysis rate is responsible for low yields and also there is formation of inhibitory products which decrease the reducing sugar yield. Further increase in incubation time does not increases the sugar yield as substrate is completely saturated with the enzyme and also the decrease in enzyme activity upon prolonged incubation 96 h may be due to irreversible adsorption of enzyme to substrate or due to feedback inhibition/denaturation of enzymes, resulting from the variation of pH and lesser cellular metabolism with ageing during enzymatic hydrolysis (Xin and Geng, 2010) [31]. El-Sayed *et al*. (2017) [6] studied the optimal conditions for hydrolysis process of cellulase 145U at 60 ºC, pH 5.3 and 120 rpm rotation obtained maximum yield of sugar (272.82 mg/g). Trivedi *et al.* (2013) [29] carried out the optimization of enzymatic hydrolysis by using commercial cellulase with enzyme dosage of 2% by varying the hydrolysis period from 6 h to 42 h. Thus enzyme dosage of 2% (w/v) with incubation period for 36 h was found optimum with reducing sugar yield of 168.15 ± 6.3 mg/g for hydrolysis of algal biomass. Further increase in incubation period to 42 h showed a decline in reducing sugar yield to 151.21±2.2 mg/g. Ahmad (2015) [1] optimized the process parameters, hydrolysis period and pH for enzymatic hydrolysis of algae *Gracilaria verrucosa*. The results indicated that the optimum condition of the enzymatic hydrolysis was pH 5.5 and 48h with reducing sugar yield of 40.28 mg/ml as similar to our findings. Classical approach i.e. one variable at a time (OVAT) used for optimization of enzymatic hydrolysis has resulted in statistically significant increase in the reducing sugars yield of untreated and pretreated *Rhizoclonium* sp. algal biomass (Fig. 1). After

optimization of process parameters, the maximum reducing sugars reported from untreated and pretreated biomass were 140.72 and 195.84 respectively, at enzymatic ratio 5:3:1:1, 12.5 ml/g enzyme dosage, 45 ºC temperature and incubation period 48h. From these treatments analysis it can be concluded that *Rhizoclonium* sp. is the suitable algae to produce bioethanol, since it was easy to break down obtaining higher monosaccharide concentration compared to the rest of microalgae.

Table 1: Effect of enzymatic ratio on saccharification of microwave pretreated and untreated *Rhizoclonium* biomass

S. No	Different Enzymatic ratio (cellulase: amylase: xylanase: pectinase)	Reducing sugars from Microwave pretreated algal biomass		Reducing sugars from Untreated algal biomass	
		mg/ml	mg/g	mg/ml	mg/g
1.	4:3:1.5:1.5	3.17 ± 0.09	63.36 ± 0.34	1.28 ± 0.13	25.6 ± 0.34
2.	4:4:1:1	3.46 ± 0.23	69.12 ± 0.57	1.90 ± 0.28	38.08 ± 0.05
3.	3:5:1:1	2.74 ± 0.12	54.72 ± 0.41	1.56 ± 0.05	31.36 ± 0.53
4.	6:2:1:1	4.48 ± 0.17	89.6 ± 0.49	1.79 ± 0.12	35.84 ± 0.40
5.	7:1:1:1	2.89 ± 0.046	52.84 ± 0.21	1.68 ± 0.17	33.6 ± 0.12
6.	5:3:1:1	6.14 ± 0.81	122.80 ± 0.17	3.16 ± 0.09	63.28 ± 0.16
	C.D.	0.71	0.91	0.50	0.98
	S.E.(m)	0.23	0.30	0.16	0.31

Table 2: Effect of enzyme dosage on saccharification of microwave pretreated and untreated *Rhizoclonium* biomass

S. No	Different Enzyme dosage (ml/g)	Reducing sugars from untreated algal biomass		Reducing sugars from microwave pretreated algal biomass	
		mg/ml	mg/g	mg/ml	mg/g
1.	2.5	2.24 ± 0.11	28.00 ± 0.11	3.46 ± 0.23	43.20 ± 0.11
2.	5	2.56 ± 0.12	38.40 ± 0.23	3.94 ± 0.05	59.60 ± 0.34
3.	7.5	2.88 ± 0.05	50.40 ± 0.22	4.99 ± 0.38	87.36 ± 0.17
4.	10	3.44 ±0.12	68.80 ± 0.21	6.24 ± 0.13	124.80 ± 0.17
5.	12.5	4.99 ± 0.14	112.32 ± 0.17	8.16 ± 0.09	183.6 ± 0.34
6.	15	3.84 ± 0.05	96.00 ± 0.15	5.38 ± 0.20	134.40 ± 0.22
7.	17.5	3.20 ± 0.11	88.00 ± 0.28	4.23 ± 0.13	116.16 ± 0.09
8.	20	2.88 ± 0.17	86.40 ± 0.17	3.52 ± 0.23	105.6 ± 0.33
	C.D.	0.36	0.62	0.65	0.73
	S.E. (m)	0.11	0.20	0.22	0.24

Table 3: Effect of temperature on saccharification of untreated and microwave pretreated *Rhizoclonium* biomass

S. No	Different Temperature (ºC)	Reducing sugars in untreated algal biomass		Reducing sugars in microwave pretreated algal biomass	
		mg/ml	mg/g	mg/ml	mg/g
1.	30 ºC	2.47 ± 0.06	55.47 ± 0.27	3.65 ± 0.23	82.08 ± 0.046
2.	35 ºC	3.49 ± 0.17	78.48 ± 0.28	4.21 ± 0.06	94.72 ± 0.17
3.	40 ºC	3.68 ± 0.18	82.80 ± 0.17	4.57 ± 0.23	102.96 ± 0.23
4.	45 ºC	5.38 ± 0.21	116.64 ± 0.37	8.21 ± 0.12	184.68 ± 0.34
5.	50 ºC	4.88 ± 0.17	109.72 ± 0.34	6.79 ± 0.41	152.92 ± 0.49
6.	55 oC	3.74 ± 0.12	84.27 ± 0.23	5.02 ± 0.012	112.97 ± 0.044
7.	60 ºC	3.03 ± 0.017	75.67 ± 0.11	4.85 ± 0.17	109.21 ± 0.12
	C.D.	0.45	0.82	0.65	0.78
	S.E.(m)	0.14	0.27	0.21	0.25

Table 4: Optimization of incubation period for saccharification of untreated and microwave pretreated *Rhizoclonium* biomass

S. No	Different Incubation period (h)	Reducing sugars in untreated algal biomass		Reducing sugars in microwave pretreated algal biomass	
		mg/ml	mg/g	mg/ml	mg/g
1.	12 h	2.52 ± 0.06	56.64 ± 0.29	3.72 ± 0.12	83.79 ± 0.40
2.	24 h	3.19 ± 0.11	71.89 ± 0.23	3.89 ± 0.20	87.62 ± 0.17
3.	36 h	3.58 ± 0.23	80.68 ± 0.06	4.58 ± 0.33	102.96 ± 0.46
4.	48 h	6.25 ± 0.28	140.72 ± 0.41	8.70 ± 0.06	195.84 ± 0.80
5.	60 h	4.58 ± 0.27	102.96 ± 0.46	8.32 ± 0.17	187.2 ± 0.16
6.	72 h	5.07 ± 0.17	114.13 ± 0.07	8.17 ± 0.09	183.77 ± 0.17
7.	84 h	3.92 ± 0.14	88.27 ± 0.16	4.87 ± 0.12	109.61 ± 0.11
8.	96 h	2.51 ± 0.12	56.51 ± 0.23	3.61 ± 0.35	81.43 ± 0.25
	C.D.	0.58	0.83	0.60	1.15
	S.E.(m)	0.19	0.27	0.20	0.38

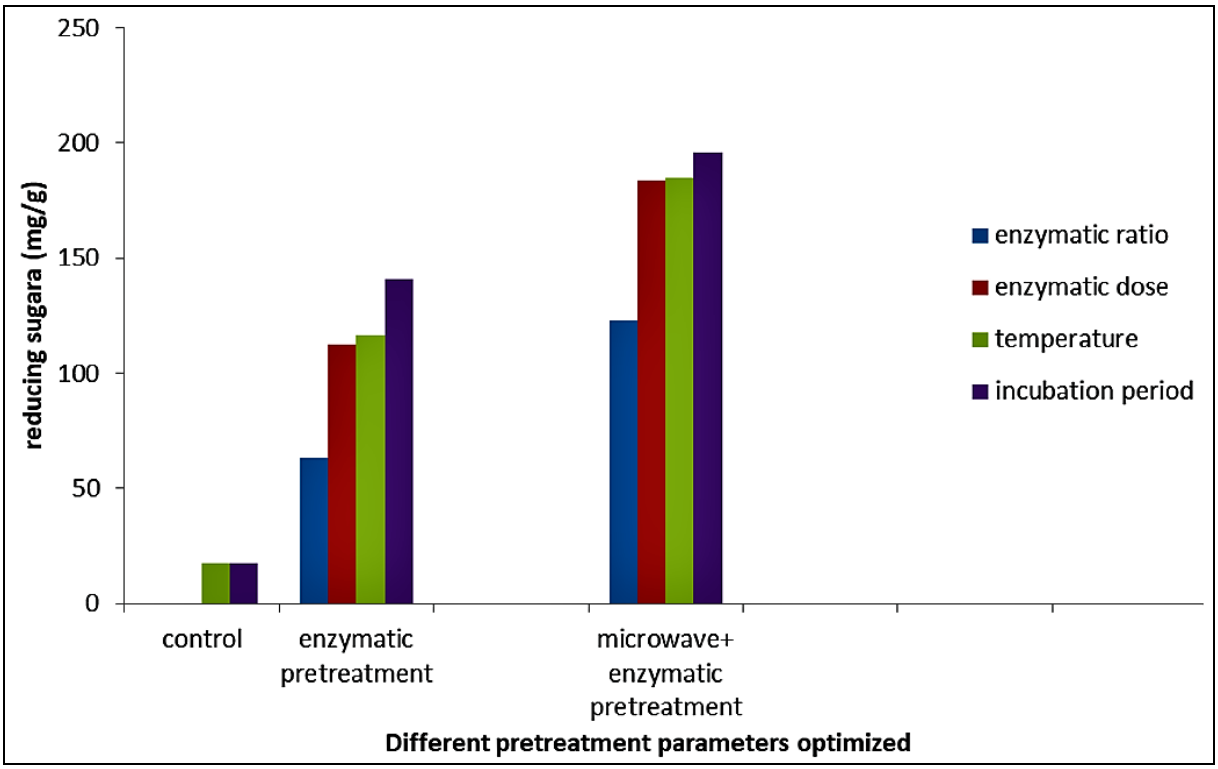

Fig 1: Overall view of increase in reducing sugar yield during optimization of different process parameters

Conclusion

This study demonstrated the feasibility of producing bioethanol from green algae *Rhizoclonium* sp. as potential feedstock. The in house enzymatic mixture from different strains containing suitable amylase, cellulase, Xylansae and pectinase composition could effectively hydrolyze *Rhizoclonium* sp. biomass for subsequent bioethanol production. The present study showed the efficiency of combined pretreatments to disrupt microalgal cell wall and hydrolyze carbohydrate chains into monosaccharides in the bioethanol production process. The enzymatic pretreatment combined with microwave pretreatment resulted in higher reducing sugar yields as compare to untreated and only enzymatic pretreated samples. To enhance the efficiency of saccharification, different factors for enzymatic hydrolysis were optimized which resulted in relatively high amount of reducing sugar yields. The best optimized conditions for hydrolysis included enzyme ratio (Cellulae: Amylase: Xylanase: Pectinase) of 5:3:1:1, enzyme dosage of 12.5 ml/g of biomass, temperature at 45 ^{o}C, incubation period for 48 h released a remarkable monosaccharide concentration of 195.84 mg/g and 140.71 mg/g in pretreated and untreated algal biomass, respectively.

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