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ORIGINAL ARTICLE

Enzymatic hydrolysis of pine needles by optimizing process parameters to enhance reducing sugars production for bioethanol production

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ABSTRACT
Fermentation of reducing sugars to produce bioethanol is one of the pathways to produce biofuels from lignocellulosic biomass. In the present work, enzymatic hydrolysis was performed on pine needle biomass to disrupt and break down complex carbohydrates into simple sugars, as a prerequisite step to produce bioethanol. A mixture of different inhouse enzymes (cellulase: xylanase) was used for enzymatic hydrolysis of untreated and pretreated biomass and different process parameters were optimized by using one factor at a time approach. The process parameters optimized for enhanced hydrolysis were microwave irradiation dose, incubation time, enzymatic dosage, enzymatic ratio and temperature. The best optimized conditions obtained were microwave dose i.e. 600 W for 4 min, incubation time of 72 h, enzymatic ratio 7.75: 4.75 (cellulase: xylanase) @ dose of 12.5 ml/g of biomass at temperature 45°C yielding 19.06 mg/g and 22.35 mg/g of reducing sugars for untreated and pretreated biomass respectively. After optimization of different process parameters for enzymatic hydrolysis maximum percent increase of 272.50 in reducing sugars was observed over untreated biomass.
***Keywords**: Fermentation, Enzymes, Optimization, Bioethanol*

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INTRODUCTION

Nowadays, the fossil fuel shortage and environmental pollution are two main challenges, which need to be addressed by our society. Since limited petroleum resources that have become increasingly depleted, petroleum oil shortages as well as rise in gasoline prices have become important factors in restricting the global economy. Fast development of a number of emerging economies has led to increase in world wide energy consumption [6]. However, the use of fossil fuels are the main cause of environmental pollution, the greenhouse effect, and climate change [7], the combustion of fossil fuels is responsible for 73% of the CO_2 production [16]. Many countries are now increasing their efforts with regard to developing renewable energy sources, which are both more economic and ecofriendly [11]. In this aspect biofuel is an alternative option and the search for renewable energy sources that reduce CO_2 emissions becomes a matter of widespread attention [2]. Lignocellulosic biomass plays a vital role in the production of bioenergy which is organic in composition and has the properties like petroleum. Lignocellulose have been taken into consideration as one form of biomass suitable to be a renewable energy source because, it is obtained from non-food biomass which can avoid the conflict between food and fuel. Lignocellulosic biomasses are basic building block of cellulose, hemicellulose and lignin [14, 15]. The production of energy of this way depends on the type, abundance and cost of biomass feedstocks, efficiency of the available processing technologies and the pattern of energy demand. The closeness of those main components (cellulose, hemicellulose and lignin) in the lignocellulosic wastes induces the necessity of pretreatment process in order to make these carbohydrates available for enzymatic hydrolysis and fermentation during biofuels production [8, 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 [4]. In contrast, enzymatic hydrolysis is an

environmentally benign process and can obtain higher glucose yields without producing inhibitory products.

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. 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 lignocellulosic biomass i.e. pine needles and different factors for enzymatic pretreatment were optimized by using one factor at a time approach (OFAT) for enhancing hydrolysis of lignocellulosic biomass.

MATERIALS AND METHODS

Collection of Biomass: Pine needles were collected from the forests of adjoining Himalayas and brought to the laboratory. Biomass was washed with tap water and dried at 60^{0}C temperature in the oven. Dried biomass was chopped into small pieces and then grinded into 2 mm sieve size and stored for the further experiments.

Enzyme production under submerged fermentation (SmF):

Cellulase production by *B. stratosphericus* N_{12} (M)

100 ml nutrient broth was seeded with 10% *B. stratosphericus* N_{12} (M) (O.D. 1.0) culture in 250ml Erlenmeyer flasks and was kept at 30 ±2°C at 120 rpm for 24 h. 100 ml of PYC (Peptone yeast extract) medium was prepared in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 10% of inoculum and incubated at kept 30±2°C for 3 days. After incubation, contents were centrifuged at 10,000 rpm for 15 min at 4°C and clear supernatant was collected for further studies. Cellulase assays with crude supernatant were performed.

Cellulase assay

The sub-enzymes of cellulase were measured by following standard assays. CMCase activity was determined by incubating 0.5ml of culture supernatant with 0.5 ml of 1.1 % CMC in citrate buffer (0.05M, pH 5.0) at 50^{0}C or 1 h. After incubation and 3 ml of 3, 5 –dinitrosalicylic acid (DNSA) reagent was added. The tubes were immersed in boiling water bath and removed after 15 min. The optical density was read at 540 nm. FPase activity was measured by Reese and Mandel method. The reaction containing 0.5ml of culture supernatant, 50 mg strips of filter paper (Whatmann no. 1) and 0.5 ml of citrate buffer (0.05 M, pH 5.0) was incubated at 50^{0}C for 1 h. After incubation and 3 ml of DNS reagent was added. The tubes were boiled in boiling water bath and removed after 15 min. The OD was read at 540 nm (Reese and Mandel, 1963). For β-glucosidase activity the reaction mixture containing 1 ml of 1mM p-nitrophenol β-D-glucopyranoside in 0.05 M acetate buffer (pH 5.0) and 100 µl of enzyme solution was incubated at 45^{0}C for 10 min. After incubation, 2 ml of 1 MNa_2CO_3 was added and the mixture was heated in boiling water bath for 15 min and OD was read at 400 nm [1].

Xylanase production by *B. altitudinis* Kd_1 (M)

100 ml nutrient broth was seeded with 12.5% *B. altitudinis* Kd_1 (M) (O.D. 1.0) culture in 250 ml Erlenmeyer flasks and was kept at 30±2°C at 120 rpm for 24 h. 100 ml of TGY was prepared in 250 ml Erlenmeyer flask and was autoclaved. After autoclaving, the flasks were inoculated with 12.5% of inoculum and incubated at kept 30±2°C for 3 days. After incubation, contents were centrifuged at 10,000 rpm for 15 min at 4°C and clear supernatant was collected for further studies. Xylanase assays with crude supernatant were performed.

Xylanase assay

To 0.5 ml of xylan solution (which is incubated overnight at 37°C), centrifuged and clear supernatant was used, 0.3 ml of citrate buffer (pH 5) was added and 0.2 ml of enzyme. The control was run with all components except the enzyme. The reaction mixture was incubated at 45°C for 10 min and then 3 ml of DNSA reagent was added and the mixture was then heated on boiling water bath for 15 min, after cooling down at room temperature, absorbance of reaction mixture was read at 540 nm [10].

Inhouse enzyme cocktail

The inhouse enzymes which were prepared had been mixed in the ratio of (3:2) i.e. 3.0 ml of cellulase from cellulase from *B. stratosphericus* N_{12} (M) (CMCase: 1.706 IU, FPase: 2.008 IU and β-glucosidase: 0.196 IU) and 2.0 ml of xylanase from *B. altitudinis* Kd_1 (M) (41.86 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis.

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Optimization of process parameters for enzymatic hydrolysis of biomass

The optimization of enzymatic hydrolysis of biomass was carried out for microwave irradiation dose, incubation period, enzyme dosage, enzymatic ratio and temperature by one factor at a time approach (OFAT).

Optimization of microwave irradiation dose

1 g untreated dried lignocellulosic biomass was taken in different petriplates and subjected to different doses of microwave irradiation i.e. 100, 300, 600 and 900 W for different time intervals of 4 min and 5 min. Sodium citrate buffer (0.05 M, pH 5.5) was added as moistening agent in 1:4 ratio. Purified enzymatic mixture of different inhouse hydrolytic enzymes (cellulase: xylanase) in 3:2 @ 5ml/g dose was employed for biomass hydrolysis at 50^{0}C temperature for 72 h of incubation period. After incubation reducing sugars were estimated [10].

Optimization of incubation period

To each 1 g untreated and microwave (600 W, 4 min) pretreated biomass was taken and to these sodium citrate buffer (0.05 M, pH 5.5) was added and autoclaved. Inhouse enzymes in the ratio of 3:2 (cellulase: xylanase) @ 5.0 ml/g dose was added to each flask under sterile conditions and incubated at optimum temperature, 50°C. The hydrolysis period was varied from 24 h, 48 h, 72 h, 96 h and 120 h for enzymatic hydrolysis. After incubation period, biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

Optimization of enzymatic dose

To each 1 g untreated and pretreated biomass sodium citrate buffer (1:4 ratio) was added and autoclaved. Inhouse enzymes in the ratio of 3:2 (cellulase: xylanase) was added in different doses i.e. 5.0 ml/g, 7.5 ml/g, 10.0 ml/g, 12.5 ml/g and 15.0 ml/g were added to each flask under sterile conditions and incubated at temperature, 50°C for 72 h. After 72 h, saccharified biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

Optimization of enzymatic ratio

To each 1 g untreated and pretreated biomass sodium citrate buffer (0.05M, pH 5.5) was added and autoclaved. Then enzymatic mixture of inhouse enzymes in different ratio i.e. 6.25: 6.25, 6.75: 5.75, 7.25: 5.25, 7.75: 4.75 and 8.25: 4.25 @ 12.5 ml/g doses were added for hydrolysis and the flasks were incubated at 45°C 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.

Optimization of temperature

To each 1 g untreated and microwave pretreated biomass sodium citrate buffer (0.05 M, pH 5.5) and autoclaved. The best selected enzymatic ratio of 7.75: 4.75 (cellulase: xylanase) with optimum enzyme dose @ 12.5 ml/g was added to each flask under sterile conditions. The flasks were incubated at different temperatures i.e. 35°C, 40°C, 45°C, 50°C and 55°C for 72 h to undergo enzymatic saccharification. After 72 h, saccharified biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

RESULTS AND DISCUSSION

In the present study, a mixture of bacterial inhouse enzymes (cellulase: xylanase) were used for enzymatic hydrolysis of untreated and pretreated biomass. Different process parameters were optimized by using one factor at a time approach for enhanced hydrolysis of biomass and to get maximum of reducing sugars. The process parameters optimized for enhanced hydrolysis were microwave irradiation dose, incubation period, enzymatic dosage, enzymatic ratio and temperature.

Optimization of microwave irradiation dose: The effect of microwave irradiation pretreatment on hydrolysis of lignocellulosic biomass along with selected enzymatic ratio of 3:2 (cellulase: xylanase) @ 5ml/g dose depicted in table 1. The highest amount of reducing sugars observed was 10.95 mg/g of biomass at microwave irradiation dosage of 600 W for 4 min after 72 h of enzymatic hydrolysis with maximum percent increase of 82.50 (fig 1). The sugar yield after hydrolysis of pretreated biomass when compared with untreated biomass, it showed a significant increase from 6.00 mg/g to 10.95 mg/g of reducing sugars respectively. Microwave pretreatment causes rapid alignment and realignment of dipoles in a polar solvent, resulting in heat generation, for alteration of cell constituents and break down of carbohydrates present in biomass [12]. A microwave dose of 100 W was not found satisfactory for release of maximum sugars from lignocellulosic biomass probably due to its inability to generate ample amount of heat required to simplify the cell structure for extraction of maximum sugars. Microwave pretreatment of biomass at 300 W increased sugars release from biomass. At higher microwave dosage of 900 W, a noticeable decrease was observed in production of reducing sugars. This decrease was

attributed to release of high heat leading to collapse of biomass tissues which hinders the enzymatic process leaving less space for action of enzyme on substrate [3].

Table1. Optimization of microwave irradiation dose for enhanced hydrolysis of lignocellulosic biomass

Sr No.	Microwave doses (Watts)	Reducing sugars (mg/g) at different time interval			
		4 min	5 min	Percent increase in reducing sugars (4 min)	Percent increase in reducing sugars (5 min)
1.	100	6.63	7.08	10.50	18.00
2.	300	10.12	9.94	68.66	65.66
3.	600	10.95	9.76	82.50	62.66
4.	900	9.77	8.65	62.83	44.16
5.	untreated	6.00	6.78		
C.D0.05		0.16	0.72		
S.E. (m)		0.09	0.23		

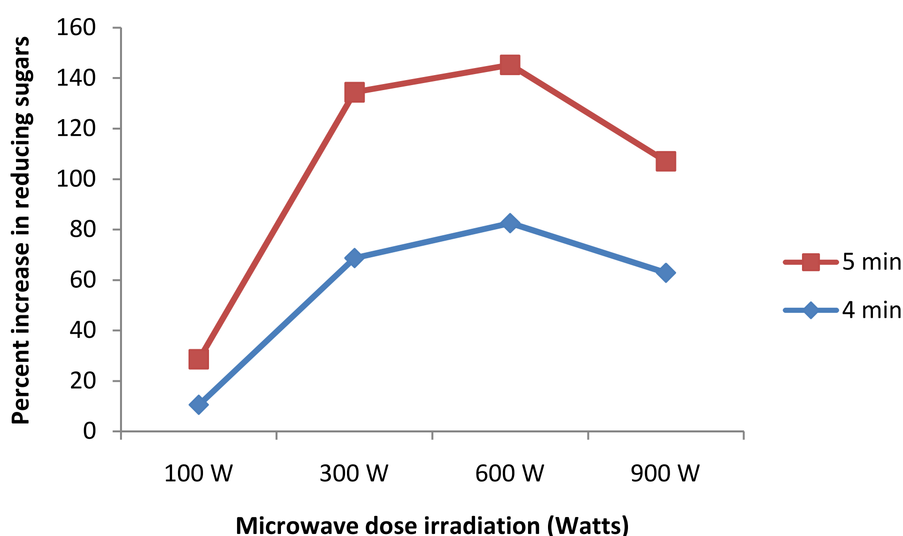

Fig1. Percent increase in reducing sugars in microwave pretreated biomass over untreated biomass

Optimization of incubation period

Reaction time had an important impact on hydrolysis and sugar formation. Table 2 shows the different time intervals employed for hydrolysis of biomass. At 72 h, the highest amount of sugars was produced as 20.31 mg/g in pretreated biomass as compared to 12.46 mg/g in untreated biomass. The reducing sugars of 8.17 mg/g and 16.54 mg/g were observed at incubation period of 24 h in untreated biomass and pretreated biomass respectively. A continuous trend of increase in reducing sugars was observed from 24 h to 72 h, afterwards reducing sugars started decreasing significantly both in untreated and pretreated biomass. The least amount of reducing sugars was observed at 120 h i.e. 4.27 and 16.35 mg/g at 96 h in untreated and pretreated biomass respectively. Maximum percent increase of 314.05 was observed in 120 h from pretreated biomass over untreated biomass (fig 2). 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 12 h 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 72 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 120 h may be due to irreversible adsorption of enzyme to substrate or due to feed back inhibition/denaturation of enzymes, resulting from the variation of pH and lesser cellular metabolism with ageing during enzymatic hydrolysis [17].

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Table 2. Optimization of incubation period for enzymatic hydrolysis of lignocellulosic biomass

Sr No.	Incubation time (h)	Reducing sugars (mg/g)		
		Untreated	Pretreated	Percent increase in reducing sugars
1.	24 h	8.17	16.54	102.44
2.	48 h	11.10	16.60	49.54
3.	72 h	12.46	20.31	63.00
4.	96 h	4.31	16.35	279.35
5.	120 h	4.27	17.68	314.05
C.D0.05		0.35	0.61	
S.E. (m)		0.10	0.21	

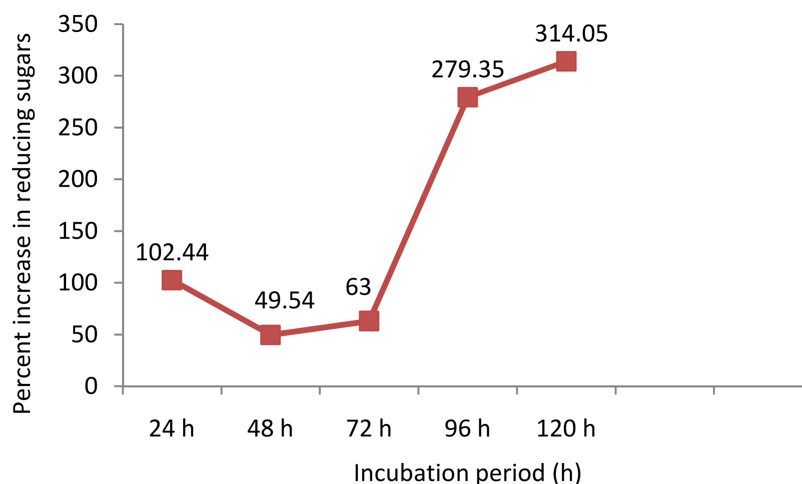

Fig2. Percent increase in reducing sugars after optimization of incubation period

Optimization of enzyme dose

Table 3 depicted the range of different enzymatic dosage (ml/g biomass) used for enzymatic hydrolysis of untreated and pretreated lignocellulosic biomass by inhouse enzymes. It also showed significant variation in the yield of reducing sugars. An increase in reducing sugars was observed with consistent increase in enzymatic dosage from 5.0 to 12.5 ml/g biomass and afterwards a significant decrease in its amount was observed. The pattern of reducing sugars increase and decrease was followed both in untreated and pretreated biomass. Maximum reducing sugar yield (mg/g) was observed at enzymatic dosage of 12.5 ml/g in untreated biomass and pretreated biomass as 19.06 and 20.56 mg/g respectively. The least amount of reducing sugars was observed at @ 2.5 ml/g i.e. 7.59 in untreated and 10.48 mg/g in pretreated biomass @ 15.0 ml/g dose. Maximum percent increase of 61.82 was achieved over untreated biomass at 7.5 ml/mg of enzyme dose (fig 3). The results showed that untreated biomass was poorly hydrolysed by the enzymatic cocktail compared to pretreated biomass. 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 [8]. High enzyme loading and prolonged hydrolysis provides complete cellulose conversion. An increase in the enzyme dosage beyond optimal level i.e. 15.0 ml/g did not improve sugar yield because of increase in rate of transglycosylation reactions and hydrodynamic instability thus counteracting the rate of hydrolysis [5].

Table 3. Optimization of enzyme doses for enhanced hydrolysis of lignocellulosic biomass

Sr No.	Enzyme dose (ml/g)	Reducing sugars (mg/g)		
		Untreated	Pretreated	Percent increase in reducing sugars
1.	5.0	7.59	10.53	38.73
2.	7.5	10.74	17.38	61.82
3.	10.0	14.90	18.12	21.61
4.	12.5	19.06	20.56	7.86
5.	15.0	9.48	10.48	10.54
C.D0.05		0.43	0.70	
S.E. (m)		0.09	0.28	

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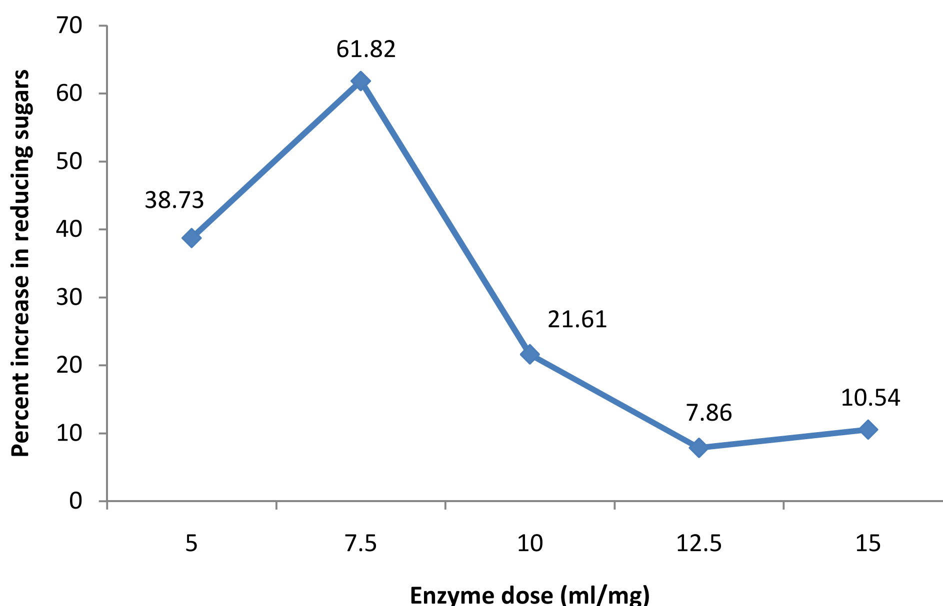

Fig 3. Percent increase in reducing sugars after optimization of enzyme dose

Optimization of enzyme ratio

The hydrolysis step is carried out by the cocktail of enzymes including cellulase and xylanase that target and degrade specific constituents of cell and release the monomeric sugars. The enzymatic ratios were designed on the basis of composition of lignocellulosic biomass which contains maximum amount of cellulose followed by hemicellulose and afterwards lignin. A series of experiments had been conducted to study the effect of different enzymatic ratios on hydrolysis of both untreated and pretreated biomass. Five different ratios were selected i.e. 6.25:6.25, 6.75:5.75, 7.25:5.25, 7.75:4.75, 8.25:4.25 and investigated for its effect on hydrolysis of biomass. Enzymatic cocktail of 7.75:4.75 revealed highest amount of reducing sugars i.e. 18.97 mg/g and 21.46 mg/g biomass in untreated and pretreated biomass respectively as shown in table 4. The enzymatic mixture of 7.75:4.75 was vital for extraction of maximum amount of monomeric sugars, as these enzymatic cocktail hydrolyzed maximum carbohydrates into monomeric sugars. Whereas maximum percent increase i.e. 19.97 was observed in 6.75:5.75 ratio over untreated biomass (fig 4). 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 6.75:5.75 was proven least effective for production of reducing sugars as up to 16.67 and 20.00 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.

Table 4. Optimization of enzyme ratio for enhanced hydrolysis of lignocellulosic biomass

Sr No.	Enzyme ratio (cellulase: xylanase)	Reducing sugars (mg/g)		
		Untreated	Pretreated	Percent increase in reducing sugars
1.	6.25:6.25	17.97	21.00	16.86
2.	6.75:5.75	16.67	20.00	19.97
3.	7.25:5.25	17.99	21.30	18.39
4.	7.75:4.75	18.97	21.46	13.12
5.	8.25:4.25	17.56	20.15	14.74
C.D0.05		0.40	0.64	
S.E. (m)		0.10	0.15	

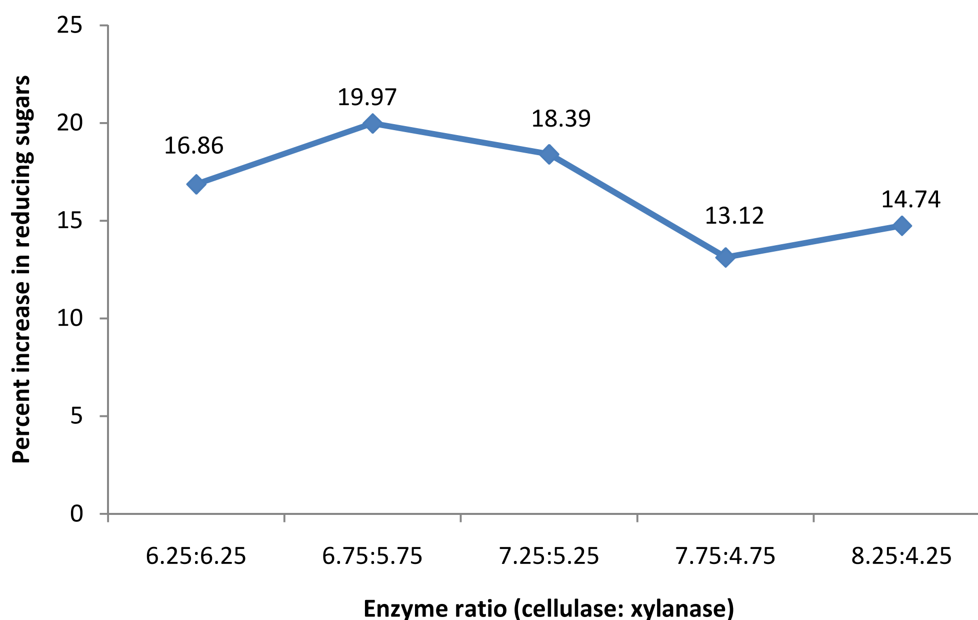

Fig 4. Percent increase in reducing sugars after optimization of enzyme ratio

Optimization of temperature

A regime of 35°C, 40°C, 45°C, 50°C and 55°C was employed for the optimization of hydrolysis temperature to produce maximum amount of reducing sugars. Data in Table 5 shows the increase and decrease in reducing sugars yield at different temperatures. The highest amount of reducing sugar of 15.86 and 22.35 mg/g biomass was observed in untreated and pretreated lignocellulosic biomass at temperature 45°C respectively. A slight decrease was observed in sugar at 55°C i.e. 9.75 and 19.85 mg/g in untreated and pretreated biomass within maximum percent increase of 103.79 (fig 5). A gradual increase was observed in reducing sugar yield with increase in temperature from 35 to 45°C beyond that, a decrease in reducing sugar was obtained in pretreated biomass. This could be attributed to performance of enzymes at a optimal temperature as they need an optimum temperature for proper catalytic function. Due to unavailability of sufficient energy required for action of enzymes on biomass, the process of hydrolysis was slow at 35°C. At higher temperature of 45°C, 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. 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[15].

Table 5. Optimization of temperature for enhanced hydrolysis of lignocellulosic biomass

Sr No.	Temperature (°C)	Reducing sugars (mg/g)		
		Untreated	Pretreated	Percent increase in reducing sugars
1.	35°C	18.57	20.00	7.70
2.	40°C	15.79	22.01	39.39
3.	45°C	15.86	22.35	40.92
4.	50°C	10.01	20.10	100.79
5.	55°C	9.74	19.85	103.79
C.D0.05		0.36	0.74	
S.E. (m)		0.11	0.25	

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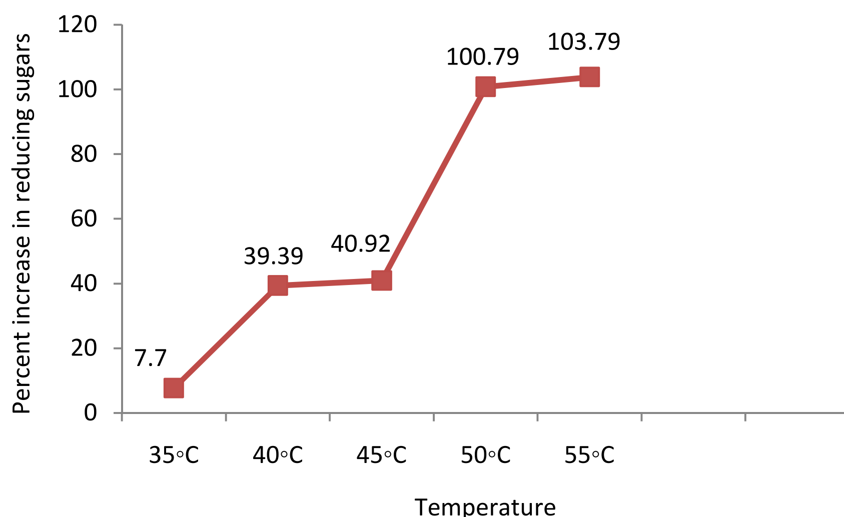

Fig 5. Percent increase in reducing sugars after optimization of temperature of enzymatic hydrolysis

After the optimization of process parameters viz. microwave dose, incubation period, enzyme dose, enzyme ratio and temperature a good appreciable increase was observed in reducing sugars with overall maximum of percent increase i.e. 272.50 from microwave pretreated biomass over the untreated biomass by optimizing process parameters by one factor at a time approach (fig 6).

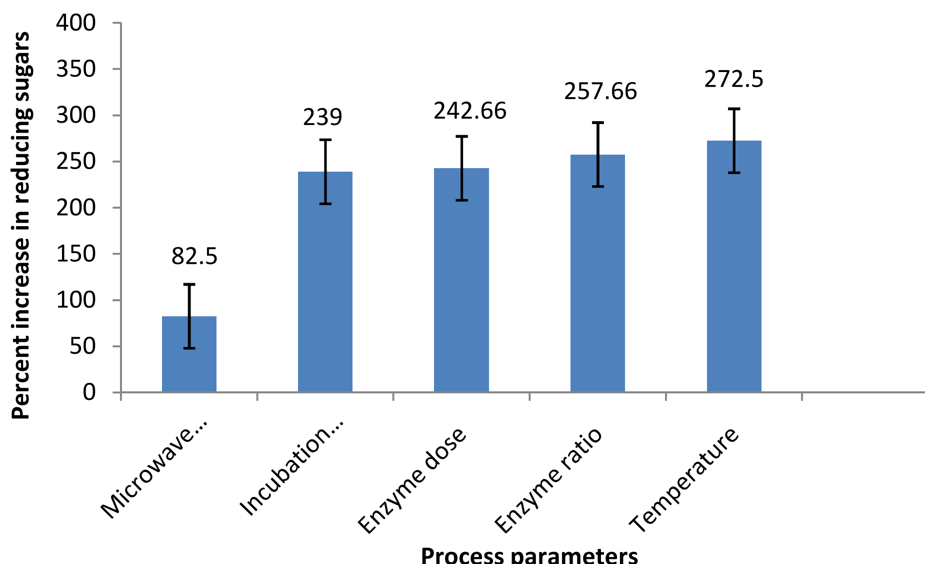

Fig 6. Overall percent increase in reducing sugars in pretreated biomass over untreated biomass after optimization of process parameters

CONCLUSION

In the present study, enzymatic saccharification of pine needle biomass was done to break down complex carbohydrates into simple sugars which will be further used for fermentation processes as a prerequisite step to produce bioethanol. A mixture of different inhouse enzymes (cellulase: xylanase) were produced under submerged fermentation and used for enzymatic hydrolysis of untreated and pretreated biomass. Different process parameters were optimized by using one factor at a time approach for enhanced hydrolysis of biomass and to get maximum of reducing sugars. The process parameters optimized for enhanced hydrolysis were microwave irradiation dose, incubation period, enzymatic dosage, enzymatic ratio and temperature. The best optimized conditions obtained were microwave dose i.e. 600 W for 4 min, incubation time of 72 h, enzymatic ratio 7.75: 4.75 (cellulase: xylanase) @ dose of 12.5 ml/g of biomass at temperature 45°C yielding 19.06 mg/g and 22.35 mg/g of reducing sugars in untreated and pretreated biomass respectively with maximum of percent increase of 272.50 for pretreated biomass over untreated biomass. The results obtained proved the effectiveness of enzymatic hydrolysis to

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enhance complex biomass break down into simple sugars for bioethanol production process from lignocellulosic biomass.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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