Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2012; 37 (1) ; 35–41.
doi: 10.5505/tjb.2012.43434

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Mart, 2012 © TurkJBiochem.com
[Published online 30 March, 2012]

Cellulase and xylanase production by co-culture of *Aspergillus niger* and *Fusarium oxysporum* utilizing forest waste

[Orman artıklarını kullanan *Aspergillus niger* ve *Fusarium oxysporum*'un birlikte kültüründe selülaz ve ksilanaz üretimi*]

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ABSTRACT

Aim: The purpose of present work was to utilize lignocellulosic forest waste for cost-effective production of commercially important enzymes i.e. cellulase and xylanase by employing co-culture of *Aspergillus niger* F_7 and *Fusarium oxysporum* F_8 in solid state fermentation (SSF).

Material and Methods: Fungal strains i.e. *A. niger* F_7 and *F. oxysporum* F_8 isolated from degrading forest litter were used as co-culture and their enzyme biosynthesis on forest waste was noticed. The prominent forest wastes i.e. *Toona ciliata, Celtris australis, Cedrus deodara* and *Pinus roxburghii* were procured from local forest and alkali (NaOH) pretreatment was given to these lignocellulosic substrates to simplify the crystalline cellulose present in them for higher yield of enzymes by fungal isolates. Two types of moistening agents i.e. tap water and modified basal salt medium (BSM) were used in the substrate in the ratio of 1:2 to evaluate their effect on enzyme production under SSF.

Results: The maximum enzyme titers i.e. 787.89 U/g of cellulase and 669.08 U/g of xylanase were observed in *C. australis* as compared to untreated biomass which was 450.02 and 398.03 U/g of cellulase and xylanase respectively when tap water was used as moistening agent. Biodegradation of forest biomass under SSF was increased many folds when moistening agent i.e. tap water had been replaced with modified basal salt media (BSM). In BSM mediated degradation of forest waste with co-culture of *A. niger* and *F. oxysporum*, extracellular enzyme activity was increased up to 2219.55 U/g of cellulase and 1012.0 U/g of xylanase in pretreated *C. australis* wood.

Conclusion: In untreated biomass enzyme production by fungi was quite low thus emphasizing the need of pretreatment for biodegradation of forest lignocellulosics. Significantly higher production of cellulase and xylanase using pretreated forest waste and modified BSM as moisture are main highlights of the present study which commercially may lead to cost effective production of these industrially important enzymes.

Key Words: *Aspergillus niger, Fusarium oxysporum*, cellulase, xylanase, lignocellulosic biomass, solid state fermentation

Conflict of interest: The authors have declared that no conflict of interest exists.

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ÖZET

Amaç: Çalışmanın amacı lignoselülozik orman artıkları kullanarak, ticari öneme sahip olan selülaz, ksilanaz gibi enzimlerin ekonomik olarak üretimidir. Bu amaçla *Aspergillus niger* F_7 and *Fusarium oxysporum* F_8 katı hal fermentasyonu kullanılarak birlikte kültüre edilmiştir.

Gereç ve yöntemler: *A. niger* F_7 and *F. oxysporum* F_8 fungal suşları bozulmakta olan orman örtüsünden izole edilerek birlikte kültürleri yapılmış ve enzim biyosentezi incelenmiştir. En yaygın orman atıkları *Toona ciliata, Celtris australis, Cedrus deodara* ve *Pinus roxburghii* vb. yerel ormandan elde edilmiş, fungal izolatlardan enzim veriminin arttırılması için lignoselülozik substratlar alkali (NaOH) ile ön muamele edilerek yapılarındaki kristal selüloz sadeleştirilmiştir. Nemlendirici ajan olarak, musluk suyu ve modifiye bazal tuz ortamı 1:2 oranında kullanılarak katı hal fermentasyonunda enzim üretimine olan etkileri değerlendirilmiştir.

Bulgular: Musluk suyu nemlendirici ajan olarak kullanıldığında en yüksek selülaz ve ksilanaz enzim titreleri *C. australis* için sırasıyla 787.89 U/g ve 669.08 U/g olarak bulunmuştur. Muamele edilmemiş biyokütle için ise 450.02 U/g selülaz ve 398.03 U/g ksilanaz değerleri elde edilmiştir. Katı hal fermentasyonu altında orman biyokütlesinin biyoparçalanımı nemlendirici ajan modifiye bazal tuz ortamı olduğunda artmaktadır. Ön muamelesi yapılmış *C. australis*'de, *A. niger* and *F. oxysporum* birlikte kültüre edildiğinde orman atıklarının modifiye bazal tuz ortamı aracılı bozunumunda ekstraselüler enzim aktivitelerinin selülaz için 2219.55 U/g, ksilanaz için 1012.0 U/g olduğu saptanmıştır.

Sonuç: Mantarlar tarafından üretilen enzim miktarının muamele edilmemiş biyokütlede oldukça az olması, lignoselülozun biyodegredasyonu için ön muamelenin gerekliliğini ortaya koymaktadır. Ön muamele edilmiş orman atıklarında ve nemlendirici olarak modifiye bazal tuz ortamı kullanıldığında selülaz ve ksilanazın fazla miktarda üretimi, endüstriyel önemi olan bu enzimlerin ekonomik bir şekilde eldesinin sağlanması bakımından önemlidir.

Anahtar Kelimeler: *Aspergillus niger, Fusarium oxysporum*, selülaz, ksilanaz, lignoselülozik biyokütle, katı hal fermentasyonu

Çıkar çatışması: Yazarlar hiçbir çıkar çatışması bulunmadığını beyan etmişlerdir.

Conflict of interest: The authors have declared that no conflict of interest exists.

***Translated by** [Çeviri] Özlem Dalmızrak

Registered: 13 September 2011; Accepted: 29 December 2011
[Kayıt Tarihi: 13 Eylül 2011; Kabul Tarihi: 29 Aralık 2011]

Introduction

In view of rising prices of crude oil due to increasing fuel demands, the need for alternative sources of bioenergy is expected to increase sharply in the coming years. Among potential alternative bioenergy resources, lignocellulosics have been identified as the prime source of biofuels and other value-added products. The primary components of lignocellulosic biomass, cellulose and hemicellulose are the most abundant organic compounds on earth and have the potential to be a renewable source for energy and chemicals. The estimated global annual production of biomass is 1×10^{11} tons, sequestering 2×10^{21} J [1].

Among lignocellulosics biomass, forest waste generated in nature is least explored for its bioconversion studies. Forest wood generally contains 42–50%cellulose, 25–30%hemicellulose, 20–25%lignin, and 5–8%extractives. This lignocellulosic pool is a major carbon sink in the forest ecosystems and accounts for roughly 20%of the terrestrial feed stock carbon storage, offering an enormous, renewable source of feedstock for biofuel production and so far there is no self-sufficient process or technology available to convert this lignocellulosic biomass for bioenergy generation [2].

The majority of glucose in lignocellulose is locked into highly crystalline cellulose polymers. Cellulose is an unbranched glucose polymer, composed of D-glucose units linked by 1, 4-β -D glycoside bond, which can be hydrolyzed by cellulolytic enzymes produced by both bacteria and fungi. Hemicelluloses consists of short, linear and highly branched chains of sugars including xylose, arabinose, rhamnose, galactose, mannose, sugar acids at high variable concentrations and lignin, a polymer of phenyl propane units linked primarily by ether bond and acts as a encrusting material. The conversion of lignocellulosic biomass typically involves a disruptive pretreatment process followed by cellulase and xylanase catalyzed hydrolysis of the cellulose and hemicelluloses to fermentable sugars [3]. Enzymatic methods of saccharification are the most preferred and use physical and chemical pretreatment processes of biomass [4]. The three types of enzymes of cellulase involved in the process of cellulose hydrolysis are endoglucanases, cellobiohydrolases, and β-glucosidases. According to standard endo- exo energy model, these enzymes cooperate in following manner: endoglucanases act randomly along the chain, producing new attack sites for cellobiohydrolases; cellobiohydrolases act as exo-enzymes, liberating cellobiose as their main product and β- glucosidases, which are not regarded as legitimate cellulases, play an important role in the process because it completes the process through hydrolysis of cellobiose and other short oligosaccharides to glucose [5]. Another most important enzyme xylanase is responsible for degradation of xylan which is a major constituent of hemicellulose fraction of biomass.

The present study is undertaken with an objective to produce cellulase and xylanase from a substrate which is inexpensive and is easily available by using hypercellulolytic and xylanolytic fungal isolates i.e. *A. niger* F_7 and *F. oxysporum* F_8 in co-culture system under SSF. The co-cultivation of microbes in fermentation has been suggested to increase the quantity of the desirable components of the cellulase complex while SSF of lignocellulosic biomass is preferred because of some of the advantages over submerged fermentation [6,7].

In the present study, saw dust of *Pinus roxburghii, Cedrus deodara, Toona ciliata* and *Celtris australis* were used as raw material for the enzyme production. In this context, the aim of the present work was to demonstrate the efficacy of SSF, employing co-culture of fungal isolates to degrade cellulose and hemicellulose rich forest waste as substrate in the production of balanced and low-cost celluloytic and xylanolytic enzyme systems that can further be used to efficiently hydrolyze lignocellulosic biomass into bioethanol and bioenergy.

Materials and methods

Collection of substrate

Four different forest species i.e. *T. ciliata, C. australis, C. deodara* and *P. roxburghii* were selected for biodegradation study depending upon their wide abundance and easy availability in local forests. The wood of these species was collected, dried in an oven and ground to mesh size of 1 mm.

Pretreatment

Lignocellulosic biomass (10 g) of each species was treated separately with 100 ml of 1%(w/v) sodium hydroxide solution for 2 h at room temperature [8], washed repeatedly with water, then dried in an oven and stored in air tight containers for the further use.

Proximate chemical composition analysis of the substrate

The chemical composition of different wood waste was analysed for holocellulose, lignin, and extractives. The wood waste was extracted with alcohol–benzene (1:2 v/v) to remove waxes, resins etc. The extractive-free wood dust was processed for chemical analysis following the Technical Association of Pulp and Paper Industry (TAPPI) protocols, (extractives- TAPPI Method T6m-59 [9]; holocellulose–TAPPI Method T9m-54 [10]; lignin–TAPPI Method T12m-59 [11]).

Fungal cultures and inoculum preparation

A. niger and *F. oxysporum*, previously isolated from the forest soil of Himalayas in northern parts of India (average height of 1275 to 2398 m) on potato dextrose medium. The isolates were identified on the basis of cultural characteristics, texture as well as microscopic structure, septate or non septate hyphae and conidias

[12]. The cultures were grown in petridish containing potato dextrose medium at 25°C for 7 days in order to get spores. These spores were scraped aseptically from the surface of agar plates and suspended in 10 ml of sterile distilled water (1×10^7 spores /ml) and this spore solution was used as inoculum.

Fermentation protocol

One ml of each spore suspension was transferred to each 5 Erlenmeyer flasks (500 ml) containing 5 g of *P. roxburghii, C. deodara, T. ciliata, C. australis* and mixed substrate (all four substrates were mixed in the ratio of 1:1:1:1) and 10 ml of moistening agent. Two types of moistening agents i.e. tap water and modified BSM (composition: 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_2Cl and separately sterilized solution of 1 M $MgSO_4$ (2 ml) and 1 M $CaCl_2$ (0.1 ml) was added after the medium was autoclaved. It was supplemented with 2%urea, 1%yeast extract, 0.1%peptone, 0.1%$NaNO_3$, 1M $CoCl_2$ (0.2 ml). Final volume was made 1000 ml) were used in the present study in fixed ratio of 1:2 i.e. substrate: moisture [13].

Enzyme extraction

After 10 days of incubation, 50 ml of phosphate buffer (0.1 M, pH 6.9) was added to the flasks and these were kept under shaking for 1 h. The flask contents were filtered using muslin cloth and the process was repeated twice. The filtrate was centrifuged at 2800 g for 15 min at 4°C and the supernatant was collected for further studies.

Analytical methods

Enzyme assays

Endoglucanase activity (carboxymethyl cellulase activity) and FPase activity (filter paper activity) in the culture supernatant were determined as described by Reese and Mandel [14]. β-glucosidase was assayed by the procedure of Berghem and Petterson [15]. Units (IU) of CMCase and FPase were defined as the μmol of glucose liberated per minute of culture filtrate. One unit of β-glucosidase was defined as the amount of enzyme liberating μmol of p-nitrophenol per minute. The cellulase activity was expressed in terms of complete enzyme activity i.e. CMCase + FPase + β-glucosidase.

Xylanase activity in the cultural filtrate was determined according to the method of Miller [16]. One unit of xylanase activity was defined as the μmol of xylose liberating per minute.

Estimation of reducing sugar and soluble protein

Reducing sugar was estimated as glucose by Miller method [16] and soluble protein was measured by Lowry's method [17].

Biodegradation index (BI)

$$BI = \frac{percentreducingsugarsreleased + percentproteinsformed}{2}$$

Biodegradation index is a standard term to measure biodegradation of lignocellulosic biomass. Percent reducing sugars released is the reducing sugars formed after biodegradation of lignocellulosic biomass. Percent protein formed is the microbial biomass produced by utilizing simple sugars in the supernatant after biodegradation [13].

Percent hydrolysis [18]

$$percenthydrolysis = \frac{totalreducingsugarsreleaed(g) \square 0.9}{weightofsubstrate(g)} \square 100$$

Results and Discussion

The fungal strains F_7 and F_8 were isolated from soil and selected among 11 strains of fungi, due to higher production of cellulase and xylanase for degradation of biomass. On PDA plates, F_7 fungus developed a long hyphae, septate spores bearing heads, globular and black while strain F_8 was purple in colour, feathery and macroconidium were sickle-shaped or oblong microconidia in chains. According to conidial morphology of these filamentous fungi, we identified isolate F_7 as *A. niger* and isolate F_8 as *F. oxysporum.*

Chemical component of different forest biomass

The composition of the untreated samples and pretreated substrate was analyzed and reported as dry weight of major components i.e. holocellulose, lignin and extractives. The holocellulose i.e. cellulose + hemicellulose content in hardwood i.e. *C. australis* (79.18) and *T. ciliata* (72.56) was high. Softwood contained less holocellulose i.e. *P. roxburghii* (67.24) and *C. deodara* (65.25) but more lignin content which probably makes its degradation comparatively difficult (Table 1). For pretreated substrates, a reduction in percentage of lignin components was observed along with the partial loss of holocellulose. In a similar study, significant loss of both cellulose and hemicellulose fractions with substantial delignification for maple wood pretreated with 1-ethyl-3-methylimidazolium acetate had been reported [19].

Biodegradation of forest waste

Biodegradation of biomass was carried out under SSF mode because it has some advantages over submerged fermentation. It could reduce the cost of downstream processing, utility of simple and cheap media for the fermentation, lower risk of contamination due to the ability of contaminants to grow in absence of free-flowing water, high concentration of the product and simple fermentation equipment as well as low effluent generation and low requirements for aeration and agitation during enzyme production [20]. SSF offers advantages over liquid cultivation especially for fungal cultures because the filaments of the fungi can penetrate deep into the substrate thus increasing surface area but in case of bacteria, it is restricted only on the outer surface of the substrate [21].

In the present study, various cheap and easily available lignocellulosics (*T. ciliata, C. australis, C. deodara* and *P. roxburghii*) have been used as carbon sources. The process of conversion of lignocellulosic biomass to sugars is complicated, so to enhance biodegradation of forest biomass, it was pretreated with alkali solution and SSF of pretreated and untreated biomass with *A. niger* F_7 was compared. When SSF was carried out with water as moistening agent, maximum enzyme activity was noticed in pretreated *C. australis* i.e. 787.89 U/g of cellulase and 669.08 U/g of xylanase as compared to untreated biomass which was 450.02 and 398.03 U/g of cellulase and xylanase respectively (Table 2). This enzyme activity was maximum when pretreated substrate was subjected to SSF using modified BSM with enzyme production reaching up to 2219.55 and 1012.95 U/g of cellulase and xylanase in *C. australis* (Table 3). Haq et al. [22] had investigated that co-culture of *A. niger* and *T. viride* gave 30-50 per cent higher production of enzymes, CMCase (2.79 U/ml/min), FPase (1.75 U/ml/min) and xylanase (189.7 U/ml/min). Ahamed and Vermette [23] investigated enhanced production of cellulase by co-culturing *Trichoderma reesei* and *Aspergillus niger* and exhibited a highly significant increase in the production of volumetric enzyme activity (98.4 U/l/h), FPase activity (7.1 U/ml) and CMCase (4.7 U/ml). Brijwani et al. [24] reported that both enzyme activities and volumetric productivities of enzyme produced were significantly higher in mixed-culture fermentation i.e. filter paper activity of 10.7 FPU/g and β-glucosidase of 10.7 U/g.

Besides enzyme activity, degradation of biomass brought about by fungal co-cultures was measured in terms of BI and percent hydrolysis where maximum BI of 11.08 and percent hydrolysis of 17.28 was reported in pretreated *C. australis* (Table 4, Figure 1).

Effect of moistening agent

The supplementation of tap water with nutrients in the form of modified BSM had resulted in an increase in the biodegradation of forest residue several folds. The impressive increase in biodegradation of forest lignocellulosics by SSF with modified BSM was due to the various supplements added to medium such as yeast extract, peptone and urea etc. in the form of nitrogen source which had a direct promotery role in growth of microorganisms and their enzyme production. Other elements like Na^{++} and Co^{++} in modified BSM probably helped in stimulization and stabilization of extracellular hydrolytic enzymes [25]. The salts had been noticed directly related to metabolism, stimulation or inhibiting enzyme production in microorganism [20].

Role of co-culture

The advantage of co-culture is more pronounced in SSF condition because the colonization of the substrate may be accomplished better in symbiotic association i.e. each species having its own niche for growth and substrate degradation. It is postulated that cellulases from different species are closely related to each other and that endoglucanase enzyme component from one fungal species can operate with exoglucanase of another atleast when both fungi have endoglucanase – exoglucanase system [26]. The explanation may be that the two enzymes in order to achieve a synergistic effect have to work together in the form of a base complex. The advantage of co-culture culture can also be due to the production of both cellulase and xylanase enzymes in the co-culture [27].

Effect of pretreatment versus lignocellulosic substrates

Significantly enhanced production of all the four enzymes (CMCase, FPase, β-glucosidase and xylanase) was observed with pretreated substrates compared to untreated one. When water was used as moistening agent, it was also found that *T. ciliata* showed highest per cent increase in enzyme activity i.e. 153.57 and lowest in case of *C. australis* i.e. 151.13 (Figure 2). The per cent increase in BI of pretreated lignocellulosic residue over untreated one has been recorded highest in *T. ciliata* (163.47%) (Fig. 3). These results emphasize the importance of pretreatment process for the higher production of cellulase. The pretreatments were found to change the chemical structure of lignocellulose by breaking the bonds, reduce lignocellulosic biomass crystallinity, render cellulose accessibility, and remove lignin, thus rendering the substrate more accessible for hydrolytic enzymes [28]. Ojumu et al. [29] has reported that the higher enzyme production may be due to high percentage of available cellulose after the pretreatment, which is the natural inducer for cellulase biosynthesis.

Overall, an interesting trend has been seen as far as biodegradation of hardwood (*C. austialis* and *T. ciliata)* and softwood (*P. roxburghii* and *C. deodara*) is concerned. Hardwood has shown better degradation as compared to the softwood. The probable reason for this behaviour may be due to more lignification of secondary walls in softwood fibers than that of hardwoods thus resulting in lesser increase in the required size range of pore volume of softwood biomass as compared to the hardwood biomass. Additionally, resins and dihydric alcohols present in the softwood also act as strong anti-microbial agents thus causing hindrance in the biodegradation process [30]. Higher glucan and xylan and lower lignin in hardwood leads to far better biodegradation as compared to softwood [31]. Among all the substrates *C. australis* was found to be the best substrate for enzyme production.

The isolates investigated in this study are fast growing as well as they have shorter fermentation period with enhanced enzyme production. Thus, we suggest these isolates as potential microbial sources for producing cellulase and xylanase under SSF. As the commercially available substrates i.e. cellulose and xylose are expensive. Therefore, in the present study, various

Table 1. Composition of different untreated and pretreated forest lignocellulosic biomass on dry matter basis

Lignocellulosic biomass	Untreated biomass			Pretreated biomass		
	Holocellulose (%)	Lignin (%)	Extractives (%)	Holocellulose (%)	Lignin (%)	Extractives (%)
T. ciliata	72.56	21.45	5.98	62.68	15.45	5.67
C. australis	79.18	12.65	5.68	69.38	5.40	4.50
C. deodara	65.25	28.35	6.10	57.78	22.00	5.87
P. roxburghii	67.24	26.40	6.20	58.54	19.2	5.80

Table 2. Activity of cellulase and xylanase by co-culture in forest biomass using water

Substrate	**Untreated biomass**			**Pretreated biomass**		
	Cellulase (U/g)	Xylanase (U/g)	Cellulase + xylanase (U/g)	Cellulase (U/g)	Xylanase (U/g)	cellulase + xylanase (U/g)
T. ciliata	199.52	171.79	371.30	538.81	402.67	941.50
C. australis	450.02	398.03	848.10	787.89	669.08	1457.00
C. deodara	111.11	57.53	168.60	224.03	124.65	348.70
P. roxburghii	122.36	72.97	195.30	258.77	172.71	431.50
Mixed substrate	189.32	157.12	346.40	510.83	359.04	869.90

Table 3. Activity of cellulase and xylanase by co-culture in forest biomass using modified BSM

Substrate	**Untreated biomass**			**Pretreated biomass**		
	Cellulase (U/g)	Xylanase (U/g)	Cellulase + xylanase (U/g)	Cellulase (U/g)	Xylanase (U/g)	Cellulase + xylanase (U/g)
*T. ciliat*a	317.62	200.56	518.20	1861.51	901.15	2763.00
C. australis	626.45	455.10	1082.00	2219.55	1012.95	3232.00
C. deodara	151.73	91.63	243.40	309.39	204.72	514.10
P. roxburghii	173.49	102.82	276.30	398.96	311.32	710.30
Mixed substrate	271.97	172.26	444.20	1349.31	798.79	2148.00

Table 4. BI of forest biomass by co-culture using water and modified BSM

Substrate	**Water**		**Modified BSM**	
	Untreated biomass	Pretreated biomass	Untreated biomass	Pretreated biomass
T. ciliata	1.67	4.40	6.12	9.02
C. australis	2.21	5.78	8.07	11.08
C. deodara	1.35	1.59	1.90	3.52
P. roxburghii	1.40	1.85	2.42	3.79
Mixed substrate	1.60	3.46	4.30	5.64

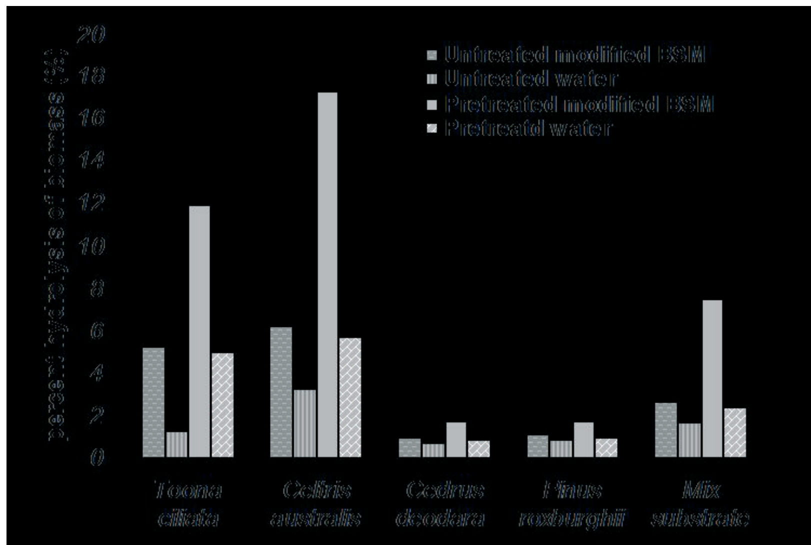

Figure 1. Percent hydrolysis of untreated and pretreated forest biomass under SSF by co-culture

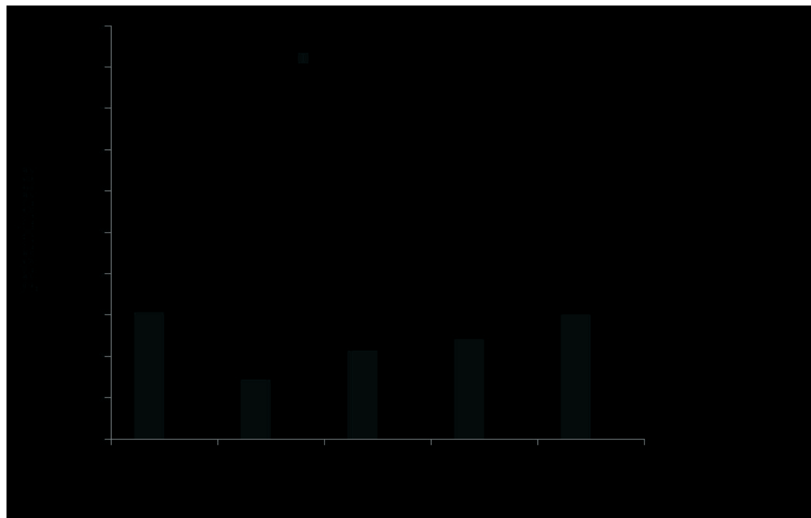

Figure 2. Percent increase in enzyme activity of pretreated biomass over untreated biomass under SSF

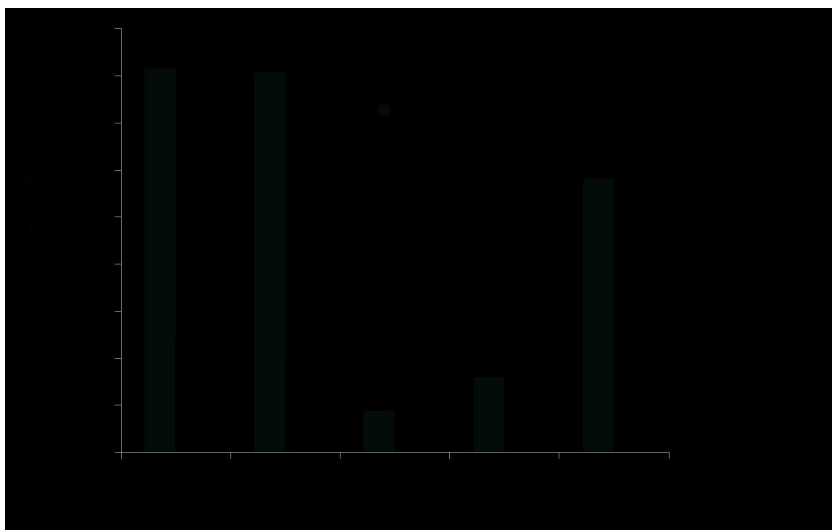

Figure 3. Percent increase in BI of pretreated biomass over untreated biomass under SSF

cheap and easily available lignocellulosics (*T. ciliata, C. australis, C. deodara* and *P. roxburghii*) have been used as carbon sources. The potential of using lignocellulosic biomass for energy production is limited because of the low rate of degradation of biomass thus laying the stress to improve the focus by using robust and more active hydrolytic enzymes. But the results indicate the suitability of using cheap and abundantly available forest biomass as solid substrate for large-scale production of cellulase and xylanase in an SSF system in order to reduce the high costs. This process therefore has a high potential for comprehensive utilization of renewable lignocellulosic resources. However, it is necessary to optimize the fermentation conditions in a fermentor to achieve the demands of large-scale production.

Acknowledgements

The financial support received from Department of Biotechnology, Govt. Of India, New Delhi, India is highly acknowledged to carry out this piece of work.

Conflict of interest: The authors have declared that no conflict of interest exists.

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