Screening and molecular identification of hypercellulase and xylanase-producing microorganisms for bioethanol production

Nivedita Sharma* and Nisha Sharma

Microbiology Laboratory, Department of Basic Sciences, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni–Solan 173 230, India

The present study projects the baseline work for bioconversion of pine needles to second generation biofuel, which deals mainly with screening, molecular identification and optimization of process parameters for cellulase and xylanase production. In total, 89 hydrolytic enzymes producing isolates were isolated from the soils and ten potential enzyme producers (seven for cellulase and three for xylanase) were subjected to secondary screening by inducing physical and chemical mutation. The wild and mutant strains of hypercellulase producers N_{12} and Kd_1 were identified as *Bacillus stratosphericus* N_{12} and *Bacillus altitudinis* Kd_1 using 16S rRNA technique. The fungal isolates RF1 and F2 were identified on the basis of 5.8 rRNA ITS technique and identified as *Rhizopus oryzae*, RF1 and *Rhizopus delemar*, F2 respectively. The mutant strains *B. stratosphericus* N_{12} (M) and *B. altitudinis* Kd_1 (M) are highly stable till 10 generations. Cellulase activity increased from 3.230 to 5.983 IU, i.e. 85.23% increase in cellulase activity was achieved. Xylanase production increased from 51.32 to 95.25 IU with 85.60% increase in production. Solid-state fermentation was also performed by potential fungal strains, i.e. *R. delemar* F2 and *R. oryzae* RF1 using pine needles as the substrate.

Keywords: Bioethanol, cellulase, solid-state fermentation, submerged fermentation, xylanase.

Introduction

THE second-generation biofuel, i.e. bioethanol has become the most promising alternative substitute for gasoline and lignocelluloses have great potential as a biomass source for bioethanol production. Lignocellulosic materials such as agricultural residues, forestry residues like pine needles and woody biomass are regarded as potential long-term alternative feedstocks for bioethanol production, since they are the most abundant reproducible resource on the earth[1]. Microorganisms play a vital role in the conversion of lignocellulosic wastes into valuable products like biofuel. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes[2]. Efficiency of hydrolytic enzyme is one of the determining factors for the saccharification of biomass into reducing sugars for bioethanol production. Thus employing efficient microbes that can produce both cellulase and xylanase may help overcome the current challenges in bioethanol production[3]. Biodiversity of microorganisms in tropical forests offers greater possibility for obtaining potential microbes for this purpose. To enhance the efficiency of a strain mutation is a promising step in which changes can be observed in the nucleotide sequence of the genetic material of an organism. For strain improvement ultraviolet (UV) radiation, ethidium bromide, N-methyl-N-nitro-N-nitrosoguanidine (NTG) and sodium azide were used as potential mutagenic agents[4]. Attention towards hydrolytic enzymes production has consistently increased over the years, due to their potential use in several industries[5]. These enzymes have various applications such as in the clarification of juices, wine industry, poultry diet, animal feed and biofuel production. Biotechnological conversion of pine needles is a potentially sustainable approach to develop novel bioprocesses and products. Pine needles are a renewable and abundant resource with great potential for bioconversion to value-added bioproducts. Microbial enzymes have become the focal biocatalysts due to their complex nature and widespread industrial applications. So significant attention has been devoted to the current knowledge of enzyme production and the challenges in research, especially in the direction of improving the production process economically of various industries. The present study was aimed to screen hypercellulolytic and xylanolytic microorganisms and generate efficient hyper enzyme producers as well as highly stable mutant strains for hydrolytic enzyme production. In our previous studies, a total of 89 microorganisms, including 84 bacteria and five fungi were isolated from soil samples collected from different sites of Himachal Pradesh, India[6]. Among them, ten hypercellulase and xylanase-producing bacteria were subjected to mutation for enhanced enzyme production and two highly stable mutant strains, i.e. N_{12} (M) and Kd_1 (M) were screened for enhanced cellulase and xylanase enzyme production;

*For correspondence. (e-mail: niveditashaarma@yahoo.co.in)

different process parameters were optimized to further increase their enzyme activity.

Materials and methods

Mutation of hypercellulolytic and xylanolytic bacteria for secondary screening

The previously isolated bacteria from soil were subjected to primary screening and ten potential isolates were subjected to mutation for secondary screening.

(i) UV irradiation (physical mutation)
- UV light of laminar airflow (Phillips TUV 30 W/ G30.T8), voltage: 30 W, distance: 15 cm and time interval: 15, 30, 45, 60 and 75 min.

(ii) Ethidium bromide (chemical mutation)
- Ethidium bromide (EtBr) concentration: 0.1–2.0 mg/ml.
- Time interval: 30, 60, 90 and 120 min.

Molecular identification of screened hypercellulase and xylanase producers

(i) 16S rRNA technique
- Wild and mutant strains of N_{12} (hypercellulase producer) and Kd_1 (hyperxylanase producer) were identified on the basis of 16S rRNA technique.

(ii) Clustal W2 analysis
- Translated nucleotide sequences were analysed for confirmation of mutation using website, i.e. www.ebi.ac.uk/services.

Submerged fermentation

Optimization of process parameters for cellulase and xylanase production by one-factor-at a time approach: Different process parameters were optimized for cellulase and xylanase production from mutant strains of *Bacillus stratosphericus* N_{12} (M) and *Bacillus altitudinis* Kd_1 (M) under submerged (SmF) fermentation.

Media optimization: Different media were used for enhanced cellulase and xylanase production.

Media used for cellulase production: PYC medium[7], basal salt medium[8], modified basal salt medium[9], Okoshi *et al.*[10] medium, Li and Gao[11] medium and Mandel and Reese medium[12].

Media used for xylanase production: Bacillus xylose salt medium, xylan medium[13], basal salt medium[8], TGY medium[14], Emmerson medium[14], X. Heck medium[15].

Temperature optimization: The temperature range 25°C, 30°C, 35°C, 40°C, 45°C and 50°C was used for the study.

pH optimization: Different pH values ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 to 9.0 were used for optimization.

Effect of different inoculum sizes: Inoculum size ranging from 1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0% to 17.5% was used for optimization.

Effect of different carbon sources: Different substrates (hexoses) ranging from cellulose, mannose, dextrose, lactose, maltose to starch were used for optimization.

Optimization of concentration of best carbon source: Different concentrations of best carbon source were studied for their optimum concentration.

Solid-state fermentation (SSF)

Cellulase and xylanase production by R. oryzae RF1 and R. delemar F2: (a) *Collection of biomass:* Pine needles were collected from the forests of Solan district and Shimla, Himachal Pradesh.

(b) *Production and extraction of enzymes:* To 5 g of each untreated and pretreated biomass, 10 ml of moistening agent, viz. Vogel's medium was added (in the ratio 1 : 6, i.e. substrate : moistening agent) in a 250 ml Erlenmeyer flask and autoclaved. Next, the flasks were inoculated with 2 ml *R. oryzae* RF1 and *R. delemar* F2 having 1×10^7 spores/ml and incubated at 50°C for 7 days in static phase and extraction of the enzymes was done by Bollag and Edelstein extraction method[16].

Results and discussion

Mutation of hypercellulase and hyperxylanase-producing isolates for secondary screening

In total, 84 bacterial isolates were obtained from soil samples in PYC (peptone yeast extract) medium having pH 6.8 and primarily screened on the basis of quantitative analysis of cellulase and xylanase under submerged fermentation. Seven bacterial isolates, i.e. N_{12} (1.476 IU), S_{12} (1.220 IU), N_5 (1.503 IU), S_7 (1.135 IU), N_{13} (2.138 IU), NS_1 (1.689 IU) and S_1 (1.639 IU) were selected for hypercellulase production on the basis of primary screening, whereas three bacterial isolates, i.e. Kd_1 (31.72 IU), SL_8 (61.54 IU) and N_{11} (29.64 IU) were selected for hyperxylanase production[6]. Two fungal isolates F2 and RF1 were also isolated from the soil and on the basis of their colour, texture and mycelia were tentatively identified as *Rhizopus* species.

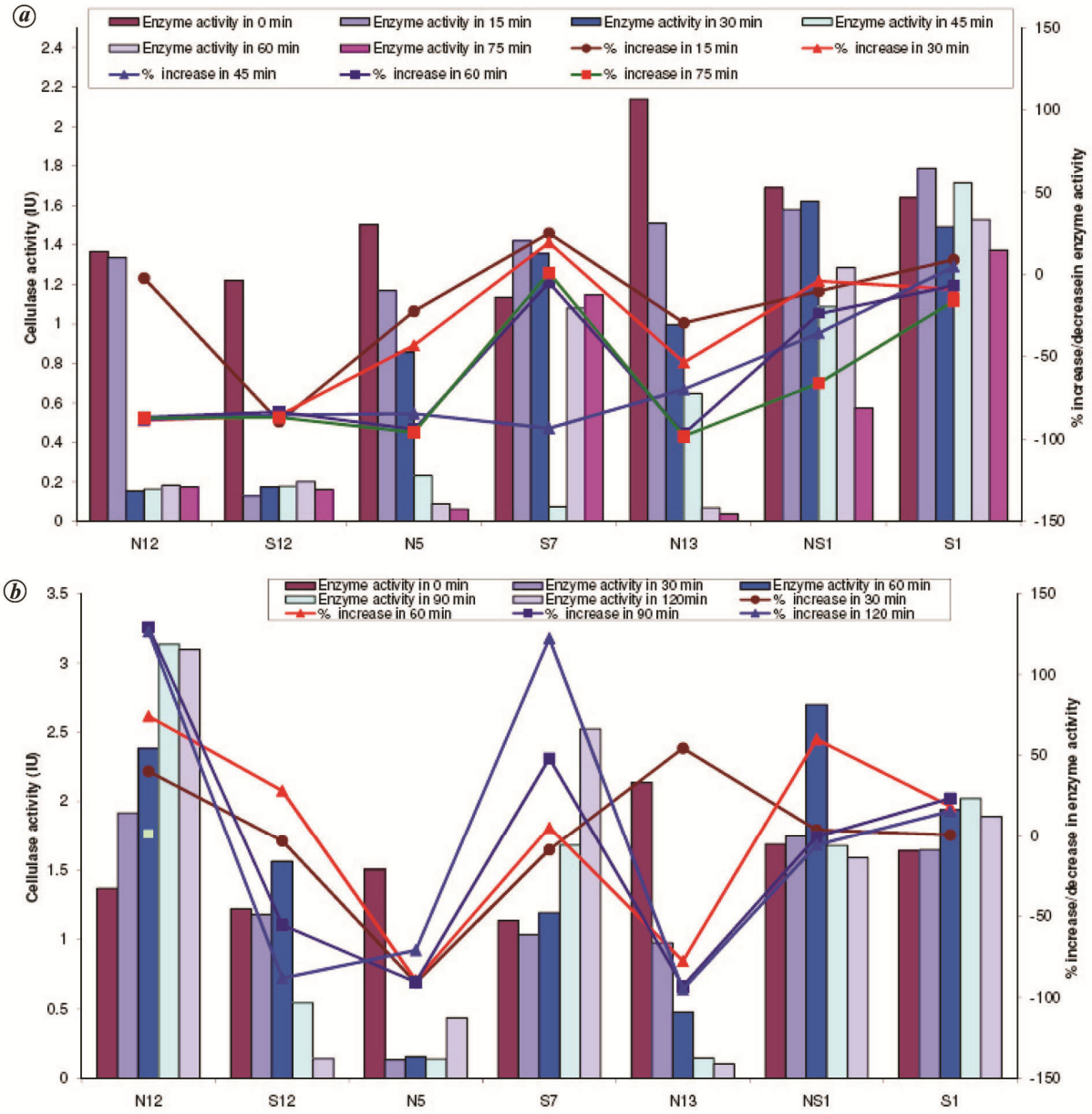

Figure 1. Overall comparison of per cent increase/decrease in cellulase activity of (***a***) UV-mutated and (***b***) EtBr-mutated strains over wild strains.

Secondary screening was done by subjecting these isolates to physical (UV radiation) and chemical mutation (EtBr). Cellulase and xylanase production was done by selected isolates at different UV doses, i.e. 0.00012 mJ/cm^2 for 15 min, 0.00024 mJ/cm^2 for 30 min, 0.00036 mJ/cm^2 for 45 min, 0.00048 mJ/cm^2 for 60 min and 0.00064 mJ/cm^2 for 75 min in PYC medium containing 0.5% cellulose for cellulase production and 0.5% xylan for xylanase production. Among different isolates N_{12} (M), S_{12} (M), N_5 (M), N_{13} (M) and NS_1 (M) showed a decrease, while S_7 (M) and S_1 (M) showed an increase in cellulase activity, i.e. from 1.135 to 1.421 IU and 1.639 to 1.787 IU respectively (Figure 1 *a*). In case of xylanase production from UV-treated isolates Kd_1 (M) and N_{11} (M) showed increase in xylanase activity ranging from 31.72 to 51.32 IU and 29.05 to 31.30 IU respectively, while isolate SL_8 (M) showed a decrease in xylanase activity (Figure 1 *b*). Based upon the results Kd_1 (M) UV-irradiated was selected for the optimization process. UV radiation have been considered important inducers for strain mutations. The pyrimidines are especially sensitive to modification by absorption of UV radiation which results in the production of thymine dimers that distort the DNA helix and block future replication[17]. In a similar study, Ghazi *et al.*[18] produced a mutant strain of *Bacillus mojavensis* PTCC1723 by inducing UV mutation which gave 3.45 times higher xylanase than the parent strain.

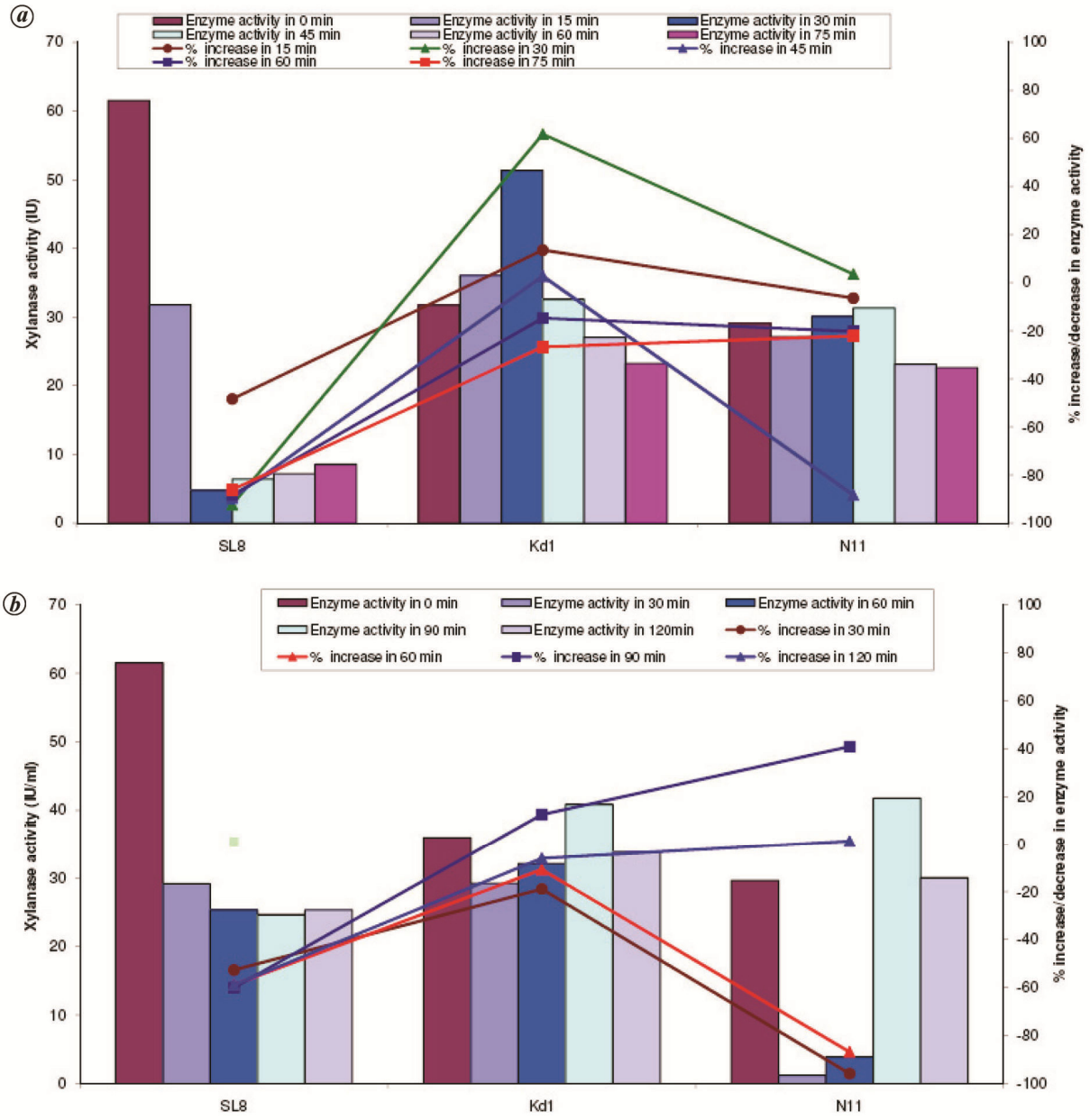

Figure 2. Overall comparison of per cent increase/decrease in xylanase activity of (***a***) UV-mutated and (***b***) EtBr-mutated strains over wild strains.

During EtBr mutation the isolate N_{12} (M) showed highest increase in cellulase activity with EtBr (90 min exposure), i.e. 1.367–3.136 IU (2.29-fold increase) and was selected for the optimization process (Figure 2 *a*) and mutant Kd_1 (M) produced 51.33 IU (1.61-fold increase) of xylanase and N_{11} (M) showed 31.30 IU (1.07-fold increase), while SL_8 (M) showed a decrease in xylanase activity upon mutation (Figure 2 *b*). Some of the isolates after mutation showed an escalation in enzyme units, while others declined compared to their respective wild strains. Figure 1 *a* and *b* shows the overall per cent increase/decrease in cellulase activity when bacterial isolates were subjected to UV and EtBr. N_{12} (M) showed 129.40%, S_{12} 28.03%, S_7 122.37%, NS_1 59.91% and S_1 23.24% increase in cellulase activity, while others showed a decrease with this treatment. On the other hand, in case of xylanase activity during mutation with EtBr, isolate Kd_1 (M) showed an increase of 13.50% and N11 40.89%, while SL_8 showed a decrease in xylanase activity (Figure 2).

In a study by Kuttanpillai *et al.*[19], *Thermomyces lanuginosus* MC 134 mutant showed a 1.5-fold increase in xylanase production on oat spelt xylan compared to wild strain. Finally, of Kd_1 (UV-mutated) for xylanase production and N_{12} (EtBr-mutated) for cellulase production were selected for further experiments on the basis of highest enzymes secreted by them.

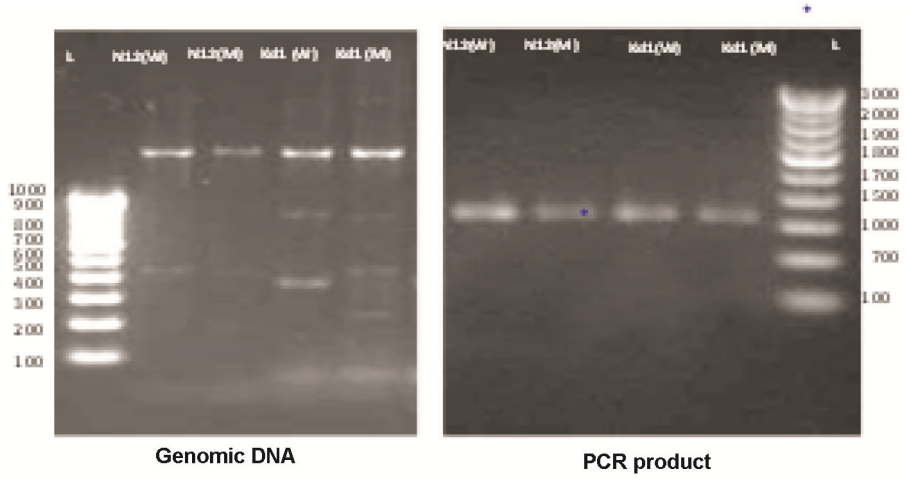

Figure 3. Molecular identification of bacterial isolates *Bacillus stratosphericus* N_{12} (W), N_{12} (M) and *Bacillus altitudinis* Kd_1 (W) and Kd_1 (M) using 16S rRNA gene technique.

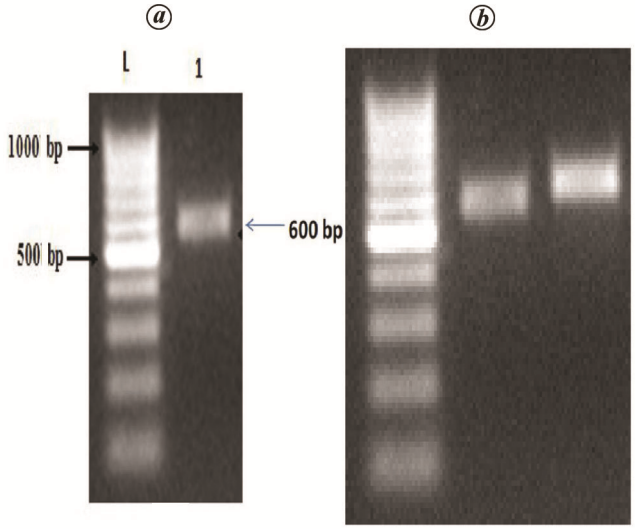

Figure 4. Molecular identification of screened fungal isolates (***a***) *Rhizopus oryzae* F2 and (***b***) *Rhizopus delemar* RF1 using ITS 5.8 rRNA gene technique.

Molecular identification of screened hypercellulolytic and xylanolytic microorganisms

Two bacterial strains with their respective mutants and two fungal strains were identified at genomic level using 16S rRNA gene and 5.8 rRNA ITS technique respectively. Genomic DNA of isolates was isolated using DNA purification kit (Banglore Genei). The sequences of these isolates have been registered under Genebank database with the following accession numbers: *Bacillus stratosphericus* N_{12} (W) [KC995116], *Bacillus stratosphericus* N_{12} (M) [KC995118], *Bacillus altitudinis* Kd_1 (W) [KC995115], *Bacillus altitudinis* Kd_1 (M) [KC995117], *Rhizopus oryzae* RF1 [KJ192199] and *Rhizopus delemar* F2 [KX5123312]. Figures 3 and 4 present phylogenetic trees of the bacterial and fungal strains with respect to other related species as inferred by neighbour joining method. The 16S rRNA gene is used for phylogenetic studies because it is highly conserved between different species of bacteria and archea, and always yields confirmed identification. The 16S rRNA gene analysis has been adapted globally as it is an authenticated approach to identify different isolates and characterize their morphological and phylogenetic position[20]. A phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 081003T should be assigned to the genus *Bacillus*, and was related most closely to the type strains of *Bacillus lehensis* (sequence similarity 99.6%), *Bacillus oshimensis* (99.4%) and *Bacillus patagoniensis* (96.6%); lower than 96.0% sequence similarity was observed with other *Bacillus* species[21].

Optimization of cellulase and xylanase by potential microorganisms under submerged fermentation

The data revealed that highest cellulase activity was found in the PYC (3.230 IU), while least enzyme production (2.211 IU) was observed in Okoshi *et al.*[10] medium. PYC medium containing carboxy-methyl cellulose (1.0%), sodium chloride (0.05%), yeast extract (0.5%), potassium hydrogen phosphate (0.3%), magnesium sulphate (0.02%) and peptone (0.5%) as growth supplements seems to have promoted extracellular cellulase production. Sodium chloride present in the medium probably helped maintain the osmotic balance of the medium while magnesium sulphate was a cofactor for a variety of metabolic reactions.

On the other hand strain *B. altitudinis* Kd_1 (M) showed maximum activity (56.83 IU) in TGY medium. This medium supported highest production of xylanase in case of *B. altitudinis* Kd_1 (M). Effect of glucose and tryptone

added in the TGY medium seem to induced high titres of xylanase. Glucose present in medium must have served as a ready utilizable carbon source leading to higher growth of *B. altitudinis* Kd_1 (M), consequently causing more synthesis of extracellular xylanase. Besides, tryptone used as nitrogen source, plays a significant role in promoting the extracellular release of enzymes. Thus medium formulation is a crucial step for designing successful laboratory experiments to enhance the yield of enzymes. It clearly reflects that medium components must satisfy the elemental requirement for cell biomass and enzyme production. A perusal of the data revealed that maximum production of cellulase was observed at pH 8.0, i.e. 3.370 IU, which was significantly higher than others statistically and minimum was observed at pH 5.0, i.e. 1.16 IU. The highest xylanase titres were observed at pH 5.5, i.e. 59.09 IU, followed by 58.72 IU at pH 6.5, which were also statistically significantly higher than others, and least xylanase titres were observed at pH 9.0, i.e. 25.48 IU. Thus *B. stratosphericus* N_{12} (M) has emerged as an alkalophile showing highest enzyme production at pH 8.0. In contrast, *B. altitudinis* Kd_1 (M) showed acidophilic nature by exhibiting maximum release of enzymes in the acidic range, i.e. 5.5. pH of the medium influences the growth of microorganisms and hence the enzyme production. Each microorganism possesses a specific pH range for its growth and activity. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism, thus lowering the overall metabolic activity[22].

Maximum cellulase titres were produced at 30°C by *B. stratosphericus* N_{12} (M), i.e. (3.789 IU), whereas least cellulase production was found at 45°C (1.082 IU). Similarly, *B. altitudinis* Kd_1 (M) also exhibited optimum temperature, i.e. 30°C (71.33 IU) for maximum xylanase production which is significantly higher than others. Temperature variation is a special feature because it can penetrate physical barrier and can have dramatic effects on the structure of macromolecules and also affect all the levels of biological adaptation[23]. Similar findings have been reported by Kumar *et al.*[24] showed increased enzyme activity of 62 U/ml at 30°C and found it to be the optimum temperature for β-glucosidase production.

Effect of inoculum size on cellulase and xylanase production was evaluated using different inoculum sizes, i.e. 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0% and 17.5% (V/V). The data revealed that optimum inoculum size was found to be 10.0% (V/V) for cellulase production showing 3.808 IU. Highest xylanase production from *B. altitudinis* Kd_1 (M), i.e. 73.44 IU was obtained at 12.5% of inoculum size having statistically significant differences over other inoculum sizes. The least cellulase and xylanase units were recorded at 15.0%. Enzyme activity was maximum at optimal level because at this point equilibrium is maintained between inoculum size and availability of substrates, while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks. Singh and Kaur[25] reported that maximum inoculum size of 10% could be used for cellulase production from *Bacillus* sp. JS14 under SSF.

Enzyme activity was measured at regular intervals up to periods of 24 to 120 h. Highest cellulase activity was measured at 48 h (4.259 IU) from *B. stratosphericus* N_{12} (M). Least enzyme production was observed at 24 h (2.34 IU) of fermentation. Statistically, enzyme production was found significantly higher at 48 h than other timings. In case of xylanase, the production was found minimum at 24 h of fermentation by *B. altitudinis* Kd_1 (M), i.e. 53.51 IU, while maximum xylanase production was noticed at 48 h, i.e. 74.45 IU. Shanmugapriya *et al.*[26] optimized the culture conditions for cellulase production by *Bacillus* sp. and observed maximum enzyme production at 48 h of incubation, pH 6.0 and temperature of 40°C. Nirmala and Sindhu[27] recorded maximum cellulase production from *Bacillus* strain at 72 h (5.0 IU/ml) of fermentation time. Shankar and Isaiarasu[28] reported maximum cellulase production at 72 h (0.5851 ± 0.006 IU/ml) from *Bacillus pumilus* EWBCM1 under varying cultural conditions.

The effect of different carbon source, i.e. cellulose, mannose, dextrose, lactose, maltose and starch for cellulase production as well as xylose, maltose, xylan, arabinose, ribose and dextrose for xylanase production were studied. Statistical analysis showed significant variation among all the substrates for enzyme titres. The cellulase units varied from 3.130 to 5.515 IU in *B. stratosphericus* N_{12} (M), whereas xylanase titre varied from 18.55 to 75.16 IU in *B. altitudinis* Kd_1 (M). Optimal level of cellulase was recorded when lactose was used as a carbon source, while maximum xylanase was produced using xylose as carbon source. The mechanism of lactose induction is considered to be due to the intracellular galactose-1-phosphate levels which are responsible for controlling the signalling of cellulase production[29]. Cellulase is an inducible enzyme which is induced in the presence of soluble saccharides. Lactose has been found as a suitable inducer of cellulase enzyme compared to other carbon sources, viz. glucose, sucrose, fructose and galactose[30]. Gupta and Kar[31] reported stimulation of xylanase production by xylose from thermophillic *Bacillus* sp. under submerged fermentation. Kapoor *et al.*[32] observed maximum xylanase production from *Bacillus pumilus* MK001 in a medium containing xylose as carbon source. There was significant variation among different substrate concentrations used for cellulase production ranging from 0.5% to 3.0% from hypercellulolytic *B. stratosphericus* N_{12} (M), and for xylanase production from hyperxylanolytic *B. altitudinis* Kd_1 (M). Optimum level of substrate concentration was found @ 2.0% for *B. stratosphericus*

Table 1. Hydrolytic enzymes (cellulase and xylanase) production under solid-state fermentation by *R. oryzae* RF1

Biomass	Total cellulase (IU)	Cellulase (U/g)	Protein (mg/g)	Specific activity	Xylanase (IU)	Xylanase (U/g)	Specific activity
Untreated	0.539	5.39	7.06	0.763	9.12	91.20	12.91
Microwave pretreated	0.913	9.13	7.75	1.178	21.90	219.00	28.25

Table 2. Hydrolytic enzymes (cellulase and xylanase) production under solid-state fermentation by *Rhizopus delemar* F2

Biomass	Total cellulase (IU)	Cellulase (U/g)	Protein (mg/g)	Specific activity	Xylanase (IU)	Xylanase (U/g)	Specific activity
Untreated	0.472	4.72	6.09	0.775	7.10	71.00	11.65
Microwave pretreated	0.801	8.01	7.06	1.134	14.99	149.90	21.10

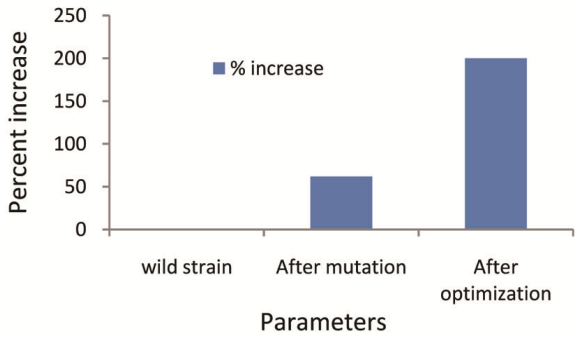

Figure 5. An overview of per cent increase in cellulase activity of *B. stratosphericus* N_{12} (M) after optimization of different parameters under SmF.

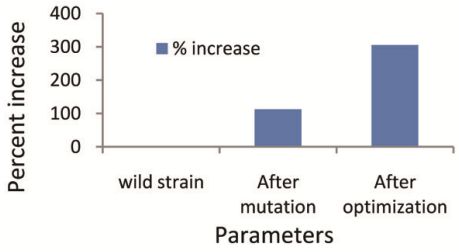

Figure 6. An overview of per cent increase in xylanase activity of wild *B. altitudinis* Kd_1 (M) after mutation and optimization of different parameters under SmF.

N_{12} (M) showing significantly higher cellulase activity of 5.983 IU, whereas least enzyme activity (3.358 IU) was recorded in 0.5% substrate concentration. The effect of substrate concentration on xylanase production from potential bacteria revealed that *B. altitudinis* Kd_1 (M) exhibited higher statistically significant xylanase activity at 2.5% substrate concentration, i.e. 95.25 IU. Minimum xylanase production, i.e. 52.60 IU was observed at 0.5% substrate concentration for this hyperxylanolytic bacteria. Figures 5 and 6 show overall per cent increase after mutation as well as optimization of different process parameters, i.e. media, pH, temperature, inoculum size, incubation time, carbon source and different concentrations of carbon source. Cellulase activity increased from 3.230 to 5.983 IU, i.e. 1.85-fold (Figure 5). With regard to per cent increase, 200.00 in cellulase activity and 305.35 increase in xylanase activity was achieved (Figure 6). Classical approach of one factor at a time (OFAT) used in the present enzyme optimization study has resulted in an impressive increase in the production of cellulase as well as xylanase, thus proving the direct utility of this technique in increasing enzyme titres.

Hydrolytic enzymes production under solid state fermentation

SSF involves the cultivation of microorganisms on a solid substrate in the near absence of free-flowing liquid. The characteristics of filamentous fungi, most notably hyphal growth, tolerance of low water activity, protein secretion and the ability to grow on a variety of low-value lignocellulosic materials provide a unique adaptability for fungi to SSF mode. In the present study, SSF was performed by *R. oryzae* and *R. delemar* using microwave pretreated pine needles biomass. Tables 1 and 2 depict cellulase and xylanase production under SSF, with maximum production using microwave pretreated biomass over untreated biomass. *R. oryzae* produced highest cellulase (9.13 U/g) and xylanase (21.90 U/g) using pretreated biomass, while *R. delemar* produced cellulase of 8.01 U/g and xylanase of 14.99 U/g. The moisture involved in the SSF process facilitates the growth and metabolic functions of the microorganisms that are typically retained within the growth matrix. The characteristics of filamentous fungi, most notably hyphal growth, tolerance of low water activity, protein secretion and the ability to grow on a variety of low-value lignocellulosic materials provide a unique adaptability for moulds to SSF mode.

Conclusion

The present study was carried out to isolate, screen and identify the most efficient cellulolytic and xylanolytic microorganisms from the soil. Mutation of hypercellulase and xylanase producers, enzyme production and optimization were performed with selected strains to recommend their use for industries. The bacterial isolates were screened for hypercellulase and xylanase production by inducing mutation with UV radiation/ethidium bromide with an apparent aim to enhance enzyme production. Two new mutant strains, i.e. N_{12} (M) and Kd_1 (M) were generated which were highly stable from ten generations and exhibited appreciable increase after mutation (1.476–3.136 IU in cellulase and 31.72–51.32 IU in xylanase production respectively). They were identified as *B. stratosphericus* N_{12} (M) and *B. altitudinis* Kd_1 (M). Optimization of different process parameters for cellulase and xylanase production for mutant strains, *B. stratosphericus* N_{12} (M) and *B. altitudinis* Kd_1 (M) using OFAT approach helped achieve 200.00% and 305.35% increase in their activities respectively. Basic and applied research on microbial cellulases and xylanases generated great scientific knowledge about their enormous industrial applications. To fulfill the requirement of enzymes on the industrial-level hyper enzyme producer strains are required. The present study provides a glimpse of the dynamics of cellulase and xylanase for their enhanced production from newly generated mutant strains. There hydrolytic enzymes can be further used for the conversion of pine needles to fermentable sugars, which is a feasible process and offers potential to reduce the use of fossil fuels as well as environmental pollution.

1. Keshwani, D. R., Modeling changes in biomass composition during microwave based alkali preteatment of switchgrass. *Biotechnol. Bioeng.*, 2010, **105**, 88–97.
2. Lynd, L. R., Weimer, P. J., van Zyl, W. H. and Pretorius, I. S., Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev*., 2002, **66**, 506–577.
3. Alam, M. Z., Manchur, M. A. and Anwar, M. N., Isolation, purification, characterization of cellulolytic enzymes produced by the isolate *Streptomyces omiyaensis*. *Pak. J. Biol. Sci*., 2004, **7**, 1647–1653.
4. Sharma, V. and Singh, P. K., Strain improvement of *Bacillus coagulans* and *Geobacillus stearothermophilus* for enhanced thermostable cellulase production and the effect of different metal ions on cellulase activity. *Int. J. Eng. Sci. Technol*., 2012, **4**, 4704–4709.
5. Immanuel, G., Dhanusha, R., Prema, P. and Palavesam, A., Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Int. J. Environ. Sci. Technol*., 2006, **3**, 25–34.
6. Sharma, N. and Sharma, N., Cellulase and xylanase productions from cellulolytic and xylanolytic microorganisms isolated from soils of Northern Himalayas. *World J. Pharm. Res*., 2016, **5**, 754–764.
7. Kim, J., Hur, S. and Hong, J., Purification and characterization of alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. *Biotechnol. Lett.*, 2005, **27**, 313–316.
8. Maniatis, T., Fritsch, E. F. and Sambrock, J., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory, New York, USA, 1982.
9. Sharma, N., Bansal, K. L. and Neopaney, B., Comparison of forest waste degradation under solid state fermentation and submerged fermentation by co-culture of *Bacillus coagulans* and *Bacillus licheniformis*. *Asian J. Microbiol. Biotechnol. Environ. Sci*., 2005, **7**, 51–54.
10. Okoshi, H., Ozaki, K., Shikate, S., Oshoni Kawai, S. and Ito, S., Purification and characterization of multiple carboxymethyl cellulases from *Bacillus* sp. KSM-522. *Agric. Biol. Chem.*, 1990, **54**, 83–89.
11. Li, X. and Gao, P., CMC-liquefying enzyme, a low molecular mass initial cellulose-decomposing cellulase responsible for fragmentation from *Streptomyces* sp. LX. *Appl. Microbiol*., 1997, **83**, 59–66.
12. Vyas, A., Vyas, D. and Vyas, K. M., Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49. *J. Sci. Ind. Res*., 2005, **64**, 281–286.
13. Annamalai, N., Thavasi, R., Jayalakshmi, S. and Balasubramanium, T., Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment. *Indian J. Biotechnol*., 2009, **8**, 291–297.
14. Garg, S., Ali, R. and Kumar, A., Production of alkaline xylanase by an alkalo-thermophilic bacteria, *Bacillus halodurans*, MTCC-9512 isolated from dung. *Curr. Trends Biotechnol. Pharm*., 2009, **3**, 90–96.
15. Heck, J. X., Hertz, P. F. and Ayub, M. A. Z., Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based solid state cultivation. *Braz. J. Microbiol*., 2002, **33**, 213–218.
16. Bollag, D. M. and Edelstein, S. J., *Protein Methods*, Wiley Liss, John Wiley, New York, USA, 1993.

17. Chavan, A., Chougale, D., Lakshmikantha, R. Y. and Satwadi, S. P. R, Mutational study of *Bacillus* species for production, purification and characterization of lipase. *Int. J. Pharm. Chem. Biol. Sci*., 2012, **2**, 545–551.
18. Ghazi, S., Sepahy, A.A., Azin, M., Khaje, K. and Khavarinejad, R., UV mutagenesis for the overproduction of xylanase from *Bacillus mojavensis* PTCC1723 and optimization of the production condition. *Iran. J. Basic Med. Sci.*, 2014, **17**, 844–853.
19. Kuttanpillai, S. K., Permaul, K. and Singh, S., Inducible character of β-xylanase in a hyperproducing mutant of *Thermomyces lanuginosus*. *Eng. Life Sci*., 2009, **9**, 298–230.
20. Fontana, C. M., Favaro, M., Pelliccioni, E. S. and Favalli, P. C., Use of the Microseq 16S rRNA gene-based sequencing for identification of bacterial isolates that commercial automated systems failed to identify correctly. *J. Clin. Microbiol.*, 2005, **43**, 615–619.
21. Chen, J., Zhang, L., Zhan, P., Wang, Y., Ai, B and Wang, G., Optimization of simultaneous saccharification and co-fermentation process for ethanol production from poplar wood. In International Conference Agricultural and Biosystems Engineering, Hong Kong, 2011, vol. 1, pp. 291–294.
22. Willey, J. M., Sherwood, L. M. and Woolverton, C. J., *Prescott, Harley and Kleins' Microbiology*, McGraw Hill, Boston, USA, 2008, 7th edn.
23. Hickey, D. A. and Singer, G. A., Genomic and proteomics adaptation to growth at high temperature. *Genome Biol*., 2004, **5**, 1–7.
24. Kumar, D. J. M., Sudha, M., Devika, S., Balakumaran, M. D., Kumar, M. R. and Kalaichelvan, P. T., Production and optimization of β-glucosidase by *Bacillus* sp. MPTK 121, isolated from dairy plant soil. *Ann. Biol. Res.*, 2012, **3**, 1712–1718.
25. Singh, J. and Kaur, P., Optimization of process parameters for cellulase production from *Bacillus* sp. JS14 in solid substrate fermentation using response surface methodology. *Braz. Arch. Biol. Technol*., 2012, **55**, 505–512.
26. Shanmugapriya, K., Saravana, P. S., Krishnapriya, Manoharan, M., Mythili, A. and Joseph, S., Isolation, screening and partial purification of cellulase from cellulase producing bacteria. *Int. J. Adv. Biotechnol. Res*., 2012, **3**, 509–514.
27. Nirmla, P. and Sindhu, A., Production of endoglucanase by optimizing the environmental conditions of *Bacillus circulans* on submerged fermentation. *Int. J. Appl. Eng. Res*., 2011, **2**, 472–481.
28. Shankar, T. and Isaiarasu, L., Cellulase production by *Bacillus pumilus* EWBCM1 under varying conditions. *Middle East J. Sci. Res*., 2011, **8**, 40–45.
29. Seiboth, B., Gamauf, C., Pail, M., Hartl, L. and Kubicek, C. P., The D-xylose reductase of *Hypocrea jecorina* is the major aldose reductase in pentose and D-galactose catabolism and necessary for beta-galactosidase and cellulase induction by lactose. *Mol. Microbiol.*, 2007, **66**, 890–900.
30. Sadhu, S., Saha, P., Sen, S. K., Mayilraj, S. and Maiti, T. K., Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. *Springer Plus*, 2013, **2**, 1–10.
31. Gupta, U. and Kar, R., Optimization and scale up of cellulase free endo-xylanase production by solid state fermentation on corn cob and by immobilized cells of a thermolerant bacterial isolates. *Jordan J. Biol. Sci*., 2008, **1**, 129–134.
32. Kapoor, M., Lavanya, M. N. and Kuhad, R. C., Cost effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. *Biochem. Eng. J.*, 2008, **38**, 88–97.

ACKNOWLEDGEMENTS. We thank the National Mission on Himalayan Studies, Ministry of Environment, Forest and Climate Change, Government of India and G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Almora for financial support.

doi: 10.18520/cs/v120/i5/841-849