Indian Journal of Experimental Biology
Vol. 58, August 2020, pp. 563-570

Production of xylanase under submerged fermentation from *Bacillus firmus* HS11 isolated from Sikkim Himalayan region

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Received 30 January 2020; *revised 11 March 2020*

Xylanase producing microorganisms isolated from high altitude soil show activity at broad window of temperature, and can have variety of industrial applications. Here, we explored production of xylanase under submerged fermentation from *Bacillus firmus* HS11 isolated from Sikkim Himalayan region. We isolated 472 bacterial strains high altitude soil samples (1120-4272 m) from Sikkim and screened for xylanase production. Among them five strains that showed higher xylanase activity were identified by 16s rRNA gene sequence analysis. Comparatively, *Bacillus firmus* HS11 showed highest activity at 10, 37 and 50°C, and was chosen for optimization experiment. *B. firmus* HS11 showed xylanase activity of 3.35 U/mg of protein at 37°C and retained 32.43% activity at 10°C and had 79.82% higher activity at 50°C. Optimization of nutrient composition for submerged fermentation of *B. firmus* HS11 was carried out by combination of Plackett-Burman design and response surface methodology. The optimized media component for xylanase production by submerged fermentation by *B. firmus* HS11 is beechwood xylan 0.8% (w/v), $MgSO_4$ 0.04% (w/v), $CaCl_2$0.04% (w/v), peptone 0.1% (w/v), NaCl 0.3% (w/v) and yeast extracts 0.01% (w/v), resulting in enhancement of xylanase production by 7.4 folds. Xylanase having activity at broad range of temperature including lower temperature can have application in food industry.

Keywords: Beechwood xylan, Box-Behnken design, Food industry, High altitude soil, Northeast India, Optimization, Plackett-Burman design, RSM

Sikkim, one of the north-eastern states of India, has varied range of elevation ranging from 300 to 8586 meters with most of the areas least explored for microbial diversity and bioprospection[1,2]. Microbial diversity of high altitude soil of this region is least explored for production of carbohydrate active enzyme except for few reports on amylase producing bacteria from fermented foods[3,4]. Higher altitude is an important factor which defines microbial population and their metabolism, which can result in production of enzymes and metabolites of industrial importance[4]. Exploration of microorganisms from high altitude soil samples of Sikkim Himalayan region can result in production of enzymes with specific biochemical properties and catalytic ability, which can find application in food and industrial bioprocesses[2,4,5].

In bioprocessing industries, application of biocatalyst is hindered due to limitation of enzymes to act at particular temperature. Enzymes having ability to act at low temperature can avoid the undesirable chemical reactions that may happen at high temperature[5]. Application of cold active enzymes will also help in preserving the heat liable compounds in the reaction mixture[5,6]. Xylanase are major enzymes hydrolysing hemicellulose by cleaving glycosidic bonds to potent simple sugars with immense utility[7]. Xylanase are known for their broad range of applications especially in food industry and also in other biotechnological sectors, such as pulp and paper production and feed manufacture[5,8,9]. Due to increase in requirement of xylanase in feed, food, paper and pulp industry, the estimated market share of this enzyme is slated to achieve 500 million dollars by 2023[9]. Screening of microorganisms from high altitude Sikkim Himalayan region may result in identification of strain for production of xylanase with activity at wide range of temperatures. Though Sikkim lies in an ecological hotspot[8], the microbial diversity with respect to enzyme production has been poorly studied, and thus reports on carbohydrate active enzymes are rare.

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Nutrient compositions of medium for submerged fermentation can affect enzyme production for different microorganisms even at species and strain level[10,11]. Therefore, it is necessary to optimize media composition for specific strain applied for enzyme production. Xylanase has been produced by different bacteria using both submerged and solid state fermentation[11]. Statistical methods such as Plackett-Burman design and response surface methodology has been used for optimization of condition for enzyme production has resulted in higher activity in comparison to conventional methods analyzing one factor at a time[10,12]. Response surface methodology (RSM) has advantage over conventional method that it can analyze the interaction between two independent factors responsible for higher production of enzymes. RSM gives collective result of all the independent variables on their interaction during fermentation process[10]. In the present study, we explored new bacterial strains from high altitude region of Sikkim Himalayan region for production of xylanase and optimisation of conditions using statistical approach including Plackett-Burman design and RSM for enhancing xylanase production.

Materials and Methods

Chemicals and sample collection

All the media and chemicals used in the study were of analytical grade and commercially available in India. Soil samples (20) were collected from different districts of Sikkim (East Sikkim, West Sikkim, North Sikkim and South Sikkim) with altitude ranging from 1120-4272 m above sea level. Soil samples were collected randomly 10-15 cm beneath the surface using sterile spatula and were packed in sterile poly bags. Isolation of bacterial strains was carried out using standard protocol of serial dilution in saline (0.85% NaCl) and spread plating in Nutrient agar (NA) medium (Hi-Media, India). The plates were incubated at 30°C for 48 h and morphologically distinct colonies were purified and stored in glycerol stock (25% v/v) at –80°C till further analysis.

Plate screening for potential xylanase producing isolates

Screening for xylanase producing microorganisms was carried out using beechwood xylan by plate based method[13]. The isolates were inoculated on media supplemented with beechwood xylan (2 g/L), peptone (5 g/L), yeast extract (1.0 g/L), NaCl (5 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.2 g/L), Agar (20 g/L) and congo red (0.2 g/L) at pH 7. The inoculated plates were incubated for primary screening at two different temperatures (30 and 37°C) for 48 h. After incubation, positive isolates were selected based on the ability of the isolates to produce a clear halo zone as a result of hydrolysis of beechwood xylan to xylose around the bacterial colony. Isolates showing positive results for xylanase activity on plate assay were taken for spectrophotometric analysis.

Xylanase assay

Selected xylanase producing isolates were assessed for production of xylanase under submerged conditions. Inoculum for production medium was prepared by growing the selected isolates on nutrient broth for 12 h at 30°C. Xylanase production under submerged condition was carried out by inoculating 1% (v/v) inoculum in production medium. Xylanase production was assessed by growing selected isolates in medium supplemented with beechwood xylan (5 g/L), peptone (5 g/L), yeast extract (1.0 g/L), NaCl (5 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.2 g/L) and the pH of the medium was maintained at 7. Assay for xylanase activity was carried out using 1% beechwood xylan as substrate dissolved in 100 mM Sodium phosphate buffer (pH 7) following 3,5-dinitrosalicyclic acid (DNS) method for determination of reducing sugar[14]. The reaction mixture containing 0.5 mL of enzyme extract, 0.5 mL of 1% beechwood xylan was incubated at 37°C for 30 min and the reaction was stop by adding 3 mL of DNS reagent followed by boiling for 5 min. The absorbance was checked at 540 nm using spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The net xylose content after the reaction was calculated using xylose standard curve prepared following DNS method[14]. The total protein content of the supernatant was estimated following Lowry's method for total protein estimation[15]. One unit of xylanase activity was defined as amount of enzyme which liberated 1.0 µg of xylose per minute from xylan.

Effect of temperature variation on xylanase activity

Selected five isolates were analyzed for xylanase activity at three different temperatures to select the isolate having xylanase activity at wide range of temperatures. The crude enzyme was incubated with equal volume of 1% (w/v) beechwood xylan at pH 7 and incubated at different temperatures (10, 37 and 50°C). Xylanase activity was measured spectrophotometrically and calculated as described earlier[14]. The isolate showing better activity at all the temperatures was selected for optimization experiment.

Molecular identification of xylanase producing isolates

Isolation of genomic DNA of five xylanase producing isolates was done from 18 h grown cultures on Nutrient broth using DNA isolation kit (Promega, USA). Amplification was done using 16s rDNA universal primers, forward primer 8F 5′AGAGTTTGATCCTGG CTCAG-3′ and reverse primer 1541R 5′AAGGAGGTG ATCCAGCCGCA-3′. PCR condition for amplification was followed as methodology described by Rai *et al*.[3]. The PCR conditions were as following: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1.0 min, annealing at 65°C for 1.0 min, elongation at 72°C for 30 s and final elongation at 72°C for 7 min in thermal cycler (C1000 TouchTM Thermcycler, Biorad, Singapore). The PCR products were separated in agarose gel (1.5% w/v) and DNA bands were visualized in gel imaging system (ChemiDoc^{TM}XRS+, Biorad, USA). The PCR products were purified using PCR purification kit (Qiagen, Germany) and sequenced following Sanger sequencing at Eurofins Scientific (Bangalore, India). The 16S rDNA sequences obtained were compared and a phylogenetic tree for all the five isolates following maximum likelihood method was prepared using MEGA X software[16].

Statistical optimization for enhanced xylanase production

Plackett-Burman

Six independent parameters such as xylan, peptone, yeast extract, NaCl, $MgSO_4$ and $CaCl_2$ with fix inoculum size of 1% (v/v) 12 h grown pre-inoculum were selected for Plackett-Burman design to screen out important parameters that influence xylanase production [17]. An experimental design consisting of 12 run orders was designed with each parameters represented at two concentrations, high and low denoted as (+1) and (−1) levels respectively (Table 1). Effect of individual parameters was determined by applying the equation:

$$E = \Sigma(Mi(+1) - \Sigma Mi(-1))/N$$

where E is the effect of parameters estimated, ΣMi (+1) and ΣMi (−1) are the response of individual parameter with high and low concentrations and N is the total number of runs. All the parameters for xylanase production using *B. firmus* HS11 were carried out at 50 mL reaction mixture.

Response surface methodology (RSM)

Further optimization of significant parameters identified from Plackett-Burman design responsible for production of xylanase was optimized through RSM[12]. Box-Behnken design was selected for optimization of three significant parameters, peptone, yeast extract and NaCl for xylanase production. Peptone and yeast extract content were taken as 0.1, 0.3 and 0.5% (w/v) and 0.01, 0.055 and 0.1% (w/v), respectively. Similarly, NaCl contents were taken as 0.1, 0.2, and 0.3% (w/v) of the total media. An experimental design of 15 run orders with three levels was generated and analysed using Minitab software version 15.1.10. All the parameters for xylanase production using *B. firmus* HS11 were optimized using 50 mL reaction mixture. The combination and levels of individual independent variables for Box-Behnken design is given in Table 2.

Results and Discussion

Microbial xylanase from unique habitat have attracted researchers across the globe for their

Table 1 — Experimental independent variables and their response at two levels used for the production of xylanase by *Bacillus fimrus* strain HS11 using Plackett-Burman design

Run Order	Xylan	Peptone	NaCl	Yeast Extract	$MgSO_4$	$CaCl_2$	Xylanase activity
1	8	8	2	3	0.4	0.1	4.02
2	8	2	2	0.5	0.4	0.4	31.71
3	2	2	2	3	0.4	0.4	22.38
4	2	2	2	0.5	0.1	0.1	24.60
5	2	8	8	0.5	0.4	0.1	5.18
6	2	8	8	3	0.1	0.4	5.63
7	8	2	8	3	0.1	0.4	16.10
8	8	8	8	0.5	0.4	0.4	8.68
9	2	8	2	0.5	0.1	0.4	3.57
10	8	8	2	3	0.1	0.1	5.49
11	8	2	8	0.5	0.1	0.1	17.16
12	2	2	8	3	0.4	0.1	9.84

Concentration of individual parameters is represented in g/L and xylanase activity is represented as U/mg of protein/min

Table 2 — Box-Behnken experimental design and xylanase activity (U/mg of protein/min) of *Bacillus fimrus* HS11

Run Order	Peptone	NaCl	Yeast Extract	Xylanase activity
1	3	3	1	5.79
2	5	1	0.55	10.19
3	3	3	0.1	8.32
4	3	1	0.1	7.15
5	1	2	1	17.71
6	5	3	0.55	5.45
7	3	2	0.55	4.85
8	3	1	1	6.31
9	1	3	0.55	24.74
10	5	2	1	6.04
11	5	2	0.1	8.13
12	3	2	0.55	8.84
13	3	2	0.55	7.81
14	1	2	0.1	19.72
15	1	1	0.55	10.85

Concentration of individual parameters is represented in g/L and xylanase activity is represented as U/mg of protein/min

biotechnological application in food, feed, paper and pulp industries[5,18]. Soil samples were collected from different places with altitudes ranging from 1120-4272 m covering different districts of Sikkim. A total of 472 bacterial strains were isolated from 20 soil samples collected from different parts of Sikkim. Among 472 isolates, it was observed that 171 isolates showed xylanase activity in plate based screening by producing halo clear zone around the colony on congo-red stained beechwood xylan plates. Based on zone diameter of isolates at different temperature, a total of 64 isolates were selected for quantitative analysis of xylanase activity. Among 64 isolates, five isolates that showed higher activity was selected for molecular identification and further studies. The sequences obtained after sequencing of selected five xylanase producing isolates were compared with other 16S rDNA sequences in BLASTn genebank, aligned with multiple sequence alignment (MUSCLE) and phylogenetic tree of all the isolates was drawn using MEGA X software (Fig. 1). The five potential isolates identified based on 16Sr DNA sequence analyses are *Bacillus firmus* HS11, *Bacillus mycoides* HS1, *Pseudomonous helmanticensis* HS2, *Bacillus mobilis* HS10 and *Bacillus subtilis* HS12. The sequences of selected five isolates based on xylanase activity were submitted to NCBI GenBank. The accession numbers obtained were MN857708 (*B. mycoides* HS1), MN857709 (*P. helmanticensis* HS2), MN857717 (*B. mobilis* HS10), MN857718 (*B. firmus* HS11) and MN857718 (*B. subtilis* HS12). *Bacillus* spp. and *Pseudomonous* spp. have been studied for production of enzymes with mesophilic and thermophilic nature with specific catalytic activities[19]. Novel strains of same species from unique environments can have interesting catalytic properties, which can have industrial importance.

Quantitative enzyme activity of selected five isolates was performed at three different temperatures to select isolate having activity at broad range of

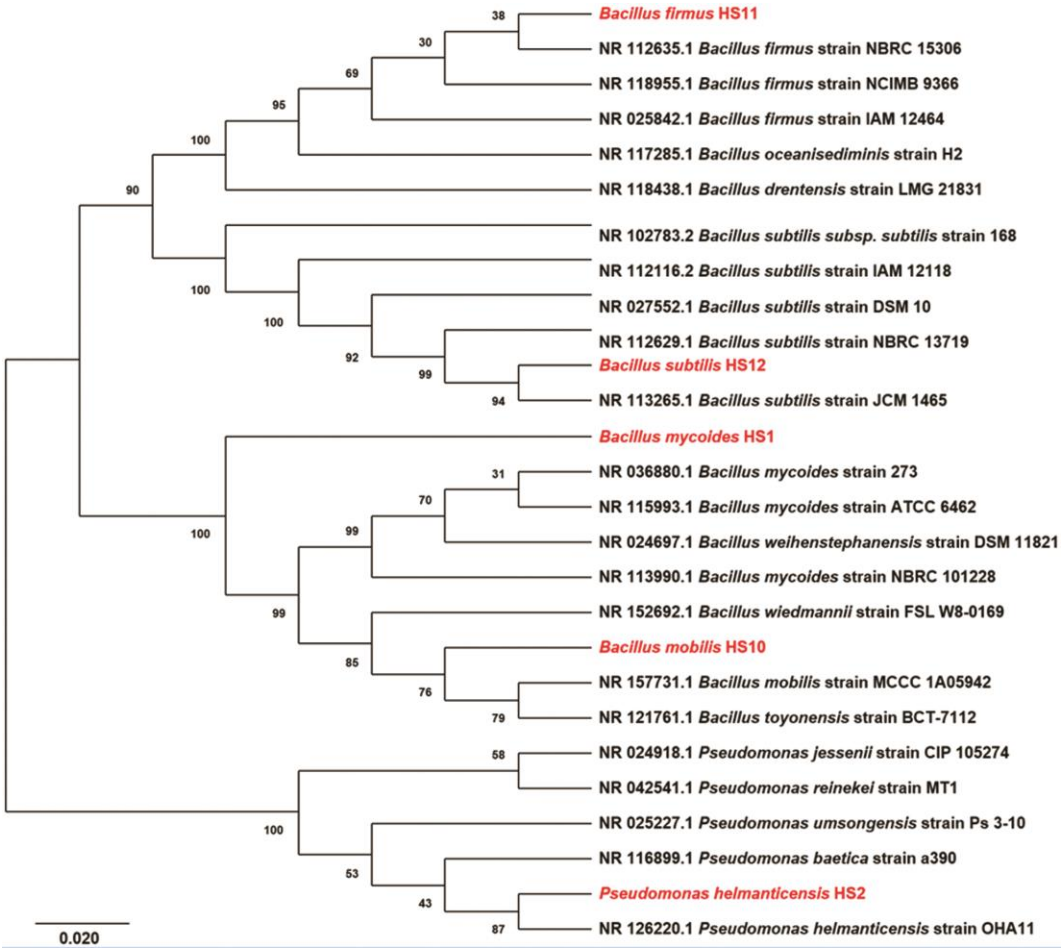

Fig. 1 — Phylogenetic tree showing the evolutionary relationships among xylanase producing bacteria isolated from high altitude soil of Sikkim Himalayan region.

temperatures (Fig. 2). The temperature range was selected from 10-50°C to select xylanase active at low temperature as well as thermophilic conditions for having wide range of applications in bioprocessing industries. Most of the studies have reported xylanase activity between 40-60°C and few reports are available on xylanase active at low temperature[5,18]. Xylanase active at low temperature has applications in food industry as the enzyme has shown to improve dough properties and enhancement of bread volume[6,5]. Xylanase activity at lower temperature can also find application in saline food processing industries[20]. Xylanase activity of the selected five isolates ranged from 1.07 to 2.26, 1.34 to 3.35 and 1.8 to 6.02 µg of xylose released/mg of protein/min at 10, 37 and 50°C, respectively. Among the selected isolates, *B. firmus* HS11 showed highest activity 37°C and retained 32.43% activity at 10°C and showed 79.82 % higher activity at 50°C (Fig. 2).

Xylanase activity at wide range of temperatures can have wide range of applications in several industrial bioprocesses. Therefore, *B. firmus* HS11 was selected for further optimization experiment using Plackett-Burman design followed by Box-Behnken Model. Xylanase from *Bacillus spp.* has been studied and preferred among other bacterial xylanase for industrial use due to their rapid growth rate[12]. *B. subtilis*, *Bacillus circulans* and *Bacillus pumilus* are among the most promising strains of *Bacillus* used for production of xylanase in industrial scale[21]. Fermentation of hydrolysate obtained through statistically optimized enzymatic saccharification of chilli post-harvest residue resulted in production of 168 IU/mL of xylanase by *B. pumilus*[22]. Optimization of fermentation condition is needed as individual

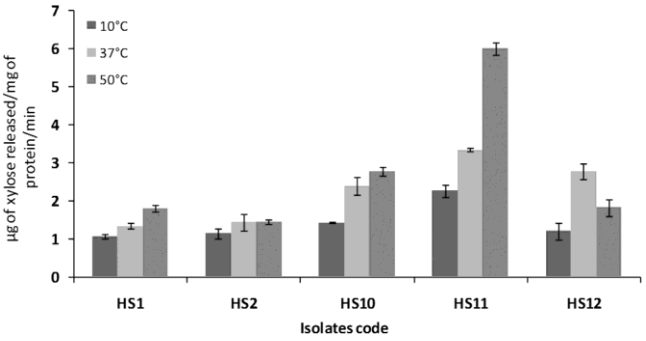

Fig. 2 — Xylanase activity (µg of xylose released/mg of protein /min) of selected isolates at three different temperatures (10, 37 and 50°C). [HS1, *Bacillus mycoides* HS1; HS2, *Pseudomonous helmanticensis* HS2; HS10, *Bacillus mobilis* HS10; HS11, *Bacillus fimrus* HS11; and HS12, *Bacillus subtilis* HS12]

nutrient effects the enzyme production[10,11]. Higher production of any enzyme by modulating nutrient composition can result in lowering its cost of production. Enzyme production was carried out by submerged fermentation as it requires less fermentation time apart from high nutrient availability and sufficient supply of oxygen[11,23]. In the present study, we optimized the condition using Plackett-Burman design followed by response surface methodology. Plackett-Burman design was applied as an initials statistical approach in identifying crucial parameters affecting production of xylanase by engineered *E. coli* harboring xylanase gene of *Thermotoga neopolitana* DSM-4359[24]. For xylanase production six parameters beechwood xylan, peptone, NaCl, yeast extract, $MgSO_4$, and $CaCl_2$ composition with a fix inoculum size of 1% (v/v) 12 h grown pre-inoculum were selected for Plackett-Burman design to identify the influence of each parameter in production of xylanase by *B. fimrus* (Table 1). Inoculum size of 1% (v/v) from overnight grown culture has shown to produce maximum xylanase by *B. pumilus* VLK-1[25].

Two concentrations were evaluated for each parameter (high and low) and designated as level +1 and level −1, respectively. After the analysis of 12 runs order experiment, it was found that three parameters, peptone, yeast extract and NaCl had significant effect in xylanase production (Table 1). Higher concentrations of these parameters had negative effect on the production of xylanase and were therefore selected for further optimization through RSM. Low concentration of beechwood xylan (0.2 % w/v) had negative impact on xylanase production while higher concentration of beechwood xylan (0.8%) along with high concentration of $MgSO_4$ and $CaCl_2$ had positive impact (Table 1). Maximum xylanase production by *Bacillus tequilensis* strain ARMATI was obtained with xylan concentration between 0.5-1.5%[12]. In another study, $MgSO_4$ and $CaCl_2$ were found to have positive impact on production of xylanase by *Penicillium* sp. WX-Z1[10]. Effect of selected parameters on xylanase production by *B. firmus* HS11 is presented in Fig. 3. Optimization of parameters by Plackett-Burman design led to enhanced xylanase production from 3.2 U/mg to 31.71 U/mg of protein. Screening of parameters by Placket-Burman design helped in understanding the impact of individual parameters for the enhanced production of xylanase and selection of parameters for further optimization through RSM.

Box-Behnken design consisting of 15 runs order was considered for optimization of three significant parameters: peptone, yeast extract and NaCl for enhanced production of xylanase by *B. firmus* HS11 (Table 2). Optimization of conditions resulted in xylanase activity between 4.85 U/mg to 24.74 U/mg of protein (Table 2). Analysing the experimental data, it was observed that media composition with 0.8% beechwood xylan along with other minimal amount of salt and nitrogen source has positive effect in xylanase production. Peptone and NaCl were observed to have significant effect on the production of xylanase by *B. firmus* HS11. Concentration of NaCl is an important factor which has shown to effect production of xylanase using *B. subtilis* and *B. megaterium*[11]. *B. subtilis* and *B. megaterium* were analysed for xylanase production at different concentration of NaCl ranging from 0.2-1% and resulted maximum xylanase production at 0.2 and 0.8%, respectively[11]. In another study, *B. megaterium* BM07 strain showed highest xylanase activity with 0.8% NaCl concentration. Yeast extract has been reported to stimulate xylanase production during submerged fermentation in *Bacillus* species[11,26]. Increase in yeast extract (0.1%) concentration as nitrogen source resulted in decrease in xylanase production by *B. firmus* HS11 (Table 2).

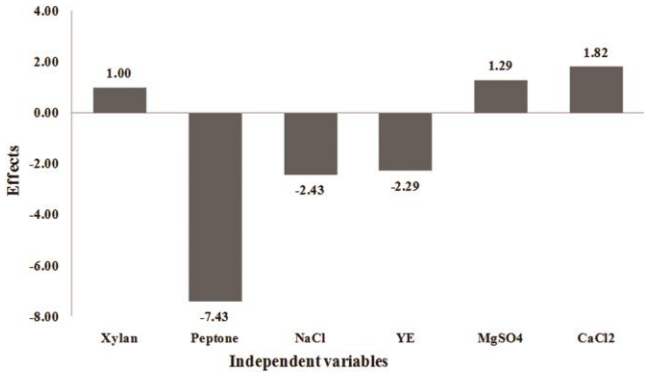

Fig. 3 — Effect of independent variables on xylanase production by *Bacillus fimrus* HS11 using Plackett-Burman design

In our study, optimum concentration for xylanase production by *B. firmus* HS11 was observed at media having 0.3% (w/v) NaCl concentration. Coefficient of determination (R^2) of the model was 96.07%, which indicated that the model could explain 96.07%, of the variation in response. Surface plot was plotted to show production of xylanase in relation to interaction between variables and to determine the optimum level of individual variables for optimal production of xylanase (Fig. 4 A-C). Three surface plots were plotted, each plot describing the interaction between different levels of two variables (peptone, NaCl and yeast extract) while keeping one of the variable hold at fixed zero level[27]. In optimization of enzyme production, Plackett-Burman followed by Box-Behnken design are the most commonly used statistical designs[28]. The optimized condition for the production of xylanase by *B. firmus* HS11 was 0.8% (w/v) beechwood xylan, 0.04% (w/v) $MgSO_4$, 0.04% (w/v) $CaCl_2$, 0.1% (w/v) peptone, 0.3% (w/v) NaCl and 0.01% (w/v) yeast extracts. The optimization plot obtained by Box-Behnken design reflected the concentration of three independent factors such as yeast extract, peptone and NaCl responsible for enhanced production of xylanase by *B. firmus* HS11 (Fig. 5). On optimization of condition using Plackett-

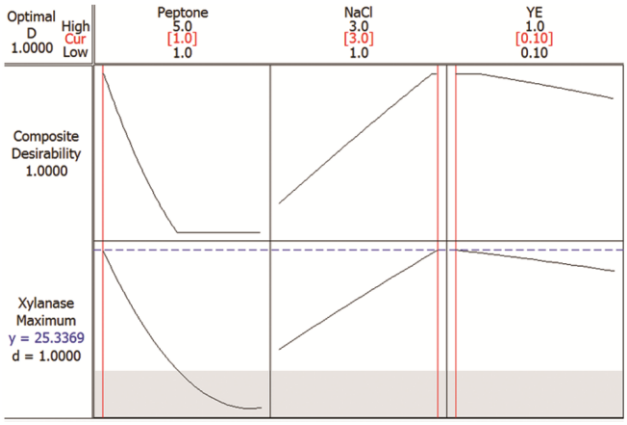

Fig. 5 — Optimization plot of production of xylanase by *Bacillus fimrus* HS11.

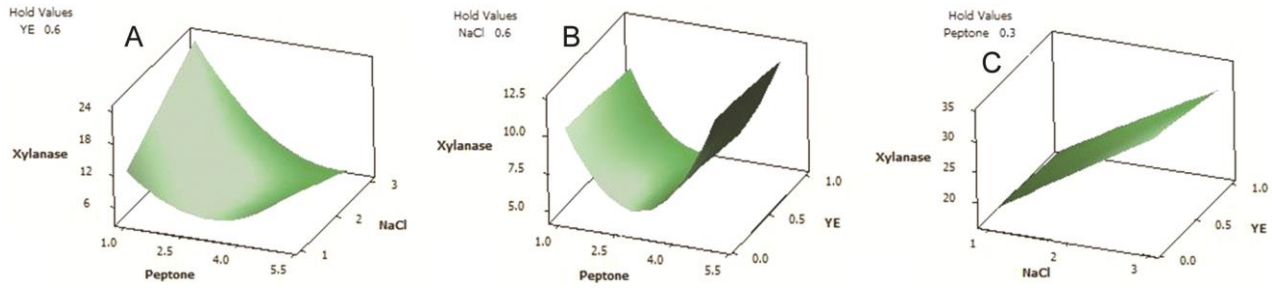

Fig. 4 — Surface plot of showing the interaction of (A) NaCl and peptone; (B), peptone and Yeast extract (YE); and (C) Yeast extract and NaCl

Burman design followed by Box-Behnken model xylanase production by *B. firmus* hs11 was enhanced by 7.4 folds. In the present study, enhancement of xylanase production was higher compared to earlier study on optimization of xylanase production by *B. circulans* D1[29].

Conclusion

Soil samples from high altitude region of Sikkim were used for isolation of xylanase producing microorganisms. Among the isolates, *Bacillus firmus* HS11 was selected for optimization of nutrient composition by statistical approach for enhanced xylanase production as it had highest activity at broader range of temperature. Nutrient compositions were optimized for submerged fermentation by combination of Plackett-Burman design and Box-Behnken Model, which resulted in enhancement of xylanase production by 7.4 folds. Further work on purification and characterization of xylanase along with its substrate specificity would help in defining its application in food and feed industry. Xylanase from unexplored sources such as Sikkim Himalayan region may have interesting biochemical properties applicable for bioprocess industries.

Acknowledgement

The authors thank the Forest Department, Govt. of Sikkim for the permission for sample collection from high altitude region of Sikkim. We also thank Department of Biotechnology, Govt. of Indian for the financial support for carrying out our research.

Conflict of Interest

Authors declare no conflict of interests.

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