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Screening and molecular identification of microbial strain *Bacillus axarquiensis* P6 from algal biomass for production and optimization of multiple enzymes of biotechnological interest

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Abstract
This study is a first of its own kind for isolation of multiple enzyme producers from biomass of *Rhizoclonium* sp. algae collected from different sites of Himachal Pradesh. One of the isolated 19 strains, the isolate P6 exhibited zones of hydrolysis for cellulase, xylanase and amylase activity indicating its potential for multiple enzyme production. Isolate identified based on their morphological and physicochemical characteristics and 16s rRNA sequencing. Phylogenetic analysis based on the results of 16S rRNA gene sequencing revealed that P6 was in close identity to *Bacillus axarquiensis.* Then different parameters for enhanced enzyme production were optimized using classical one factor at a time approach (OFAT). The effects of different media, medium pH, temperature, inoculum size and incubation period for multiple enzyme production were studied. The enzyme production was found maximum at pH 8.0 for cellulase and amylase while pH 5.0 for xylanase, temperature 30 ºC and incubation time 72 h by multiple enzyme producer *Bacillus axarquiensis* P6. Overall, the results of the present study demonstrate that the genus *Bacillus axarquiensis* P6 is a potential candidate for multiple enzyme production and can be used for various biotechnological purposes as hydrolysis of biomass for biofuel production.

Keywords: Multiple enzymes, *Bacillus axarquiensis*, optimization

1. Introduction

Functional integration and the cooperative effect of multienzymes over individual counterparts have demonstrated its important role in the production of different commodities in food and feed industries. Programmes to select new microorganisms for enzyme production are increasing around the world. Microbial enzymes are routinely used in many environment friendly and economic industrial sectors (Hoondal *et al*. 2012) [17]. Microbes are the best source of enzymes as they allow an economical technology with low resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Dalvi and Anthappan 2007) [7]. Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. Different enzymes for commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have become a focus of research (Malvessi and Silveira 2014; Phutela *et al*. 2015) [29, 35].
Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food fermentation, textile to paper industries (Lin *et al*., 2007; Pandey *et al*., 2012) [27]. Complex cellulolytic enzymes, which convert cellulase to glucose, have been shown to be extracellular product of several microorganisms. Cellulase hydrolyzes-1, 4 glucosidic bonds in cellulase and its derivatives while it acts on the non-substituted cellulase leading to the formation of cellobiose, which can be further hydrolyzed by -glycosidase. The enzyme cellulase is also useful economically as it has the ability to convert het biomass into fuels and basic chemicals. Cellulase decomposing bacteria and fungi are widely distributed in the marine environment and they play an important role in mineralizing organic matter and also influencing the productivity of the sea (Kadota, 2006). Microbial xylanases have attracted great attention due to their biotechnological uses and potential application in various industrial processes, such as bioconversion of lignocelluloses material to fermentative products,

clarification of juices, improvement of the consistency of beer and the digestibility of animal feed stock (Butt *et al*. 2008) [5], and production of acidic oligosaccharides having potential pharmacological benefits (Christakopoulos *et al*. 2013) [6]. Bacteria are inherent part of the physical environment of algae both in the natural and laboratory environments (Gallacher and Smith, 2009) [11]. The physiological and ecological relevance of the algal-bacterial interactions in the biogeochemical cycling has been found to inhibit each other's growth and in the biogeochemical cycling has been reported earlier (Lovejoy *et al.* 2013; Imai *et al*. 2011) [28, 18]. The algae can serve as rich reservoir of industrially important enzymes producing microorganisms. Multiple enzyme activities in supernatant produced through fermentation facilitates the production of enzyme activities with high titer values with synergetic effects. A selection of précised fermentation conditions is utmost important for achieving these multiple enzyme activities from a microbial supernatant. However, currently there is still no appropriate bioprocess with a well-established bioengineering approach for simultaneous production of multienzymes through a complex microbial culture system. Therefore, the least explored and highly probable source of hydrolytic enzyme producing microorganisms, *Rhizoclonium* sp. algal biomass used in current study was selected for isolation of multiple carbohydrases producing microorganisms. In the present study, attempts were made to isolate and optimize different process parameters for bacterial strain capable of producing appreciable levels of multiple carbohydrase i.e. cellulase, xylanase and amylase.

2. Materials and methods

2.1 Collection of sample

Rhizoclonium sp. algal biomass collected from different sites of Himachal Pradesh in clean polythene bags and was brought to the laboratory.

2.2 Isolation and screening of multiple enzyme producing microorganisms: To algal biomass, 1% solution of cellulose, xylan and starch powder was added separately. The enriched samples have been serially diluted and plated on to nutrient agar medium supplemented with different substrates with initial pH of 7.0 and incubated at 37 ºC for 2-3 days. The pure cultures were obtained and maintained at 4 ºC on nutrient agar medium.

2.3 Phenotypic and Biochemical identification: The conventional morphological and biochemical tests of all isolates as described by Aneja (2003) [2] were performed. The isolates were characterized morphologically including colony morphology, cell shape, Gram's reaction and culture conditions based on Bergey's manual of systemic bacteriology. Different biochemical tests were performed on bacterial isolates for classifying them according to their biochemical features. The tests include catalase, indole test, MR/VP test and H_2S production and carbohydrate fermentation test by standard methods.

2.4 Qualitative assay of multiple enzymes: An extensive screening of bacterial isolates capable of exhibiting appreciable levels of cellulolytic, xylanolytic and amylolytic activity was done. The degradation of cellulose and xylan was estimated by flooding plates hydrolyzing media with 0.1% congo red for 15 min and then washed with 1 N NaCl (Teather and Wood 1982) [43]. Starch hydrolysis test was performed on amylase medium and incubated at 37 ºC for 72 h. The zone of hydrolysis was observed (Shaw *et al*. 1995) [39].

2.5 Quantitative analysis: The bacterial isolates were screened for the production of extracellular enzymes i.e. cellulase (CMCase, FPase, β-glucosidase), xylanase and amylase (α-amylase, β-amylase) activities. Each bacterial isolate was grown in nutrient broth at 37 ºC for 24 h. As soon as the substantial growth was observed. Then 5 ml of inoculum was added to 45 ml of nutrient broth supplemented with 1% of cellulose, xylan and starch powder. The inoculated flasks were then incubated at 37±2 ºC for 24h. Then, the cell-free supernatant was recovered by centrifugation (10,000 rpm, 10 min at 4 °C) and the clear supernatant was used to determine the different enzyme activities.

2.6 Enzyme assays

2.6.1 Cellulase assays: CMCase, FPase and β-glucosidase assays was determined as described by Grazek (1987) [15] and (Berghem and Petterson, 1973) [4] using 1% CMC, 50 mg filter paper strips and 1mM ρ-nitrophenyl ß-D-glucopyranoside as substrate respectively. The released reducing sugars were quantified using glucose and ρ-nitrophenol standard curves as a reference. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of glucose/ρ-nitrophenyl ß-D-glucopyranoside released per min under assay conditions.

2.6.2 Xylanase assay: xylanase activity was determined using 1% (w/v) oat spelt xylan in 0.055 mM sodium acetate buffer pH 4.0 as substrate (Miller, 1959) [31]. The released reducing sugars were quantified using a xylose standard curve as a reference. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of xylose per min under assay conditions.

2.6.3 Amylase assay: α-amylase and β-amylase assays was determined as described by Xiao *et al*. 2006 using 0.2% starch as substrate. The released reducing sugars were quantified using soluble starch and glucose standard curves as a reference. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of starch/glucose released per min under assay conditions.

2.7 Molecular identification: The genotypic identification of the selected isolate P6 was done by the 16S rRNA gene sequencing method. Briefly, genomic DNA was extracted using a genomic purification kit DNA prep kit (Banglore Genei, India Pvt. Ltd. The universal primers used for amplification were 8F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'. The PCR analysis was carried out with a volume of 25 µl mixture in a thermo cycler. It consisted of 35 cycles, 92 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min. Sequence for 16S rRNA was compared with the sequences available in public database National Center for Biotechnology Information (NCBI). Based on the BLAST results sequences for other species were retrieved. All sequences were aligned using Clustal W and the phylogenetic tree was constructed (Tamura *et al*. 2007) [42]. Genetic relationships were inferred from neighbor-joining nucleotide alignment after 1000 bootstrap replicates. On the basis of 16S rRNA gene technique, isolate P6 was identified as *Bacillus axarquiensis* and had been registered under the accession number |MF443874|.

2.8 Optimization of growth parameters for multiple enzyme production

To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield of enzyme without increasing the cost of production (Gangadharan *et al.*, 2008) [12]. Since, the growth and enzyme production of the organisms are strongly influenced by different process parameters viz. temperature, pH, inoculum size, media types, incubation period etc. have been optimized by using Classical One Factor at a Time (OFAT) approach.

2.8.1 Effect of different media: Effect of different media i.e**.** PYC Medium (Kim *et al*. 2005) [23], Basal Salt Medium (Maniatis *et al*. 1982) [30], Okoshi *et al*. Medium (Okoshi *et al*. 1990) [32], Li & Gao Medium (Li & Gao 1997) [26], Mandel and Reese Media (Vyas *et al*. 2005) [45] were studied for cellulase production, Bacillus Xylose Salt Medium (Annamalai *et al*. 2009) [3], Xylan Medium (Annamalai *et al*. 2009) [3], Basal Salt Medium (Maniatis *et al*. 1982) [30], TGY Medium (Garg *et al*. 2009) [13], Emmerson Medium (Garg *et al*. 2009) [13] for xylanase production, Babu and Satyanarayana (1993), Kwan *et al*. (1993) [25], Starch Agar medium (Saxena *et al*. 2007) [38], Modified starch medium (Jyoti *et al*. 2011) [19], Modified yeast medium (Fooladi and Sajjadian 2010) [10] for amylase production were studied.

2.8.2 Effect of different pH: The effect of pH for multiple enzyme production was performed by varying pH of the medium from 5.0, 6.0, 7.0, 8.0 and 9.0 whereas the other parameters were unaltered. Flasks were seeded with inoculum and incubated at 37 °C for 72 h and enzymes were assayed.

2.8.3 Effect of different temperature: Effect of temperatures ranging from 25, 30, 35... 45 °C at 120 rpm on multiple enzyme production was examined. Flasks were seeded with inoculum and incubated for 72 h. The enzyme activities were estimated.

2.8.4 Effect of different inoculums size: Optimum inoculum concentration for multiple enzyme production was studied by varying the inoculums concentration (7.5% to 17.5%) were added to the enzyme production medium and incubated at 30 °C for 72 h and the enzymes were assayed.

2.8.5 Effect of different incubation period: Effect of incubation time ranging from 24h, 48h… 144h at 120 rpm on multiple enzyme production was examined. Flasks were seeded with inoculum and incubated at 30 °C. The enzyme activities were estimated.

3. Results and Discussion

3.1 Isolation and Screening of multiple enzyme producing bacteria: In total 19 isolates were obtained from algal biomass collected from different sites of Himachal Pradesh. The isolated strains were screened for multiple enzyme production using nutrient agar media supplemented with different substrates. Out of total, nine bacterial isolates screened for different hydrolytic activities were further characterized.

3.2 Identification of Isolated bacterial strain

3.2.1 Biochemical identification: Preliminary identification of the screened bacteria have been done on the basis of their morphological, physiological, biochemical and metabolic characteristics. Morphological fingerprinting of the colonies revealed that 47% were creamish yellow in color, 26% were creamish white, 5% were yellow and 11% were mucoid while 11% were creamish in color. As far as shape of the colonies was concerned, 26% of the colonies were circular in form, 37% were punctiform, 5% were filamentous and 21% were irregular while 11% were spindle. On the basis of the colony elevation and margin, 42% of the isolates were raised, 37% were flat while 11% were convex whereas 37% were entire, 37% were undulated, 21% were lobate and 5% were curled. Gram staining of all these bacterial isolates differentiated them into gram +ve and gram-ve. Among these 19 isolates, 78% were found to be gram +ve and the rest 22% were found to be gram -ve. All the isolates were found to be H_2S negative. Among 9 isolates, 6 were VP positive and only 1 was MR positive. Seven isolates were found to be positive for citrate utilization and 8 isolates for catalase production. Among all, 5 isolates were found to be positive for indole test. All the bacterial isolates were tentatively identified and were placed under the group of Genus *Bacillus* as per Bergey's Manual of Determinative Bacteriology, 7th Edn.

Table 1: Isolation of different hydrolytic enzyme producing bacterial isolates from *Rhizoclonium* sp. algal biomass and their morphological characteristics

Source of isolation	Isolate name	Zone test				Morphological Characters				
		Amylase	Xylanase	Cellulase	Pectinase	Form	Margin	Elevation	Color	Texture
Algal biomass	P1	-	+	-	-	Circular	Entire	Raised	Creamish white	Smooth
Algal biomass	P2	+	-	-	-	Filamentous	Undulate	Flat	Creamish yellow	Smooth
Algal biomass	P3	+	-	+	-	Circular	Undulate	Raised	Creamish yellow	Rough
Algal biomass	P4	-	+	-	-	Punctiform	Entire	Raised	Creamish	Smooth
Algal biomass	P5	-	-	-	-	Circular	Undulate	Raised	Creamish yellow	Smooth
Algal biomass	P6	+	+	+	-	Punctiform	Undulate	Flat	Creamish white	Rough
Algal biomass	P7	-	+	+	-	Punctiform	Entire	Raised	Creamish white	Smooth
Algal biomass	P8	-	-	-	-	Circular	Entire	Raised	Mucoid	Smooth
Algal biomass	P9	-	-	-	+	Circular	Undulate	Convex	Yellowish	Smooth
Algal biomass	P10	-	+	+	-	Punctiform	Entire	Raised	Mucoid	Smooth
Algal biomass	P11	-	-	-	-	Spindle	Curled	Raised	Creamish Yellow	Rough
Algal biomass	P12	-	-	-	-	Irregular	Lobate	Flat	Creamish Yellow	Rough
Algal biomass	P13	-	-	-	-	Spindle	Lobate	Flat	Creamish Yellow	Smooth
Algal biomass	P14	-	-	-	-	Irregular	Lobate	Flat	Creamish	Smooth
Algal biomass	P15	-	-	-	-	Punctiform	Entire	Convex	Creamish Yellow	Smooth
Algal biomass	P16	-	+	+	-	Irregular	Undulate	Flat	Creamish white	Rough
Algal biomass	P17	-	-	-	+	Punctiform	Entire	Flat	Creamish white	Rough
Algal biomass	P18	-	+	-	-	Irregular	Lobate	Convex	Creamish yellow	Smooth
Algal biomass	P19	-	+	-	+	Puctiform	Undulate	Convex	Creamish yellow	Smooth

3.2.2 Genotypic Identification: Molecular characterization of the selected multiple enzyme producing strain P6 was done at genomic level by using 16S rRNA gene technique Genomic DNA of bacteria was isolated by using DNA isolation method. Further these amplified 16S rRNA sequences of the bacterial strains was blasted using online tool (mBLAST NCBI). The isolate P6 showed 98% homology with *Bacillus axarquiensis.* The 16S rRNA gene sequences of the isolate has been deposited to National Centre for Biotechnology Information (NCBI) gene bank using Bankit program and has been registered in the databases vide accession number *Bacillus axarquiensis* P6 | MF443874|. The taxonomical identification was done by the phylogenetic tree construction and the comparison of bacterial strain sequences with other homologous bacterial sequences.

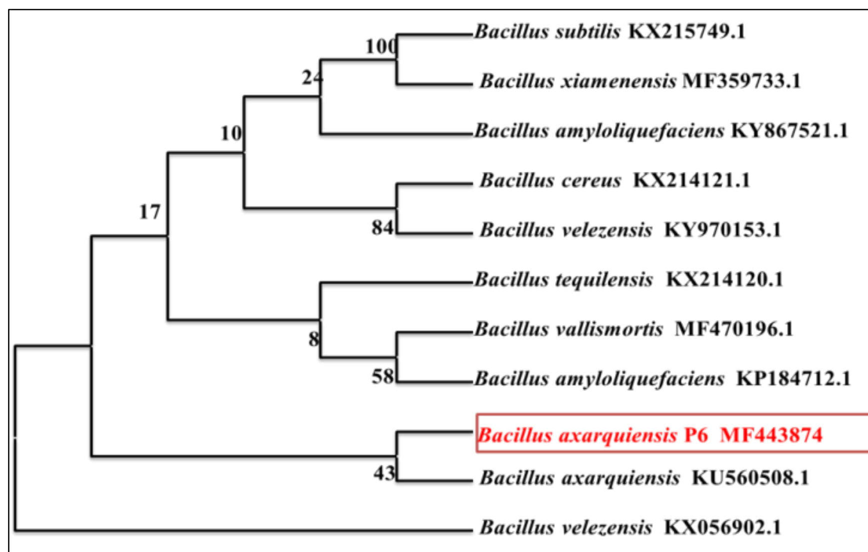

Fig 1: Neighbor-joining tree showing phylogenetic relationship of *Bacillus axarquiensis* P6 based on a distance matrix analysis of 16S rRNA sequences

3.3 Optimization of various parameters for profound enzyme activities using one factor at a time approach (OFAT

3.3.1 Effect of media: The data pertaining to effect of different media on cellulase, amylase and xylanase production has been enlisted in Table 2, 3 and 4 had revealed that highest cellulase activity was found in the Li and Gao medium i.e. 1.05 IU while least enzyme production 2.21 IU was observed in PYC medium. Highest α-amylase activity of 29.99 IU and β-amylase activity of 5.81 IU was observed. Similarly, the maximum xylanase activity was observed in Bacillus xylose salt medium i.e. 4.61 IU followed by TGY medium (3.92 IU) which was higher than other media. Least xylanase production i.e. 2.44 IU was observed in Xylan medium. Vidyalakshmi *et al.* (2014) [44] have reported the production of amylase from *Bacillus* sp. in the presence of yeast extract and peptone. *Paenibacillus* sp. N1 showed maximum xylanase production in Basal salt medium i.e. 27.20 IU at 50 °C in 5 days incubation (Pathania *et al.,* 2012) [34].

Table 2: Effect of different media on extracellular cellulase production from *Bacillus axarquiensis* P6

Media	Cellulase (IU)								
	Protein (mg/ml)	FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase)	
		Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity
PYC medium	0.98	0.07	0.069	0.39	0.40	0.37	0.37	0.83	0.85
Basal Salt medium	0.64	0.02	0.032	0.16	0.26	0.39	0.61	0.58	0.90
Okoshi *et al*. Medium	1.00	0.05	0.05	0.37	0.37	0.44	0.44	0.86	0.86
Li and Gao medium	1.15	0.05	0.04	0.60	0.52	0.41	0.35	1.05	0.92
Mandel and Reese media	1.02	0.05	0.04	0.40	0.39	0.39	0.39	0.84	0.83
C.D. $_{0.05}$	0.27	N/S	N/S	0.09	0.06	N/S	0.135	0.16	N/S
S.E.(m)	0.08	0.06	0.05	0.03	0.02	0.02	0.04	0.05	0.04

Table 3: Effect of different media on extracellular amylase production from *Bacillus axarquiensis* P6

Media	α-amylase activity (IU)*	Protein (mg/ml)	Specific activity	β-amylase activity (IU)	Protein (mg/ml)	Specific activity
Babu and Satyanarayan	27.49	2.56	10.74	5.81	2.08	2.79
Kwan *et al*. 1993 [25]	29.06	3.54	8.21	3.33	1.23	2.71
Starch medium	27.88	2.65	10.52	4.55	1.99	2.29
Modified starch medium	29.99	3.67	8.17	1.85	0.89	2.08
Modified yeast medium	25.45	2.14	11.84	3.53	1.43	2.47
C.D.$_{0.05}$	1.62	0.62	0.96	0.75	0.42	N/S
S.E. (m)	0.51	0.19	0.30	0.23	0.13	0.16

Table 4: Effect of different media on extracellular Xylanase production from *Bacillus axarquiensis* P6

Media	Xylanase activity (IU)	Protein (mg/ml)	Specific activity
Bacillus xylose salt medium	4.61	2.17	2.12
Xylan Medium	2.44	1.02	2.39
Basal salt medium	2.45	1.06	2.31
TGY medium	3.92	1.86	2.11
Emmer son medium	2.52	1.21	2.08
$C.D._{0.05}$	0.94	0.39	N/S
S.E. (m)	0.29	0.12	0.15

3.3.2 Effect of different pH: Maximum production of cellulase was observed at pH 8.0 i.e. 1.25 IU which was significantly higher than others statistically and minimum was observed in pH 9.0 i.e. 0.70 IU (table 5). The highest α-amylase activity of 32.63 IU was observed for at pH 8.0 with specific activity 7.71 IU/mg. At lower pH of 6.0 and 7.0, a respective amylase activity of 30.97 IU and 31.43 IU has been observed. The highest xylanase titers were observed at pH 5.0 i.e. 4.95 IU which was statistically significant higher than others and least xylanase titers were observed at pH 6.0 i.e. 25.44 IU (table 6 and 7).

Table 5: Effect of different pH on extracellular cellulase production from *Bacillus axarquiensis* P6

pH				Cellulase (IU)*					
	Protein (mg/ml)**	FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU/ml)	Specific activity
5.0	0.88	0.05	0.06	0.11.	0.13	0.29	0.33	0.45	0.51
6.0	0.91	0.16	0.18	0.12	0.13	0.51	0.56	0.78	0.86
7.0	1.16	0.06	0.05	0.60	0.52	0.43	0.37	1.09	0.94
8.0	1.20	0.04	0.04	0.81	0.67	0.39	0.33	1.25	1.04
9.0	0.87	0.04	0.05	0.31	0.36	0.36	0.41	0.70	0.81
$C.D._{0.05}$	0.24	0.06	0.08	0.17	0.19	0.10	0.08	0.28	0.18
S.E.(m)	0.08	0.02	0.02	0.05	0.06	0.03	0.02	0.09	0.06

Table 6: Effect of different pH on extracellular amylase production from *Bacillus axarquiensis* P6

pH	α-amylase activity (IU)	Protein (mg/ml)	Specific activity	β-amylase activity (IU)	Protein (mg/ml)	Specific activity
5.0	30.43	3.87	8.18	3.58	1.22	2.93
6.0	30.97	3.21	9.79	5.63	2.54	2.22
7.0	31.43	2.96	10.46	5.75	2.75	2.09
8.0	32.63	4.23	7.71	8.72	3.21	2.72
9.0	31.56	3.43	9.20	2.72	0.89	3.06
C.D.	N/S	0.66	1.35	0.81	0.41	0.50
S.E.(m)	0.37	0.21	0.42	0.25	0.13	0.16

Table 7: Effect of different pH on xylanase production from *Bacillus axarquiensis* P6

pH	Xylanase activity (IU)	Protein(mg/ml)	Specific activity
5.0	4.95	2.05	2.25
6.0	2.44	1.06	2.30
7.0	2.45	1.25	1.98
8.0	3.92	1.91	2.05
9.0	2.52	1.83	1.38
^{CD} 0.05	0.94	0.61	N/S
S.E.(m)	0.29	0.19	0.25

This implies that the 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. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity (Willey *et al.,* 2008) [46]. Sethi *et al.* (2015) [41] observed the effect of pH on cellulase production by *Pseudomonas fluorescens, Bacillus subtilIs, E. coli* and *Serratia marcescens* isolated from soil and showed the optimum cellulase production at pH 8.0. Several agroindustrial residues were screened as the substrate for the production of α-amylase by *Bacillus subtilis* strain PS03 and pH 8 was found to be optimum for maximum α-amylase production using (Jagadeeswari and Santhi, 2016) [37].

3.3.3 Effect of temperature: Effect of temperature on cellulase, amylase and xylanase production from *Bacillus axarquiensis* P6 has been shown in Tables 8, 9 and 10 respectively. Maximum cellulase titres were produced at 30 °C i.e. (1.77 IU) whereas; least cellulase production was found at 45 °C (0.79 IU). Highest amylase activity i.e. α-amylase activity 33.86 and β-amylase activity of 11.94 IU at an optimum temperature of 30 °C which was significantly higher than others. Similarly temperature i.e. 30 °C (5.59 IU) was found optimum for maximum xylanase production which is higher than others. Minimum xylanase was produced at 45 °C (2.56 IU).

Table 8: Effect of different temperature on extracellular cellulase production from *Bacillus axarquiensis* P6

Temperature (°C)	Protein (mg/ml)	Cellulase (IU)							
		F Pase activity		CM Case activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity
25	1.00.	0.22	0.22	0.53	0.53	0.54	0.54	1.28	1.28
30	1.17	0.29	0.24	0.91	0.77	0.58	0.49	1.77	1.51
35	0.97	0.20	0.21	0.79	0.82	0.52	0.53	1.51	1.57
40	0.49	0.06	0.12	0.68	1.38	0.48	0.98	1.22	2.48
45	0.65	0.06	0.09	0.33	0.50	0.41	0.63	0.79	1.23
$CD_{0.05}$	0.26	0.08	0.05	0.22	0.48	N/S	0.20	0.44	0.60
S.E.(m)	0.08	0.03	0.02	0.07	0.15	0.06	0.06	0.14	0.19

Table 9: Effect of different temperature on extracellular amylase production from *Bacillus axarquiensis* P6

Temperature (°C)	α-amylase activity (IU/)*	Protein (mg/ml)**	Specific activity***	β-amylase activity (IU)*	Protein (mg/ml)**	Specific activity***
25	31.80	3.16	10.06	5.84	1.98	2.95
30	33.86	4.56	7.43	11.94	3.87	3.09
35	32.41	3.98	8.14	8.44	2.43	3.47
40	29.52	2.54	11.62	5.92	2.10	2.82
45	26.24	2.21	11.87	4.75	1.55	3.06
$C.D._{0.05}$	1.29	0.59	1.33	1.08	0.51	N/S
S.E. (m)	0.40	0.19	0.42	0.34	0.16	0.23

Table 10: Effect of different temperature on xylanase production from *Bacillus axarquiensis* P6

Temperature (°C)	Xylanase (IU)*	Protein (mg/ml)**	Specific activity***
25	4.84	2.05	2.36
30	5.59	2.60	2.15
35	4.39	1.97	2.23
40	3.65	1.32	2.77
45	2.56	1.01	2.53
$C.D._{0.05}$	1.09	0.39	N/S
S.E. (m)	0.34	0.12	0.17

Ziyanda *et al.* (2016) [48] observed that the optimal temperature for cellulase activity was 25 °C with enzyme activity of 198 U/ml and cellulase activity decreased with increasing temperature until 45 °C after which a gradual increase was observed up to 50 °C from *Micrococcus* sp. On the other hand, optimal temperature for xylanase production was achieved at 25 °C with enzyme activity of 1007 U/ml. As temperature increased from 25 °C, xylanase activity declined to about 650 U/ml at 45 °C and then increased to about 765 U/mL at 50 °C. *Bacillus* sp. showed maximum amylase titer (90.42 ± 2.1 U/ml) at 30 °C. Further increase in the temperature to 40 and 50 °C inhibited the amylase production indicating the organism mesophilic in nature (Khusro *et al.* 2017) [22].

3.3.4 Effect of inoculum size: The size of inoculum plays an important role in the fermentation of enzymes. Different inoculum sizes studied for enhanced enzyme production were 7.5, 10.0…, 17.5.0% (v/v). Optimum inoculum size was found to be 12.5% (V/V) for cellulase, α-amylase, β-amylase and xylanase production from *B. axarquiensis* P6 revealing 1.99 IU, 33.975 IU, 15.36 IU and 6.84 IU respectively (table 11, 12 and 13). Highest enzyme production was obtained at 12.5% of inoculum size but was not statistically significant over other inoculum sizes. The least cellulase, α-amylase, β-amylase and xylanase units were recorded at 17.5%. In contrary to our findings, variable inoculum sizes have been reported by workers. Agarwal *et al.* (2014) [1] studied the optimization of cellulase enzyme by *Pseudomonas aeruginosa* MTCC 464. The effect of various inoculum size of 1-10% was tested. The maximum cellulase activity (18.83 U/g) was found at 10% v/v using 5% of saw dust with temperature of 30 °C and pH of 7.2 ± 0.2. *Simair et al.* (2017) [40] achieved highest amylase concentration when 10 (% v/v) of 24 h old *Bacillus* sp. BCC 01-50 was inoculated into the fermentation medium. Kumar *et al.* (2017) [24] observed the effect of different inoculum sizes for *B. amyloliquefaciens* SH8 and 10% showed maximum xylanase titers of 48.91 IU/ml.

Table 11: Effect of different inoculum size on extracellular cellulase production from *Bacillus axarquiensis* P6

Inoculum Size (%)	Protein (mg/ml)	Cellulase (IU)							
		FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme activity (IU)	Specific activity	Enzyme activity (IU)	Specific activity	Enzyme activity (IU)	Specific activity	Enzyme activity (IU)	Specific activity
7.5	1.78	0.19	0.11	0.45	0.25	0.45	0.25	1.09	0.61
10.0	1.95	0.28	0.14	0.90	0.46	0.57	0.29	1.75	0.89
12.5	1.08	0.21	0.19	0.49	0.45	0.49	0.45	1.99	1.10
15.0	1.03	0.15	0.15	0.36	0.35	0.39	0.38	0.90	0.87
17.5	0.89	0.060	0.07	0.33	0.37	0.36	0.40	0.75	0.84
$C.D._{0.05}$	0.35	0.12	N/S	0.32	N/S	N/S	N/S	0.64	N/S
S.E. (m)	0.11	0.04	0.03	0.10	0.08	0.09	0.09	0.20	0.13

Table 12: Effect of different inoculum size on extracellular amylase production from *Bacillus axarquiensis* P6

Inoculum Size (%)	α-amylase activity (IU)	Protein (mg/ml)	Specific activity	β-amylase activity (IU)*	Protein (mg/ml)	Specific activity
7.5	32.80	3.54	9.27	6.49	1.54	4.21
10.0	33.79	4.76	7.09	11.65	3.52	3.31
12.5	33.95	4.43	7.64	15.36	4.78	3.21
15.0	29.46	2.98	9.89	4.76	1.01	4.71
17.5	27.69	2.32	11.93	3.80	0.98	3.88
C.D. $_{0.05}$	1.39	0.62	1.10	0.91	0.33	0.53
S.E. (m)	0.44	0.19	0.35	0.29	0.10	0.17

Table 13: Effect of different inoculum size on xylanase production from *Bacillus axarquiensis* P6

Inoculum size (%)	Xylanase activity (IU)	Protein (mg/ml)	Specific activity
7.5	1.38	1.44	0.96
10.0	5.40	1.93	2.79
12.5	6.84	2.25	3.04
15.0	2.78	1.76	1.58
17.5	1.76	0.98	1.79
C.D.$_{0.05}$	0.89	0.37	0.45
S.E.(m)	0.28	0.12	0.14

Enzyme activity is maximum at optimal level because at this point because 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 (Deb *et al.* 2013) [8].

3.3.5 Effect of incubation period: To determine the effect of incubation period on enzyme production from *B. axarquiensis*, enzyme activities were measured at regular intervals from 24h to a period of 144 h. The effect of incubation time on cellulase, amylase and xylanase production has been evaluated in Table 14, 15 and 16. Highest enzyme activities were measured at 72 h i.e. 2.13 IU cellulase, 37.90 α-amylase, 15.98 β-amylase and xylanase 7.20 followed by gradual decline on either side. Least enzyme production was observed at 144 h of fermentation time for all he enzymes. Statistically enzyme production was found significantly higher at 72 h than others timings. At 72 h, the cell population in the culture reaches at its peak which leads to a stable microbial association with the substrate and resulted in maximum enzyme production. The decline in the activity afterwards could be due to various reasons. Cellulase, amylase and xylanase produced by *B. axarquiensis* P6 were growth associated, reaching to maximum after 72 h at exponential peak and enzyme production decreased up to 144 h.

Table 14: Effect of different incubation time on extracellular cellulase production from *Bacillus axarquiensis* P6

Incubation Time	Cellulase (IU)								
	Protein (mg/ml)	FPase activity		CMCase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity	Enzyme Activity (IU)	Specific activity
24h	0.86	0.06	0.06	0.34	0.39	0.41	0.48	0.81	0.94
48h	1.05	0.19	0.18	0.45	0.43	0.41	0.39	1.05	1.00
72h	2.82	0.22	0.08	0.94	0.33	0.57	0.20	2.13	0.61
96h	1.36	0.20	0.15	0.58	0.42	0.49	0.36	1.27	0.95
120h	1.08	0.14	1.29	0.49	0.45	0.44	0.40	1.06	0.98
144h	0.89	0.05	0.06	0.36	0.40	0.36	0.40	0.77	0.86
C.D.$_{0.05}$	0.58	0.04	0.15	0.24	N/S	N/S	N/S	0.44	N/S
S.E.(m)	0.19	0.01	0.05	0.08	0.09	0.11	0.09	0.14	0.15

Table 15: Effect of different incubation period on extracellular amylase production from *Bacillus axarquiensis* P6

Incubation period (h)	α-amylase activity (IU)	Protein (mg/ml)	Specific activity	β-amylase activity (IU)	Protein (mg/ml)	Specific activity
24	33.64	3.94	8.54	7.09	1.76	4.03
48	35.78	4.89	7.32	8.94	3.78	2.37
72	37.90	4.71	8.05	15.98	4.96	3.08
96	32.60	3.05	10.68	6.67	1.17	5.70
120	30.61	2.95	10.38	5.10	1.16	4.39
144	25.89	1.98	13.07	5.37	1.02	5.26
C.D.$_{0.05}$	1.23	0.37	0.82	0.76	0.56	0.67
S.E. (m)	0.39	0.12	0.26	0.24	0.18	0.22

Table 16: Effect of different incubation time on xylanase production from *Bacillus axarquiensis* P6

Incubation period (h)	Xylanase activity (IU)	Protein (mg/ml)	Specific activity
24	2.33	1.74	1.34
48	3.16	2.33	1.36
72	7.20	3.55	1.86
96	4.23	2.76	1.53
120	3.16	1.98	1.59
144	2.17	1.11	1.96
C.D.$_{0.05}$	0.88	0.49	0.31
S.E. (m)	0.28	0.16	0.09

Afterwards incubation beyond 72 h resulted in a decreased enzyme activity that could be due to depletion of nutrients available causing a stressed microbial physiology eventually resulting in an inactivation of enzyme (Flores *et al.* 1997) [9]. Another reason could be the catabolite repression i.e. the increase in the production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production (Premila, 2013) [36]. Gautam *et al.* (2014) [14] studied the cellulase production optimization from *Bacillus* sp. The maximum yield of exoglucanase (1.64 U/ml) and endoglucanase (1.84 U/ml) activity was obtained after 4 days. Khusro *et al.* (2017) [22] observed the highest amylase production by *Bacillus* sp. (98.56±2.2 U/ml) after 72 h of incubation period. Different agro-residues were evaluated as substrates in solid state fermentation for xylanase production by a thermotolerent *Bacillus* isolate. Maximum xylanase production of 74.96±5.2 U/gds took place at 45 ºC after 72 h (Gupta and Kar 2015) [16].
The optimization of these fermentation factors particularly physical and chemical parameter are of primary importance in the development of any fermentation process owing their impact on the yield practicability and economy of the process. Optimization of different process parameters after media optimization, cellulase activity from 1.05 to 2.13 IU, α-amylase activity from 29.99 to 37.90 IU, β-amylase activity from 5.81 to 15.98 IU and xylanase activity from 4.61 to 7.20 IU were increased. As far the percent increase is concerned, 102.86 percent increase in cellulase activity, 26.37 percent increase in α-amylase activity, 175.04 percent increase in β-amylase and 56.18 percent increase in xylanase activity were achieved. Classical approach for one Variable at a time (OVAT) used in the present enzymes optimization study has resulted in an increase in the production of different enzymes proving the direct utility of this technique in increasing enzyme titers.

4. Conclusion

In the present study a multiple enzyme producing bacteria was isolated and screened from out of 19 isolates, isolated from *Rhizoclonium* sp. algal biomass collected from different sites of Himachal Pradesh. Laboratory scale optimization of different conditions for enhanced eznyme production by carried out by classcical one factor at a time approach (OFAT). It can be concluded from the above studies, *Bacillus axarquiensis* P6 based on its capability to produce multiple enzymes could be considered as useful source of multiple enzyme production and has the potential for industrial applications and this genus is first time reported for multiple enzyme production. Present investigation on the determination of optimal process parameters for *Bacillus axarquiensis* P6 for its better enzyme activity has yielded important results, indicating that the strain can be used for large scale industrial production of the enzymes. However, these parameters have to be tested in mass cultures in automated incubators so as to confirm the optimum conditions for enzyme production for industrial application.

5. References

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