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Isolation of potential novel cellulolytic and xylanolytic bacteria and optimization of their cultural conditions for enhanced production of cellulase and xylanase

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Abstract

The aim of the present study is to demonstrate potential cellulolytic as well as hyperxylanolytic bacteria and optimization of culture conditions for enhanced enzyme activity. Cellulolytic and xylanolytic bacteria were isolated from diverse sources viz; soil, sludge, rice fields etc. by standard serial dilution and pour plate method. Bacteria were further identified by morphological and biochemical tests and subjected to enzyme production. Twenty three different bacterial strains were isolated and screened for cellulase and xylanase production in submerged fermentation process. Among them, two tested bacterial strains; R2, R4 showed maximum yield for cellulase production. While SD9 showed higher xylanase production. This strain was further characterized by biochemical and morphological as well as genotypic identification and identified as *Bacillus licheniformis* R2, *Bacillus mojavensis R4*. Xylanoytic bacteria was identified as *Bacillus atropheaus* SD9. Results indicated that favourable conditions and the selection of a suitable growth medium played a key role in the production of enzyme from newly isolated *Bacillus licheniformis* R2, *Bacillus mojavensis*. Xylanoytic bacteria was identified as *Bacillus atropheaus* SD9. Due to its particular characteristics this enzyme will be used in scarification process for bioethanol production from plant biomasses.

Keywords: Cellulase, Xylanase, *Bacillus*, CMC agar

1. Introduction

Sustainable resources, which are in need of human beings, are derived from plant biomass. Cellulose is the major component of plant biomass [1]. Plants produce 4×10^9 tons of cellulose annually [2]. Cellulose is the most common organic compound on Earth. It is well known that plants are the most common source of renewable carbon and energy on the earth. Cellulose has no taste, is odourless & is hydrophilic [3]. Cellulose is derived from D-glucose units, which condense through β(1→4)-glycosidic bonds [4]. 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 [5]. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium are important parameters for the optimized production of cellulase enzymes [6]. For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot, organic matters and composts [7]. Researchers keep on working to isolate microorganisms with higher cellulase activity [8].

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Present study is aimed to isolate bacteria, which can produce more efficient cellulase enzymes in submerged fermentation.

2. Materials and methods

Isolation of cellulolytic bacteria. Cellulolytic and xylanolytic bacterial strains were isolated from soil by using serial dilutions and pour plate technique. The medium used for isolation of bacteria is Riveri's medium at pH 6.8 for 48 hours of incubation at 35˚C [9]. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4°C for further identification and screening for cellulase production.

Screening of cellulolytic bacteria. The isolates were grown at 35˚C for 3 days with rotary shaking in Erlenmeyer containing 45mL of Riveri's medium. The cell free supernatant containing the crude extracellular enzyme was collected after centrifugation of the culture in 7,000 g at 4°C for 20 min. The crude enzyme was kept in 4°C refrigerator for further analyses.

Cellulase activity was determined by using DNS (3, 5-dinitrosalicyclic acid) method [10] through the determination of the amount of reducing sugars. The mixture was incubated for 30 min at 37°C and the reaction was stopped by the addition of solution. The treated samples were boiled for 15 min, cooled in room temperature for 10 min and the optical density was measured at λ 540 nm. A unit (U) of cellulase activity was defined as the amount of enzyme that produced reducing sugars corresponding to 1 µmol glucose equivalents from carboxymethylcellulose per minute under the assay condition.

Identification of Screened Bacteria. The screened isolates R2, R4 and SD9 were tentatively identified on the basis of their phenotypic and biochemical characteristics in our research Laboratory as per Aneja (2003) [32] as *Bacillus sp.*

Genotypic identification

Molecular characterization using 16s rRNA PCR technique. R2, R4 and SD9 showing high enzyme production were identified at genomic level using 16S rRNA techniques.

Isolation of genomic DNA. The genomic DNA of the strain was isolated according to Genei DNA isolation kit.

PCR amplification of 16s rRNA region. PCR amplification was done to confirm the identity of the bacterial strains, the small sub-unit 16s rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'TACCTTGTTACGACTT3') primers to get an amplicon size of 1500 bp. Amplifications were carried out in 20 µl reaction mixture consisting of 10 x buffer, 2.0 µl ; 2.0 µl; 2.0 mM dNTPs, 2.0 µl ; 3.0 U/µl Taq DNA polymerase, 0.2 µl ; 100 ng/µl of each primer, 1.0 µl ; template DNA, 1.0 µl and sterilized distilled water 12.8 µl in a Biorad (USA) thermalcycler using the PCR conditions 95°C for 2 min (denaturation), 55.0°C (Strain R2, and R4), 52.3°C Strain SD9) for 1 min (annealing) and 72°C for 3 min (extension). The total number of cycles was 40, with the final extension of 72°C for 10 min. The amplified products (50 µl) were size separated on 1 % agarose gel prepared in 1% TAE buffer containing 0.5 µg/ml ethidiumbromide and photographed with the gel documentation system (Biorad, USA). A 100 bp DNA ladder (Genei) was used as molecular weight size markers.

Purification of the PCR product. The PCR product (1500 bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µl of nuclease free water.

Nucleotide sequencing. Sequencing pattern – The PCR amplicons obtained by amplifying PCR products was diluted in Tris buffer (10 mM, pH 8.5). The dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 ng/µl), the sequencing reaction required 8 µl DNA. The primer used in all sequencing reactions was 16 SF (5'AGAGTTTGATCCTGGCTCAG3') at a concentration of 3 µM. Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA).

Effect of process parameters on cellulase and xylanase production

Media. Five different media viz. Basic Liquid mineral Medium, Cellulolytic medium, CMC broth

medium, CMC production medium, Cellulose powder medium were studied to monitor their effect on cellulase production. For xylanase production, Basic enzyme production, TGY, Basal salt medium, Calik et al and synthetic medium were used.

Incubation period. Flask containing 45 ml of production medium were inoculated with 5.0 ml of seed culture and incubated at 37°C with constant shaking at 150rpm. Following incubation for various time interval (24, 36, 48 and 60 and 120 h), the culture filtrate was centrifuged and enzyme activity was determined.

Effect of pH and Temperature on Enzyme Activity. Cellulase and xylanase production was studied at pH values ranging from 4.0 to 9.0. Each flask containing 45 ml production medium at different pH was inoculated with 5.0 ml of 18 h old inoculum and incubated at 37°C for 48 hr in rotatory shaker incubator at 150 rpm.

The optimum temperature at optimum pH was determined by incubating the flasks containing 5 ml of inoculum at different temperatures ranging from 25 to 50°C. The enzyme activity was assayed in culture filtrate.

3. Results and Discussions

Cellulose is the main building block of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this carbon to the environment [11]. Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sourcesfor isolation of cellulolytic microorganisms [12]. About one fifth of fresh water and soil samples yield cellulose degrading bacteria after enrichment but some samples did not bear such kind of bacteria [13]. This is due to existence of microenvironments where different growth conditions for cellulose degrading bacteria are present. These bacteria are generally found in well manure soils [14].

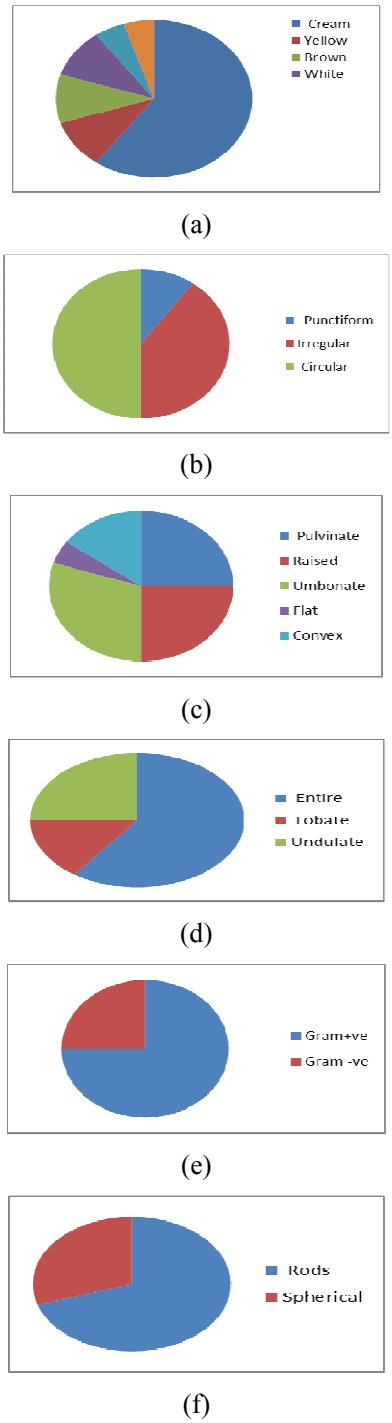

Figure 1a. Morphology of cellulolytic and xylanolytic bacterial isolates on basis of a) color b) configuration c) elevation d) margin e) Grams reaction f) shape

Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars [15] but the need for newly isolated cellulose degrading microorganism still continues [16].

In the present study, twenty cellulolytic bacteria were isolated from various sites. These bacteria were screened for cellulase production in submerged fermentation process using Riveris medium. Among all these bacterial strains; R2, R4 gave better yield of 0.484 and 0.529 IU/ml respectively as shown in Table 2. R2 and R4 isolates were further identified by using morphological and biochemical tests (Fig.1a and 1b).

Colonies were white and cremish in color, glistening; margins were entire, convex and circular and 1-2 mm in diameter at 30˚C. When scraped with a loop, colonies were slimy or viscous and tend to clump. Fresh culture of this isolate consists of Gram positive, slender and rod shaped cells but the older cultures contain coccoid cells. Microscopic examination of these isolate revealed that they were gram positive and catalase +ve.

Researchers studying on cellulolytic activity have isolated various bacteria from different environmental sources. Hatami et al. 2008 [17] isolated aerobic cellulolytic bacteriaf rom forest and farming soils and determined their ability to decompose cellulose. In another study, Otajevwo and Auyi, 2010 [18] isolated *Pseudomonas* sp. and *Serratia* sp. from soil samples, which have greater ability to produce cellulase enzyme.

Genotypic identification. Sequences of isolates so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search (mBLAST, NCBI) for the R2 and R4 showed 99 % homology with nucleotide sequence of *Bacillus licheniformis* R2 and *Bacillus mojavensis* R4 strain. The 16SrRNA sequences of these isolates have been registered under gene bank databases with accession numbers i.e. *Bacillus licheniformis* R2 [KJ 588781], *Bacillus mojavensis* R4 [KJ 588787], *Bacillus atropheaus* SD9 [KJ 590121].

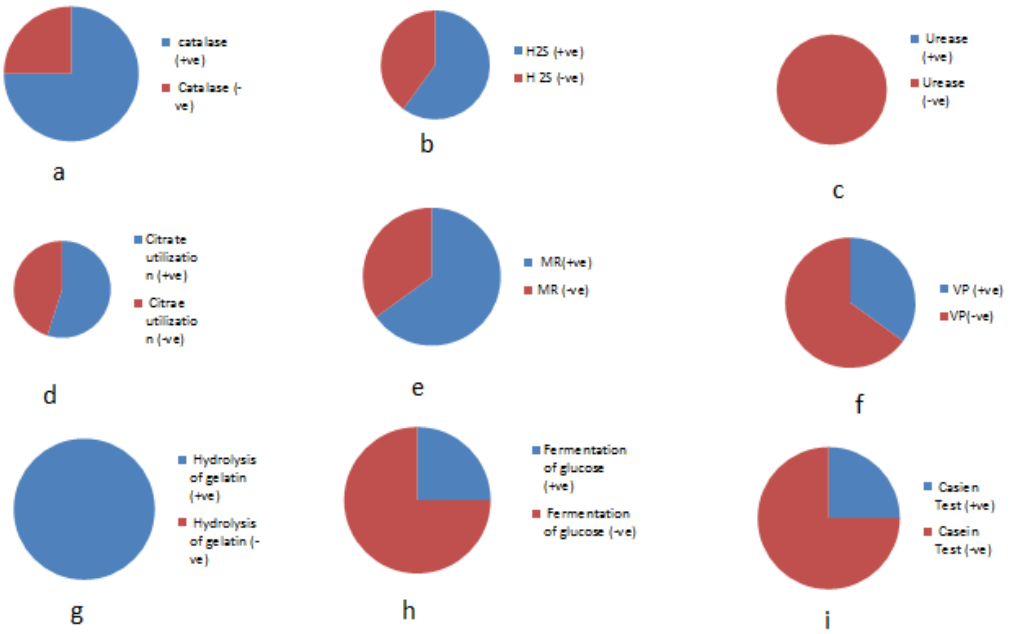

Figure 1(b). Biochemical characteristics of cellulolytic and xylanolytic bacterial isolates (a) Catalase test (b) H2S production (c) Urease test (d) Citrate utilization (e) MR- test (f) VP-test (f) Casein test (g) Gelatin Hydrolysis (h) Fermentation of glucose (i) casein test

Table 1. Screening of bacterial isolates for hypercellulase and xylanase production

S. No.	Isolate name	Protein (mg/ml)	CMCase activity		FPase activity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase		Proteins (mg/ml)	Xylanase		Total enzyme (cellulase + xylanase)	Total Proteins (mg/ml)
			Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity		Enzyme activity	Specific activity		
1	S1	1.58	0.155	0.098	0.127	0.080	0.029	0.018	0.311	0.196	1.38	2.65	1.92	0.576	2.96
2	S2	2.12	0.129	0.060	0.140	0.066	0.028	0.013	0.297	0.140	1.12	1.06	0.94	1.357	3.24
3	S3	1.43	0.070	0.048	0.138	0.096	0.025	0.017	0.233	0.162	1.30	0.68	0.52	0.913	2.73
4	S4	1.89	0.135	0.071	0.029	0.020	0.034	0.017	0.198	0.104	1.52	3.94	2.59	2.788	3.41
5	S5	1.09	0.014	0.012	0.033	0.030	0.034	0.031	0.081	0.074	1.31	0.91	0.69	0.991	2.40
6	S6	1.20	0.015	0.012	0.020	0.016	0.014	0.011	0.049	0.040	1.62	2.68	1.65	2.729	2.85
7	S7	1.58	0.083	0.052	0.023	0.014	0.016	0.010	0.122	0.077	1.23	3.44	2.78	3.562	2.81
8	SD8	2.85	0.076	0.026	0.048	0.016	0.120	0.042	0.244	0.085	1.02	4.07	3.97	4.314	3.87
9	SD9	3.39	0.144	0.042	0.124	0.036	0.110	0.032	0.378	0.111	1.11	4.31	3.87	4.688	4.50
10	SD10	1.20	0.019	0.015	0.020	0.016	0.018	0.015	0.057	0.047	1.10	2.75	2.50	2.807	2.30
11	SD11	1.89	0.027	0.014	0.029	0.015	0.090	0.047	0.146	0.077	1.08	8.22	7.59	8.366	2.97
12	SD12	1.00	0.013	0.013	0.023	0.023	0.057	0.057	0.093	0.093	1.12	6.77	6.00	6.863	2.12
13	R1	1.53	0.055	0.035	0.052	0.033	0.080	0.052	0.187	0.122	1.04	6.26	5.98	6.447	2.57
14	R2	3.32	0.170	0.051	0.130	0.039	0.122	0.036	0.422	0.127	1.45	2.72	1.87	3.142	4.77
15	R3	1.01	0.020	0.01	0.027	0.026	0.117	0.115	0.164	0.162	1.12	2.25	2.00	2.414	2.13
16	R4	1.00	0.092	0.092	0.402	0.402	0.112	0.112	0.522	0.522	1.12	2.48	2.21	3.002	2.12
17	C1	1.76	0.076	0.043	0.124	0.070	0.110	0.062	0.310	0.176	1.35	4.83	3.56	5.140	3.11
18	C2	1.00	0.014	0.014	0.125	0.125	0.090	0.09	0.219	0.219	2.26	1.56	0.69	1.779	3.26
19	C3	1.21	0.034	0.028	0.103	0.085	0.026	0.021	0.163	0.134	1.07	3.23	3.00	3.393	2.28
20	C4	1.90	0.020	0.10	0.090	0.047	0.024	0.012	0.134	0.070	1.03	2.06	2.00	2.194	2.93

Table 2. Effect of different pH on extracellular cellulase production from *Bacillus licheniformis* R2 under SmF

pH	Cellulase								
	Protein (mg/ml)	CMCase activity		Fpaseactivity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
4.00	0.42	0.18	0.42	0.19	0.45	0.14	0.33	0.51	1.21
5.00	0.48	0.22	0.45	0.21	0.43	0.15	0.31	0.58	1.20
6.00	0.72	0.40	0.55	0.22	0.30	0.10	0.13	0.82	1.13
7.00	0.59	0.42	0.71	0.22	0.37	0.10	0.16	0.70	1.18
8.00	0.46	0.22	0.47	0.20	0.43	0.12	0.26	0.54	1.17
9.00	0.40	0.27	0.67	0.25	0.62	0.08	0.20	0.60	1.50

Table 3. Effect of different pH on extracellular cellulase production from *Bacillus mojavensis* R4 under SmF

pH	Cellulase								
	Protein (mg/ml)	CMCaseactivity		Fpaseactivity		β-glucosidase		Cellulase (CMCase + FPase + β-glucosidase	
		Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity	Enzyme activity	Specific activity
4.00	0.46	0.25	0.54	0.25	0.54	0.09	0.19	0.59	1.28
5.00	0.32	0.19	0.59	0.24	0.75	0.12	0.37	0.55	1.71
6.00	0.84	0.38	0.45	0.34	0.40	0.17	0.20	0.89	1.05
7.00	0.61	0.30	0.49	0.33	0.54	0.12	0.19	0.75	1.22
8.00	0.50	0.39	0.78	0.18	0.36	0.09	0.18	0.66	1.32
9.00	0.28	0.15	0.53	0.15	0.53	0.10	0.35	0.40	1.42

Table 4. Effect of different pH on xylanase production from *B. atropheaus SD9* under SmF

pH range	Protein(mg/ml)	Xylanase(IU)	Specific activity
4.0	4.50	7.61	1.69
5.0	**5.00**	**8.04**	**1.60**
6.0	5.12	6.61	1.29
7.0	4.98	6.56	1.31
8.0	4.89	5.50	1.12
9.0	4.90	4.85	0.98

Optimization of cultural and nutritional conditions for cellulase and xylanase production

Effect of Different media. The data pertaining to effect of different media on cellulase and xylanase production detailed in Fig. 2-3 had revealed that highest cellulase activity was found in the CMC liquid medium for both isolates i.e. *Bacillus licheniformis* R2 and *Bacillus mojavensis* R4 0.484 IU/ml and 0.529 IU/ ml respectively. Least enzyme production was shown by basic liquid mineral medium i.e. 0.12 and 0.15 IU/ml respectively. For xylanase, synthetic medium showed maximum production (7.99 IU/ml) while minimum xylanase production (2.00 IU/ml) was shown by basic enzyme production medium (Fig. 4).

Utilization of CMC as carbon source is best for cellulase production as reported by Das et al. 2010 [19] for *Bacillus* sp (3.028µg/mg/min). Shabeb et. al., 2010 showed that addition of cellulose, filter paper, CMC, starch or cellobiose to the fermentation medium favoured cellulase production by *Cellulomonas* sp, *Clostridium* and *Bacillus* sp. *subtilis*.[21, 20]

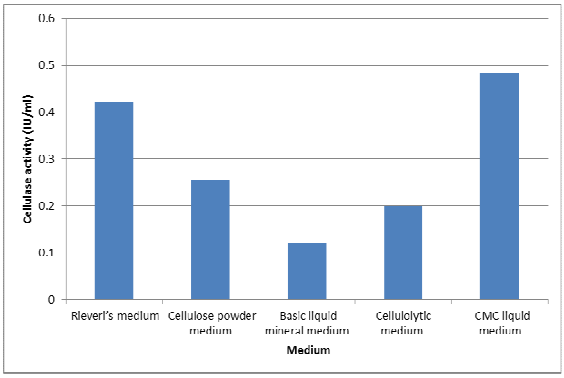

Figure 2. Effect of media on cellulase production from *B.licheniformis R2*

The present findings are in accordance with Jaradat et al. 2008 [22] who also achieved maximum cellulose production in a medium containing ammonium chloride as a nitrogen source. Vyas et al. 2005 [23] reported that the best inorganic nitrogen source for exoglucanase as well as endoglucanase activity is $(NH_4)_2SO4$. Similar findings were also obtained by Balamuruganet al. 2011 [24]. Addition of $(NH_4)_2PO4$ or tryptone to the molasses mediumen hances the cellulase production by *B.*

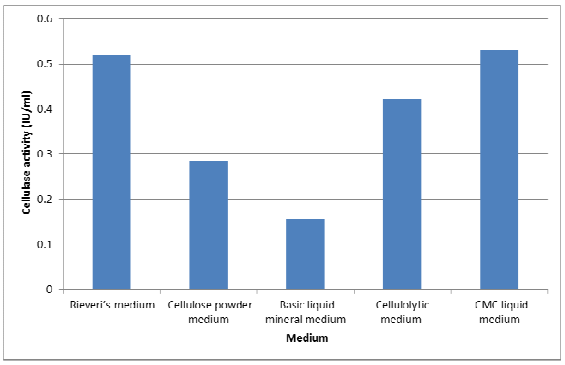

Figure 3. Effect of media on cellulase production from *B.mojavensis R4*

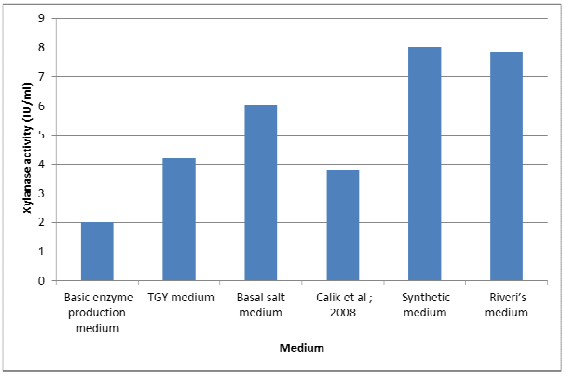

Figure 4. Effect of media on xylanase production from *B. atrophaeus* SD9

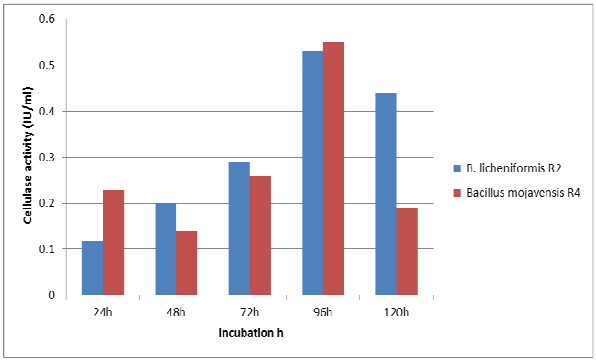

Figure 5. Effect of incubation time on cellulase production from *B.licheniformis* R2 and *B.mojavensis* R4

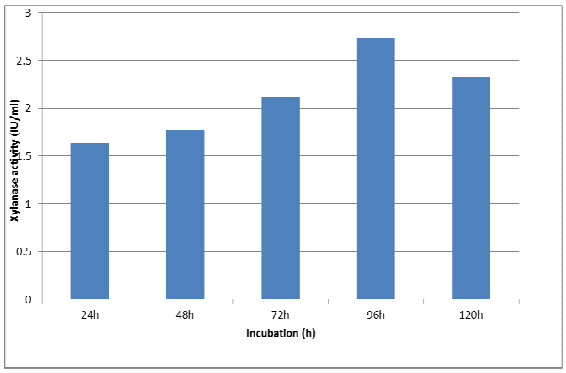

Figure 6. Effect of incubation time on xylanase production from *B.atropheaus* SD9

When the cell population in the culture reaches at its peak 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 produced by *B. licheniformis* R2 and *B. mojavensis* R4 as well as xylanase produced by *B. atropheaus* SD9 were growth associated, reaching to maximum at exponential peak. A decline in the enzyme afterwards may be because of proteolysis or due to depletion of nutrients available to the strain, causing a stressed microbial physiology resulting in an in activation of enzyme [27].

Effect of pH. Table 3 and 4 revealed the effect of pH on the medium for cellulase and xylanase production of cellulase and xylanase production was estimated at 4.0,….9.0. In these results, isolates R2 *Bacillus licheniformis* and R4 *Bacillus mojavenis* had the optimum pH 6 where the highest cellulase activities were 0.82 and 0.89 IU/ml, respectively which was significantly higher than others statistically and minimum was observed in pH 9 enzyme activity was 0.58 and 0.40 IU/ml respectively.

It was evident from the data in pH 5.00 (8.04 IU/ml) showed maximum xylanase production while minimum xylanase (4.85 IU/ml) was shown at pH 9.00 Table 5.

Since 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 their overall metabolic activity.

If the cultivation of the organisms is carried out at an unfavorable pH, it may limit their growth as well as enzyme production. Enzymes have an optimum pH in which their activity is maximum and at higher and lower pH values, their activities decreases because pH of the culture medium affects the availability of certain metabolic ions and permeability of cell membranes.

Effect of Temperature. Ko et al. (2010) [25] reported that temperature is one of the most important physical variable factor affecting enzyme activity. When the temperature increases up to optimum temperature, enzyme reaction rate increase due to the increasing of kinetic energy. Increasing in kinetic energy will accelerate the movement of vibration, translation, and rotation of both enzyme and substrate. This will cause enzyme and substrate to react. Effect of temperature on cellulase and xylanase production from hypercellulolytic and xylanolytic bacteria has been shown in Fig. 7.

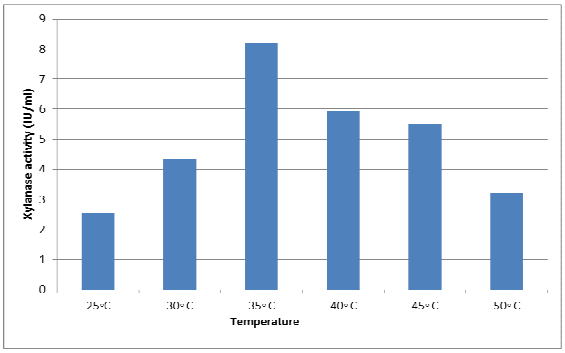

Figure 7. Effect of temperature on xylanase production from *B. atropheaus* SD9

Isolates *Bacillus licheniformis* R2 and *Bacillus mojavensis* R4 showed the highest cellulase activity at 35°C 0.99, 1.07 IU/ml respectively. Similar findings have been reported by Kumar et al. (2012) [31] showing increased enzyme activity at 35°C and found it as optimum temperature for cellulase production. In case of xylanase, *Bacillus atropheaus* SD9 maximum production at 8.22 IU at 35°C while minimum production at 2.54 IU/ml at 25°C (Fig. 8). Temperature plays an important role in enzymatic reactions.

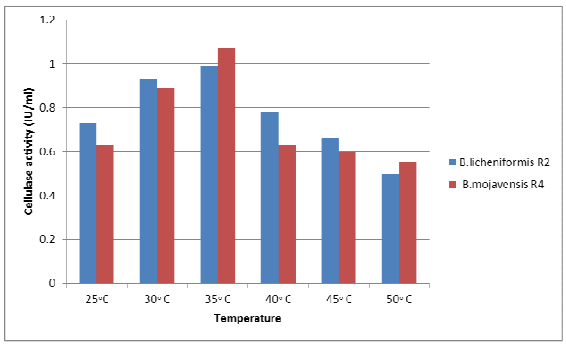

Figure 8.Effect of temperature on cellulase production from *B.licheniformis* R2 and *B.mojavensis* R4

Microorganisms grow slowly at a temperature below or above the normal temperature because of reduced rate of cellular production. If the growth temperature is too high but not lethal, there may be permanent induction of target protein expression [6]. At lower temperature, substrate transport across the cells is suppressed and lowest product yield are attained. At higher temperature, the maintaince energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway resulting in maximum production [29].

Optimum temperature recorded for maximum cellulase was 25°C for mutant *Bacillus pumilus* BpCRI6 [30], 30°C for *Cellulomonas* sp. and *Bacillus pumilus* [28]. Ingledew (1990) [26] reported that pH 6-7 is the optimum pH for the growth of the genus of *Bacillus*.

4.Conclusion

In the current study, hyper cellulolytic and hyper xylanolytic microorganisms were isolated from different sources and their optimum conditions for cellulase and xylanase production had been evaluated for enhanced enzyme production. The enhanced production of industrially important enzymes by potential isolates would help to scale up their production and commercialization after their purification.

Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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