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Research Article

Isolation, Production, and Characterization of Thermotolerant Xylanase from Solvent Tolerant *Bacillus vallismortis* RSPP-15

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Sixty bacterial strains isolated from the soils sample in the presence of organic solvent were screened for xylanase production. Among them, strain RSPP-15 showed the highest xylanase activity which was identified as *Bacillus vallismortis*. The isolate showed maximum xylanase production (3768 U/mL) in the presence of birch wood xylan and beef extract at 55°C pH 7.0 within 48 h of incubation. The enzyme activity and stability were increased 181.5, 153.7, 147.2, 133.6, and 127.9% and 138.2, 119.3, 113.9, 109, and 104.5% in the presence of Co^{2+}, Ca^{2+}, Mg^{+2}, Zn^{+2}, and Fe^{+3} ions (10 mM). Xylanase activity and stability were strongly inhibited in the presence of Hg and Cu ions. The enzyme was also stable in the presence of 30% of *n*-dodecane, isooctane, *n*-decane, xylene, toluene, *n*-hexane, *n*-butanol, and cyclohexane, respectively. The presence of benzene, methanol, and ethanol marginally reduced the xylanase stability, respectively. This isolate may be useful in several industrial applications owing to its thermotolerant and organic solvent resistance characteristics.

1. Introduction

Xylanase (endo-1,4-β-D-xylanohydrolase) is a hydrolytic enzyme that plays an important role in depolymerization of xylan, the main renewable hemicellulosic polysaccharide of plant cell wall. It is produced by many microorganisms like bacteria [1–3], fungi [4, 5], actinomycetes [6], and yeast [7]; though enzyme from fungal and bacterial sources has dominated applications in industrial sectors, bacterial xylanases are preferred as they grow rapidly, need less space, can be easily maintained, and are accessible for genetic manipulations [8]. Bacteria, mainly *Bacillus* sp., are capable of producing alkaline thermostable xylanases. Previous reports stated that *Bacillus* SSP-34, *Bacillus stearothermophilus* strain T6, *Streptomyces, Bacillus* sp. strain NCL 87-6, *Bacillus circulans* AB 16, and *Bacillus pumilus* SV-85S were used efficiently in the production of xylanases [9–12].

Recently, interest in xylanase has evidently increased due its broad variety of biotechnological purposes such as prebleaching of pulp, improving the digestibility of animal feed stocks, alteration of cereal-based stuffs, bioconversion of lignocellulosic material and agrowastes to fermentable products, clarification of fruit juices, and degumming of plant fibers [13, 14]. Cellulase-free xylanases active at high temperature and pH are gaining importance in pulp and paper industry as they reduce the need for toxic chlorinated compounds making the bleaching process environment friendly [15]. Submerged fermentation offers various advantages over solid state fermentation, including fermentation study, greater product yield, and easier scale-up of process. In this study, we isolate an extracellular thermosolvent tolerant xylanase from an alkalophilic strain of *Bacillus vallismortis* RSPP-15 from soil in the presence of organic solvents. After that, we optimized physicochemical and nutritional parameters for better xylanase production for industrial application.

2. Materials and Methods

2.1. Isolation, Screening, and Identification of Thermosolvent Tolerant Xylanase Producing Bacteria. The soil samples were collected aseptically from different sites of pulp and paper industry of Faizabad to isolate xylanase producing bacteria. One-gram soil was suspended in 9.0 mL sterile distilled water, agitated for a minute. Then 0.1 mL suspension was spread

over birch wood xylan agar plates (pH 7.0) containing 1.0% xylan (birchwood); 0.5% ammonium sulphate, and 2% agar. The inoculated plates were overlaid with 7.0 mL of organic solvents (ethanol, propanol, cyclohexane, toluene, butanol, methanol, and isopropanol) and incubated at 55°C, till sufficient growth appeared. After sufficient growth incubated plates were overlaid with Congo-Red solution (0.1%) for 10 min and then washed with 1 N sodium chloride solution for destaining. If a strain was xylanolytic, it started hydrolyzing the xylan present in the surrounding and in the zone degradation there was no red color formation. Selection was done as per colonies with and without clear and transparent zone as xylanase producing and xylanase nonproducing strain, respectively. Bacterial colonies showing clear zones were selected, streaked twice on xylan agar plates for purification, and maintained as pure culture over xylan agar slants (pH 7.0, 4°C). The isolate having maximum clearance zone was selected for further studies. The selected bacterial isolate RSPP-15 was identified by morphological and biochemical characterization as per Bergey's Manual of Systematic Bacteriology [16]. The identity of RSPP-15 was authenticated from Institute of Microbial Technology (IMTECH), Chandigarh, India, based on the phenotypic (16S rDNA) and biochemical tests. The bacterial isolate RSPP-15 was grown on xylan nutrient agar slants at 55°C for 24–48 h. The fully grown slants were stored at 4°C and were subcultured every two weeks.

2.2. Crude Enzyme Preparation and Enzyme Assay. The culture was grown in a 150 mL Erlenmeyer flask that contained 50 mL of basal medium containing 2.0% xylan and 0.5% ammonium sulphate. The pH of the medium was adjusted to 7.0 prior to sterilization. The flask was inoculated and incubated at 55°C for 24 h for sufficient growth. The crude enzyme was filtered and centrifuged at 12000 rpm for 10 min and enzyme assay was carried out. Xylanase was assayed by measuring the reducing sugar released by reaction on birchwood xylan. Xylanase assay was done by Nelson [17] and Somogyi [18] methods using a reaction mixture consisting of 500 μL of substrate solution (1.0% birchwood xylan in 1.0 M phosphate buffer, pH 7.0.), 100 μL of the enzyme solution, and 1 mL of volume maintained by adding 400 μL distilled water. The reaction mixture was incubated for 10 min at 55°C. Reaction was stopped by adding 1 mL of alkaline copper tartrate solution and incubated in boiling water bath for 10 min and cooled; then arsenomolybdate solution was added for color stabilization. Optical density of each sample with reaction mixture was taken at 620 nm in a spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 μg of glucose min/mL.

2.3. Biomass Determination. Bacterial cells in broth were harvested by centrifugation (10000 rpm for 10 min at 4°C), washed with distilled water, and dried in an oven at 80°C until reaching a constant weight. The biomass was reported in the form of dry cell mass (g/L).

2.4. Optimization of Physicochemical and Nutritional Parameters for Xylanase Production. The various process parameters influencing xylanase production were optimized individually and independently of the others. The optimized conditions were subsequently used in all the experiments in sequential order. For the optimization, the basal medium was inoculated and incubated at different temperatures, namely, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80°C under the standard assay conditions. The samples were withdrawn at every 8 h interval up to 72 h to study the effect of incubation period. The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 4.0 to 11.0 at 55°C using different suitable buffers at concentration of 100 mM citrate buffer (pH 4.0–6.0, 1 M), phosphate buffer (7.0-8.0), Tris-HCl buffer (pH 8.0-9.0), and glycine-NaOH (10–11.0) under standard assay conditions. The growth medium was supplemented with different carbon sources, namely, fructose, glucose, lactose, soluble starch, sucrose, birchwood xylan, sugarcane bagasse, wheat bran, rice bran, rice husk, and maize bran (at the level of 2%, w/v). Different organic nitrogen sources (beef extract, gelatin, casein, malt extract, peptone, and yeast extract, 0.5% w/v) and inorganic nitrogen sources (sodium nitrate, ammonium nitrate, ammonium chloride, potassium nitrate, ammonium sulphate, and urea, 0.5% w/v) were also used for enzyme production. Thereafter, optimized carbon and nitrogen sources were further optimized at different concentrations.

2.5. Effect of Metal Ions on Enzyme Activity and Stability. The effect of various metal ions on enzyme activity was investigated by using $FeSO_4$, $CaCl_2$, NaCl, $MgCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $CoCl_2$, $HgCl_2$, and $NiCl_2$ at a final concentration of 5 mM and 10 mM. The enzyme was incubated with different metals at 55°C for 1 h to study metal ion stability of the enzyme and assayed under standard assay conditions. The enzyme activity was measured by conducting the reaction at temperature 55°C and pH 7.0. The activity of the enzyme was considered as 100% under standard assay conditions.

2.6. Effect of Organic Solvent on Xylanase Stability. Cell free supernatant having maximum xylanase activity was filtered with nitrocellulose membrane (pore size 0.22 μm) and incubated with 30% (v/v) of different organic solvents, namely, *n*-dodecane, *n*-decane, isooctane, *n*-octane, xylene, *n*-hexane, *n*-butanol, cyclohexane, *n*-heptane, benzene, toluene, ethanol, methanol, and propanol for 7 days in screw crapped tubes at 55°C and 120 rpm. The residual xylanase activity was estimated against the control, in which solvent was not present.

2.7. Characterization of Crude Enzyme

2.7.1. Effect of Temperature on Enzyme Activity and Stability. The influence of temperature on activity of xylanase was studied by incubating the reaction mixture at different temperatures (35–100°C). The enzyme was incubated at different temperatures, 35–100°C, for 1 h to study the stability of the enzyme. The residual xylanase activity was determined by

performing the reaction at temperature 55°C and pH 7.0. The activity of the enzyme was considered as 100% under standard assay conditions.

2.7.2. Effect of pH on Enzyme Activity and Stability. The effect of pH on xylanase activity was measured in the pH range of 4 to 10, using the appropriate buffers at concentration of 100 mM (4.0–6.0, sodium acetate; 6.0–8.0, sodium phosphate; 8.0–10.0, Tris-HCl) under standard assay conditions. To evaluate the stability as a function of pH, 100 μL of the purified enzyme was mixed with 100 μL of the buffer solutions and incubated at 55°C for 1 h; then, aliquots of the mixture were taken to determine the residual xylanase activity (%) under standard assay conditions.

2.8. Statistical Analysis. Each experiment was performed thrice in triplicate, and mean standard deviation for each experimental result was calculated using the Microsoft Excel.

3. Results and Discussion

3.1. Isolation, Screening, and Identification of Thermosolvent Tolerant Xylanase Producing Bacterial Cultures. Sixty (60) bacterial isolates producing variable xylanolytic zones on birchwood xylan agar plates stained with Congo-Red solution followed with sodium chloride solution were studied. The zones of clearance by isolates reflect their extent to xylanolytic activity. Those having clearance zone greater than >1.0 cm were considered as significant isolates. Among 60 bacterial isolates, 35 bacterial isolates exhibited good xylanase activities which were reassessed by loading their culture broth in the wells on birchwood xylan agar plates which stained with Congo-Red solution followed with sodium chloride solution (pH 7.0). The culture broth having good xylanase activity cleared more than >1.0 cm zone within 4-5 h of incubation at 55°C, thereby indicating an extracellular nature of the xylanase. The isolate RSPP-15, showing maximum clearance zone diameter, was selected for further studies.

The efficient strain RSPP-15 was rod-shaped, Gram-positive, motile, aerobe, and facultative in nature. It gave positive results for acetylmethylcarbinol, catalase, and oxidase test. It grew over a wide range of pH (4.0–11), temperatures (10–85°C), and sodium chloride concentrations (0.0–12%) and was able to hydrolyze gelatin, casein, starch, and Tween 20, 40, and 80. It produced acid (acetic and lactic acid) from glucose, xylose, mannitol, and arabinose. It gave positive test for citrate utilization and nitrate reduction. The strain was halotolerant as it grew in the presence of 0.0–12% sodium chloride. On account of morphological and biochemical characteristics, it was identified as *Bacillus* sp. by MTCC MTECH, Chandigarh (India). Analysis of 16S rDNA sequence revealed its 99.3% homology with *Bacillus vallismortis* strains, and it was designated as *Bacillus vallismortis* RSPP-15. The 16S rDNA sequence was submitted to GenBank [JQ: 619483]. The strain RSPP-15 was in the same cluster of phylogenetic tree (Figure 1) with different strains of *Bacillus vallismortis*. However, the 16S rDNA sequence analysis indicates that it is a different and novel strain of *Bacillus vallismortis*.

3.2. Effect of Temperature on Xylanase Production. Influence of temperature on xylanase production in submerged fermentation is one of the important parameters. Figure 2 depicted that the maximum enzyme production (560 U/mL) was obtained at 55°C with 2.3 g/L biomass production while minimum (119.8 U/mL) production was observed at 35°C. It retained its 80% activity at 75°C. Similar results for optimum temperature for xylanase activity of *Bacillus aerophilus* KGJ2 have been reported by Gowdhaman et al. [19]. Xylanase with similar temperature optima had been reported from *Bacillus licheniformis* in the broad range of 40°C to 100°C [20]. Our observations showed that xylanase from *Bacillus vallismortis* RSPP-15 could be useful for industrial applications at the temperature range of 35–70°C. Most of workers have reported that xylanase of *Bacillus* sp. retained its 100% activity at 70–80°C [8, 21]. Thermal stabile xylanase finds potential applications in many industries [22]. Xylanase enzyme produced by *B. vallismortis* RSPP-15 shows interesting characteristics and properties and it appears to be a prospective candidate for application in feed and food industries.

3.3. Effect of Different Incubation Periods on Xylanase Production. Just after optimization of temperature for xylanase production in the liquid medium, incubation period was optimized for enzyme production. The results clearly indicated that *B. vallismortis* RSPP-15 shows maximum 689.2 U/mL enzyme production with 2.3 g/L biomass production within 48 h of incubation (Figure 3). Further increase in the incubation period did not increase the enzyme production but the stability of enzyme is 87% in 72 h. Similarly, Nagar et al. [23] and Kamble and Jadhav [24] reported that the highest enzyme titer from other *Bacillus* spp. was recorded at 48 h and 72 h. In contrast to our results, Kumar et al. [25] reported that xylanase production by *B. pumilus* VLK-1 was maximum (29318 IU/g) in 96 h, after which a gradual decrease was observed. It may be due to denaturation or decomposition of xylanase owing to interaction with other components in the medium, as it is reported elsewhere [24]. Incubation time depends on the characteristics of the culture, growth rate, and enzyme production. Thus our strain produced xylanase within 48 h of incubation and it is thus better than reported by the other workers mentioned above.

3.4. Effect of Initial pH on Xylanase Production. Initial pH of the medium is playing a vital role in enzyme production. To study the effect of initial pH on xylanase production, medium was adjusted using different buffers. It was observed that the maximum xylanase production (756.9 U/mL) with 2.5 g/L biomass production by strain *B. vallismortis* RSPP-15 was achieved at pH 7.0. Xylanase production was also remarkable at pH 6.0–9.0, while the production was less at pH 10.0-11.0 (Figure 4). The enzyme retained its 89% activity at pH 9.0, indicating an alkaliphilic nature of the *B. vallismortis* RSPP-15. Similar pH optimum for xylanase production from *Bacillus* sp. was reported by Guha et al. [26].

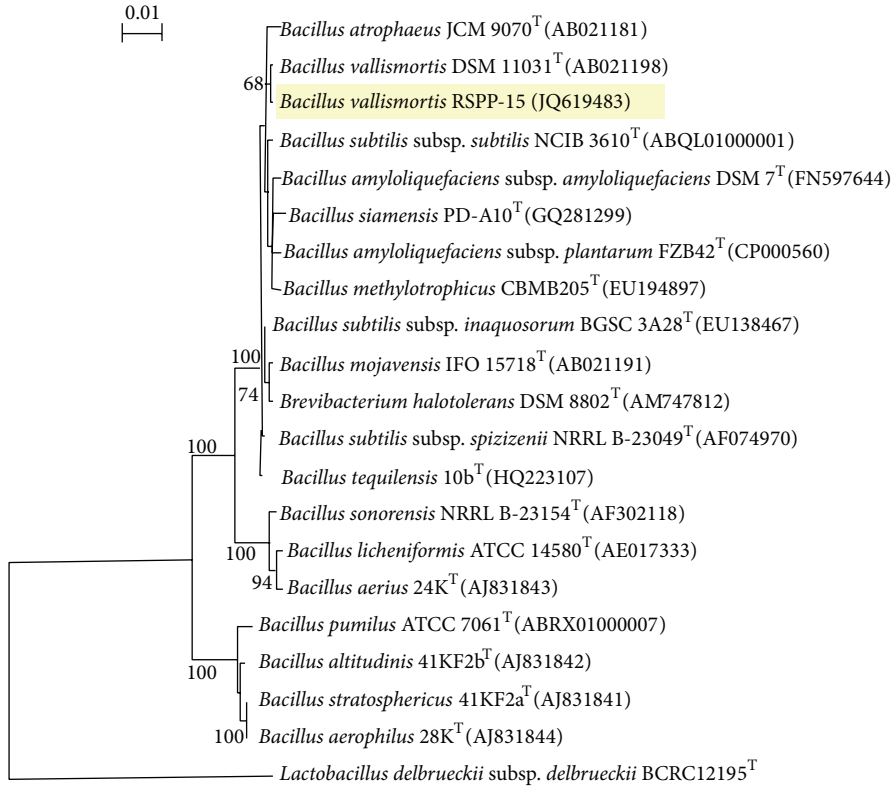

Figure 1: Phylogenetic tree showing relation between strain RSPP-15 and other *Bacillus* strains.

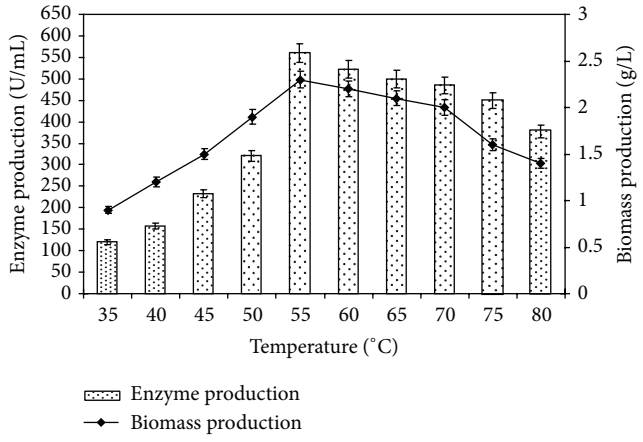

Figure 2: Effect of temperature on xylanase production. The flasks were inoculated with culture in the medium and were incubated at different temperatures (35–80°C) for 48 h at pH 7.0. For enzyme activity reaction mixture was incubated at 55°C for 15 min and reaction was conducted as standard assay method. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

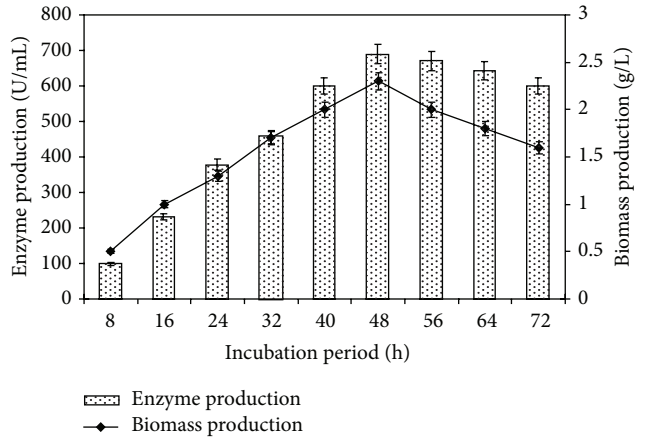

Figure 3: Effect of incubation periods on xylanase production. The flasks were inoculated with culture and were incubated at different incubation periods (8–72 h) at initial pH 7.0, 55°C. For enzyme activity the reaction was assayed at respective incubation periods at 55°C for 15 min. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines [27]. Growth of microorganisms is vastly affected by the medium pH as pH influences the transport of nutrients as well as the enzymatic systems in microorganism [11]. If the pH of the medium is unfavorable, the growth and xylanase production may be restricted due to substrate inaccessibility [19].

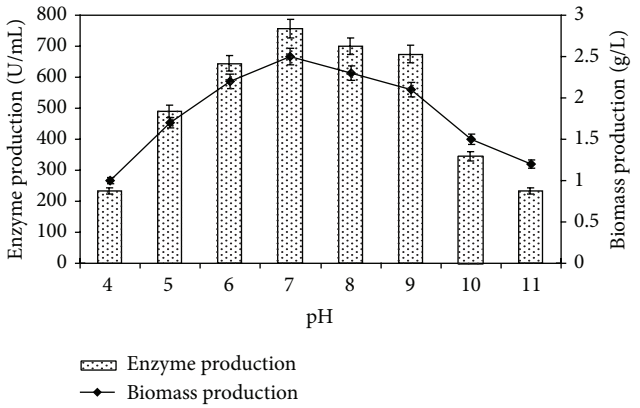

Figure 4: Effect of pH on xylanase production. The flasks were inoculated with culture and were incubated at different pH (4–11) for 48 h at 55°C. For enzyme activity the reaction was assayed at respective pH with buffers (100 mM) at 55°C for 15 min. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

3.5. Effect of Carbon Sources and Their Concentrations on Xylanase Production. Various carbon sources, namely, starch, sugarcane bagasse, birchwood xylan, wheat bran, rice bran, rice husk, glucose, fructose, lactose, maltose, and sucrose, at a concentration of 2.0% (w/v) were individually tested in the basal medium at their optimal temperature, incubation period, and pH to observe the effect on enzyme production by *B. vallismortis* RSPP-15. Out of these carbon sources, birchwood xylan was found the best for xylanase production (980 U/mL) with 2.6 g/L biomass production followed by sugarcane bagasse (923 U/mL) within 48 h (Figure 5). Similarly, Garg et al. [28] and Guha et al. [26] reported that *Bacillus halodurans* MTCC 9512 and *Bacillus* sp. gave the highest enzyme yield with birchwood xylan followed by sugarcane bagasse.

B. vallismortis RSPP-15 showed considerable enzyme production with fructose, lactose, and sucrose (Figure 5). Several workers also reported that most of *Bacillus* spp. showed considerable enzyme production in the presence of sucrose, fructose, and lactose [19, 24]. *B. vallismortis* RSPP-15 also showed considerable enzyme production in the presence of wheat bran, rice bran, and maize bran (Figure 5). Similar result was achieved from *Bacillus* sp. in the presence of wheat bran and rice bran as reported by Guha et al. [26]. *B. vallismortis* RSPP-15 showed minimum enzyme production in the presence of glucose (Figure 5). Garg et al. [28] also observed no xylanase production by *B. halodurans* MTCC 9512 when medium was supplemented with glucose. The production was repressed in the presence of glucose suggesting the possible regulation via catabolite repression. The expression of genes encoding extracellular hydrolytic enzymes such as xylanase is generally activated by specific substrates [29].

In another set of the experiment, different concentrations of birchwood xylan in the medium were tested for xylanase production at the same growth conditions at which carbon sources were evaluated. *B. vallismortis* RSPP-15 showed

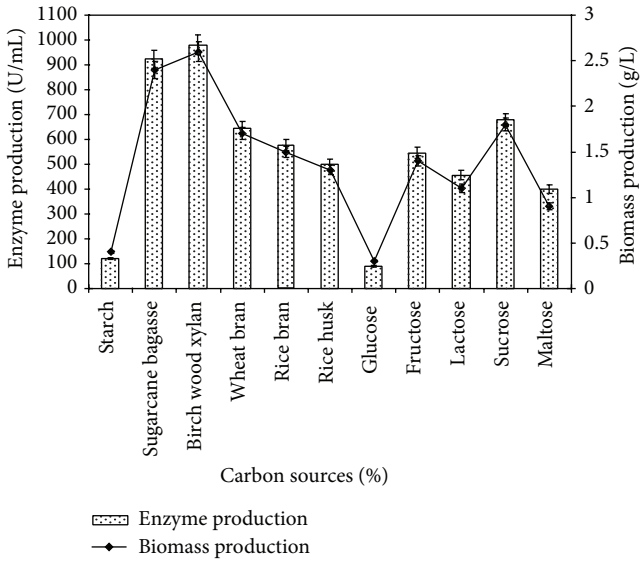

Figure 5: Effect of different carbon sources on xylanase production. Test flasks contained different carbon sources in the medium at a level of 2% (w/v). The flasks were inoculated with culture and incubated at 55°C for 48 h at pH 7.0. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

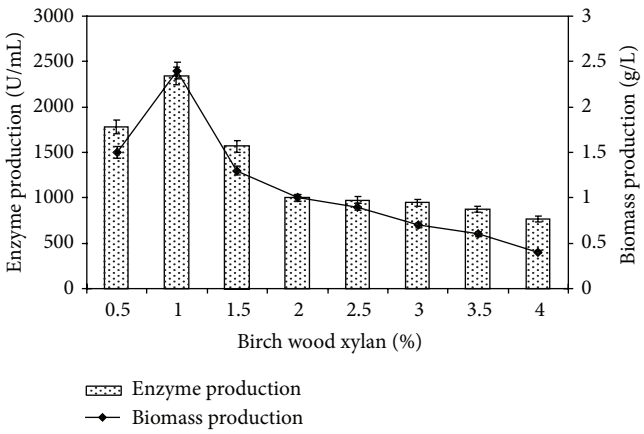

Figure 6: Effect of different concentrations of birch wood xylan on xylanase production. Test flasks contained different concentrations of birch wood xylan (0.5–4.0%, w/v) in the medium. The flasks were inoculated with culture and incubated at 55°C for 48 h at pH 7.0. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

2340 U/mL xylanase production with 2.4 g/L biomass production at 1% birchwood xylan; above this concentration enzyme production was slightly decreased (Figure 6). Similarly, Guha et al. [26] reported that the highest xylanase activity was obtained when xylan was used at 1% concentration and enzyme level was decreased with further increase in xylan concentration, yet some other workers reported that 0.5% xylan showed maximum enzyme production [19, 24].

3.6. Effect of Nitrogen Sources on Xylanase Production. Inorganic and organic nitrogen sources, namely, peptone, beef extract, yeast extract, malt extract, gelatin, casein, urea, sodium nitrate, ammonium nitrate, potassium nitrate, ammonium sulphate, and ammonium chloride, at the rate of 0.5% (w/v) were used in the basal medium for xylanase production (Figure 7). The enzyme production by the isolate was maximum in beef extract amended medium (3245 U/mL) followed by peptone, ammonium chloride, and ammonium sulphate. Similar observations were also reported by Swarnalaxmi et al. [20] and Gowdhaman et al. [19]. Haddar et al. [30] also reported that ammonium chloride favored growth and enzyme secretion by bacterial strains followed by yeast extract and soy peptone. Other nitrogen sources like urea showed inhibitory effect on xylanase production of *B. vallismortis* RSPP-15. Gowdhaman et al. [19] have already reported that supplementation of urea at 5 g/L concentration resulted in a decrease in xylanase production.

Different concentrations of beef extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 7.0, 8.0, 9.0, and 1.0%, w/v) in the medium were also tested for xylanase production at the same growth condition at which nitrogen sources were evaluated. *B. vallismortis* RSPP-15 showed higher enzyme production (3768 U/mL) with 2.6 g/L biomass production at 0.3% beef extract concentration; increasing further the concentration, enzyme production was reduced (Figure 8).

3.7. Effect of Metal Ions on Enzyme Activity and Stability. In this experiment, maximum xylanase production was reported in the presence of Co^{2+} (10 mM) followed by Ca^{2+}, Mg^{+2}, Zn^{+2}, and Fe^{+3}. In this experiment, maximum enzyme activity (3768 U/mL) considered 100% xylanase activity. Results suggest that xylanase showed maximum relative activity (181.5, 153.7, 147.2, 133.6, and 127.9%) and stability (138.2, 119.3, 113.9, 109, and 104.5%) in the presence of Co^{2+}, Ca^{2+}, Mg^{+2}, Zn^{+2}, and Fe^{+3} ions, respectively. Some other researchers also reported that Co^{2+}, Ca^{2+}, Mg^{+2}, Zn^{+2}, and Fe^{+3} ions strongly stimulated xylanase activity [31, 32]. The enzyme activities were enhanced in the presence of metal ions, which may be due to the alteration of structural conformation of the enzyme [33]. Xylanase activity was slightly inhibited by Mn^{2+} (Table 1). Xylanase was strongly inhibited in the presence of Cu^{2+} and Hg^{2+}. Similar results were observed in case of *Bacillus subtilis* [34], *Bacillus halodurans* PPKS-2 [35], and *Simplicillium obclavatum* [36]. It has been reported that the xylanase activity was inhibited by Hg^{2+} ion, which might be due to its interaction with sulfhydryl groups of cysteine residue in or close to the active site of the enzyme [37]. The inhibition of xylanase by Cu^{2+} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity.

3.8. Effect of Organic Solvents on Xylanase Stability. In another approach, the effect of various organic solvents (30%, v/v) on xylanase stability was also investigated for 7 days,

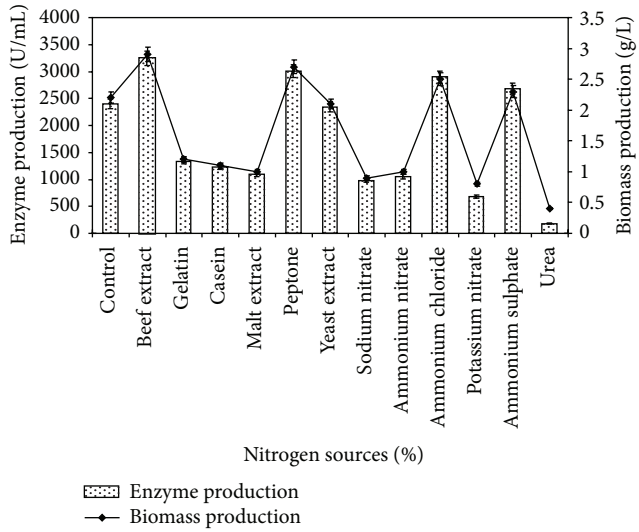

Figure 7: Effect of different nitrogen sources on xylanase production. The control flask does not contain any nitrogen sources. Test flasks contained different nitrogen sources in the medium at a level of 0.5% (w/v). The flasks were inoculated with culture and incubated at 55°C for 48 h at pH 7.0 with 1.0% birch wood xylan. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

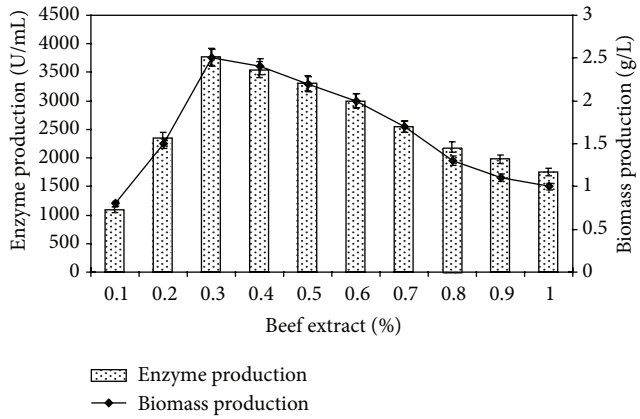

Figure 8: Effect of different concentrations of beef extract on xylanase production. Test flasks contained different concentrations of beef extract (0.1–1.0%, w/v) in the medium. The flasks were inoculated with culture and incubated at 55°C for 48 h at pH 7.0. Error bars presented mean values of ± standard deviation of triplicates of three independent experiments.

and the results are depicted in Table 2. The xylanase of *B. vallismortis* RG-01 is extraordinarily stable in the presence of all organic solvents under study. It was observed that, except benzene, methanol, and ethanol, presence of other solvents enhanced the xylanase activity. After incubation with *n*-dodecane, isooctane, *n*-decane, xylene, toluene, *n*-hexane, *n*-butanol, and cyclohexane, the xylanase activity increased to 230.8, 137.7, 219.8, 107, 190.5, 194.7, 179.3, and 111.6%, respectively. The presence of benzene, methanol, and ethanol marginally reduced the xylanase with residual activities of

Table 1: Effect of metal ions on enzyme activity and stability.

Metal ions	Concentration (mM)	Residual activity (%)	
		Activity	Stability
Control		100.0	100.0
$CaCl_2$	5	104.4	135.2
	10	153.7	119.3
$NiCl_2$	5	101.8	99.1
	10	110.4	100.5
$FeSO_4$	5	112.2	126.3
	10	127.9	104.5
$MgCl_2$	5	110.9	125.6
	10	147.2	113.9
$CuSO_4$	5	62.4	53.5
	10	50.7	42.5
$HgCl_2$	5	41	47.7
	10	21	29
$MnCl_2$	5	90.9	83.9
	10	85.9	80.2
NaCl	5	106.2	101.3
	10	121.9	115.9
$ZnSO_4$	5	102.9	128.4
	10	133.6	109
$CoCl_2$	5	125.4	107.8
	10	181.5	138.2

Enzyme activity was determined at 55°C in the presence of metal ions in the reaction mixture directly and for stability enzyme was preincubated with different metal ions at 55°C for 1 h and assayed as standard assay method. The enzyme activity without incubation with metal ions was taken as 100%. Mean standard deviation for all the values is <±5.0%.

Table 2: Stability of xylanase in the presence of various organic solvents.

Organic solvents (30%)	log *P*	Residual activity (%)							
		1 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Methanol	−0.76	100	119.2	131.1	118.5	110.3	101.9	94.5	88.4
Isopropanol	−0.28	89	95.7	100	90.6	87	80	77.9	73.8
Ethanol	−0.24	89	93.6	97.9	90.2	89.2	85.5	80.8	78.3
Benzene	2.13	90	93	100	100	98	94	90	85.8
Cyclohexane	3.3	90	95	100	111.6	101.5	93	92	91.6
Acetone	−0.23	95.3	100.4	100.5	95.4	90	90	83	80
Butanol	−0.80	90.6	179.3	154.7	124.5	112.5	100.8	96.7	90
Toluene	2.5	96.7	190.5	170.6	149.9	100.7	99.0	90.6	90
Isooctane	2.9	97.5	137.7	116.8	102.5	100.6	92.7	90.5	87.9
Xylene	3.1	90	100	107	103	100	96	90	86
Hexane	3.6	98	179	194.7	179.8	147.5	119.9	104	95
n-Decane	5.6	99.8	189.3	219.8	208.4	160.9	137.8	107	90.0
n-Dodecane	6.0	100.8	207.5	230.8	219.5	200.6	160.9	140.0	100.0

Enzyme was preincubated with different organic solvents at a concentration of 30% (v/v) at 55°C for different time periods and assayed as standard assay method. The enzyme activity without incubation with organic solvent was taken as 100%. Mean standard deviation for all the values is <±5.0%.

85.8, 88.4, and 78.3%, respectively. An organic solvent stable alkaline protease has been reported from *P. aeruginosa* PseA by Gupta and Khare [38]. After 10 days of incubation with organic solvent (25%, v/v), the residual protease activities were 112, 75, 98, 92, 97, 94, 75, 90, 96, 102, and 104% in the presence of ethanol, 1-butanol, benzene, toluene, xylene, cyclohexane, hexane, heptane, isooctane, *n*-decane, and *n*-dodecane, respectively. Abusham et al. [39] also reported a protease of *B. subtilis* strain rand with enhanced activity in the presence of organic solvents (25%, v/v) of log *P* value reduced the protease activity by 37–65%. It is therefore evident from our study that xylanase of *Bacillus vallismortis*

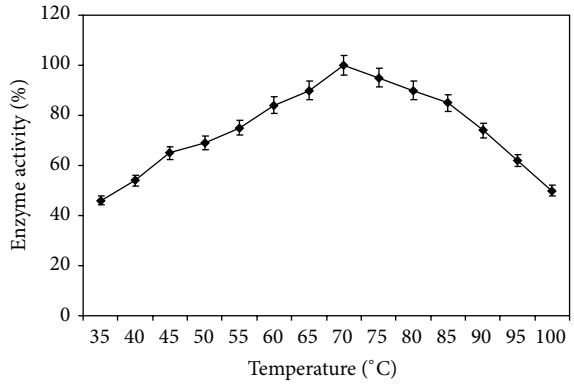

Figure 9: Effect of temperature on enzyme activity. For enzyme activity reaction mixture was incubated at different temperatures (35–105°C) for 1 h and reaction was conducted as standard assay method.

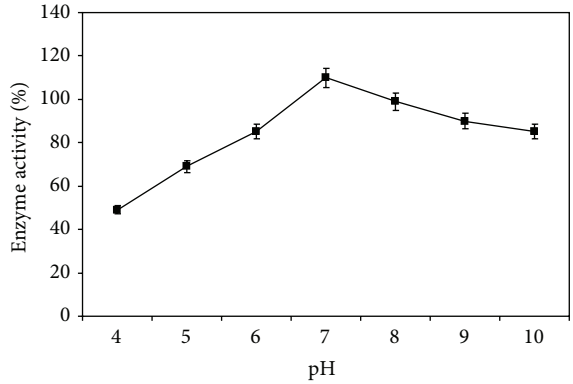

Figure 10: Effect of different pH on enzyme activity. For enzyme activity the reaction was assayed at respective pH and enzyme was preincubated with buffers (100 mM, in ratio 1 : 1) of different pH (4–10) at 55°C for 1 h and assayed by standard assay method.

RG-01 is remarkably stable in the presence of broad range of hydrophilic as well as hydrophobic organic solvents employed in this study. Hence, it is qualified for use in biotechnological applications and bioethanol production, and all its properties make it a useful tool for biobleaching in pulp and paper industry [40].

3.9. Characterization of Crude Enzyme

3.9.1. Effect of Temperature and pH on Enzyme Activity. Influence of temperature on xylanase activity is one of the important parameters. Figure 9 showed that more than 65–90% of the maximum activity was retained between 45°C to 65°C and about 100% activity was retained at 70°C. Xylanase with similar temperature optima had been reported from *Bacillus aerophilus* KGJ2 in the broad range of 30°C to 70°C [19]. Our observations showed that the xylanase from *Bacillus vallismortis* RG-01 could be useful for industrial applications at the temperature range of 45°C–70°C.

The effect of pH on enzyme activity was examined by evaluating the enzyme activity at varying pH values ranging from 4.0 to 10.0 using different suitable buffers. The crude enzyme of *Bacillus vallismortis* RSPP-15 was active at a wide range of pH from 5.0 to 9.0. It is observed that the highest xylanase activity was established at pH 7.0; on the other hand, it was found to be most stable at pH 7.0-8.0 (Figure 10). Similar pattern of pH optimum for enzyme activity was also found in *Bacillus* sp. NTU-06 [41]. Above and below of these pH values, xylanase activity decreased rapidly. Xylanase from *Bacillus vallismortis* RSPP-15 was stable in a range of pH 5.0–9.0 and at pH 10.0 approximately 85% of its activity was retained (Figure 10). The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines [27].

4. Conclusion

A thermosolvent stable xylanase is produced by a novel isolate *B. vallismortis* RSPP-15. The organism appears to have greater potential for enhanced enzyme production through optimization of nutritional and physical parameters. Tolerance against organic solvent and metal ions facilitates its use for various processes under stressed conditions. Owing to its thermotolerant nature, its xylanase may have potential uses in industries such as detergent, food, pharmaceutical, leather, agriculture, kraft pulp prebleaching process, and molecular biology techniques.

Conflict of Interests

The authors declare that they have no conflict of interests.

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