



Isolation, Identification and Partial Optimization of Novel Xylanolytic Bacterial Isolates from Bhilai-Durg Region, Chhattisgarh, India

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Received: 30 Aug. 2015; Revised: 5 Sep. 2017; Accepted: 16 Sep. 2017; Published online: 11 Aug. 2018

Background: Plant biomass and agricultural waste products disposal is a serious problem in agriculture based countries. These wastes, usually rich in xylan can be satisfactorily converted to industrially important and useful products by efficient biotechnological application of potent xylanase producing bacteria which generally have high temperature and pH optima. **Objective:** The aim was to isolate and identify xylanolytic bacterial isolates from Bhilai-Durg region of Chhattisgarh, India which was otherwise unexplored for isolation of thermoalkalophilic xylanase producing bacteria. Partial scale up of process development was performed. **Materials and Methods:** Xylanase producing bacteria were isolated from probable samples following three stages of screening procedures. The potent isolates were identified and various parameters affecting xylanase production were optimized using the conventional one-factor-at-a-time approach. **Results:** Two potent indigenous bacterial isolates belonged to genus *Bacillus* and designated as *Bacillus sp.* MCC2728 and *Bacillus sp.* MCC2727 were isolated from forest soils with the ability to degrade xylan. Significant differences were observed in their morphology and phenotypic characters amongst themselves and with its closest type strains implying the novelty of the two isolates. After optimization, maximum xylanase levels were obtained at pH 9.0, 55 °C for *Bacillus sp.* MCC2728 and 50 °C for *Bacillus sp.* MCC2727, 5% inoculum, agitation speed (150 rpm). Yeast extract and peptone are best nitrogen sources and wheat bran, the best carbon source. The GenBank/EMBL/DDBJ accession numbers of strains *Bacillus sp.* MCC2728 and *Bacillus sp.* MCC2727 are KP742971 and KT444621 respectively. Wheat bran, Yeast extract and peptone proved to be the best carbon and nitrogen sources respectively and xylose as an additive was found to be contributing to maximize the xylanase yields. **Conclusion:** Two potent thermoalkalophilic novel bacterial isolates were successfully isolated with xylan degrading ability which may be used as promising xylanase producing candidates for various industrial purposes using agricultural based waste residues.

Keywords: *Bacillus sp.*, Optimization, Phylogenetic analysis, Thermoalkalophilic bacteria, Xylanase.

1. Background

Hemicelluloses are considered as the second most abundant polysaccharide in nature after cellulose, which represents 20-35% of plant biomass on earth. The most common hemicelluloses found in plants and trees are xylan (1). This common hemicellulosic polysaccharide is distributed in many types of tissues and cells in a variety of plant species but found abundantly in woody and annual plants (2). In case of cell walls of land plants, xylan represents about 30% of

dry weight in hardwoods from angiosperms and about 12% of dry weight in softwoods from gymnosperms (3). Xylan being one of the abundant materials in plant biomass is also found in solid agricultural and agro industrial residues (4) which can be potentially used to produce various industrially useful products like biofuels, animal feed, enzymes etc. (5). Xylan has many uses in industries such as production of numerous value added products (6). Moreover, the ever growing human population has propelled mankind to use

advanced agricultural practices thereby enhancing crop production and yield. This increased demand of food crops has resulted in problems related to proper disposal of agricultural waste residues leading to pollution. The existence of such pollution problems has encouraged their utilization for various bioconversion processes and production of many industrially useful products. The efficient use of plant biomass particularly non-food lignocellulosic material as renewable resource for efficient biotechnological applications has become increasingly important in recent years (7). Looking at the present global scenario; the main aim is to find enzymes that can be integrated into industrial processes for converting biomass into valuable products. A distinct class of enzymes produced by microorganisms is xylanases. These enzymes have incurred a lot of interest in the recent times, as the substrate, xylan is of special interest as a renewable resource. It is a large component of agricultural wastes and thus can be used as a source of biomass (8). Xylanases have created a niche for themselves in the field of enzyme technology due to having an biotechnological applications. Although fungi have incurred a great interest as potent xylanase producers, but the simple nutritional requirements, high growth rate, less generation time, high temperature and pH optima, negligible cellulase activities, considerably good levels of xylanase and low protein purification costs make bacterial xylanases the preferred choice for various industrial processes (9).

2. Objective

The state of Chhattisgarh, India is known for its rich resources and biosphere which is endowed with thick and diverse vegetation. Its many virgin forests which are yet to be explored make it a biodiversity hotspot. A study was undertaken to explore industrially important xylanase producing bacteria from the rich natural resources of Bhilai- Durg region of Chhattisgarh, India as there are no published reports till date on isolation and screening of xylanolytic bacterial isolates from this region.

3. Materials and Methods

3.1. Sample Collection

Different types of samples including forest soil, dung, decaying wood and lignocellulosic material were collected from different areas of Bhilai-Durg region of Chhattisgarh state, India and immediately transferred to the sterile containers and stored at 4 °C till further use.

3.2. Isolation and Screening

One gram of each of the collected samples were

suspended in 10 mL of sterile distilled water and serially diluted till 10^{-5} dilution. 0.1 mL of the highest dilution was then spread plated on hemicellulose rich; wheat bran agar medium [5% (w/v) wheat bran, 0.5% (w/v) peptone, 0.5% (w/v) NaCl, 0.3% (w/v) yeast extract and 1.5% (w/v) agar] for the enriched growth of hemicellulose utilizing bacterial isolates by incubating for 3 days (10). Unless specified the sterilization steps were performed at 121 °C (15 lb.inch⁻²) for 20 minutes. The pH of the sterilized medium was adjusted to 9.0 with sterile 10% Na₂CO₃ solution and incubation temperature maintained at 50 °C to encourage thermoalkalophilic bacterial isolates. Bacterial isolates obtained in this preliminary screening step were sub-cultured to purity and each of the isolates were then spot inoculated on xylan agar medium [0.5 % (w/v) Birch wood xylan, 0.5 % (w/v) Yeast extract, 0.5 % (w/v) Peptone, 0.02 % (w/v) MgSO₄.7H₂O, 0.1 % (w/v) K₂HPO₄ and 2.0 % (w/v) agar] for qualitative screening of the isolates as a part of secondary screening step (11). Their ability to produce extracellular xylanase was detected by Congo red plate assay method.

3.3. Congo Red Assay

The plates after an incubation period of 3 days were placed in 1% Congo red solution and kept for 10-15 minutes and destained with 1M NaCl solution for visibility of hydrolytic clearance zone (12).

3.4. Xylanase Production

In the final process of screening, the selected isolates were subjected to submerged fermentation for determining their potential for maximum xylanase production as part of quantitative screening by growing in liquid xylan medium with same composition as xylan agar medium except agar. Twenty mL of liquid xylan medium was inoculated with 1% v/v of 18 hour old fresh culture and incubated for 3 days at 50 °C on a rotary shaker (Rivotek, India) at 150 rpm. On completion of incubation period, the production medium was processed for xylanase enzyme extraction (13).

3.5. Preparation of Inoculum

The inoculum for final screening procedure and for further enzyme production studies was raised by inoculating a loop full of 18 hour old pure culture of the bacterial isolates in 50 mL of xylan liquid medium in 250 mL Erlenmeyer flask and incubating for 3 days at temperatures (37 ± 2 °C).

3.6. Xylanase Extraction

The bacterial isolates selected through screening

procedures were used for the production of xylanase enzyme under the conditions already mentioned. Cell free extract was obtained by centrifugation (REMI C-24BL, Cooling centrifuge; India) at 10,000 ×g for 10 min at 4 °C. The supernatant obtained was used as a source of crude extracellular xylanase enzyme (10) for various analytical purposes.

3.7. Xylanase Assay

The quantity of xylanase enzyme produced by each of the isolate in liquid xylan medium was determined following the method of Bailey *et al.* (1992) with some modifications (14). 1.0 % Birch wood xylan (HiMedia, India) in 50 mM Glycine-NaOH buffer (pH 9.2) was used as substrate. 0.5 mL buffered substrate was reacted with 0.5 mL of suitably diluted crude xylanase enzyme at 55 °C. The reaction was terminated after 10 min by adding 3 mL DNS reagent (15) and boiled for 5 minutes. After cooling, the released xylose was quantified at 540 nm against reagent blank which was prepared in the same manner except that 0.5 mL buffer replaced the crude enzyme. An enzyme blank was also set up in the pattern in which the reagent was added prior to the addition of crude so that only the released reducing sugar is estimated. A standard of xylose (HiMedia, India) was prepared using stock concentration of 10 g.L⁻¹. One unit of xylanase activity was defined as 1 μmol of xylose liberated per minute per mL of enzyme preparation under standard assay conditions.

3.8. Total Soluble Protein Estimation

Total soluble protein was estimated by Lowry's method (16) using Bovine serum albumin as standard (stock concentration- 1 mg.mL⁻¹).

3.9. Identification of Bacterial Isolates

Phenotypic and molecular characterization was carried out to enable the identification of the selected bacterial isolates. The pure cultures of potent bacterial isolates screened out through the three screening procedures were sent to National center for cell science (NCCS, Pune, Maharashtra, India) for identification purpose.

3.10. Phylogenetic Analysis

The 16s rRNA sequences of the isolates were aligned with closest type strains in the database to ascertain the phylogenetic position of the isolated strains using phylogeny construction by Neighbor-joining method (17) and Kimura two-parameter model (18) was used to compute the evolutionary distances. Boot-strap phylogeny method taking 1000 replicates into account was used to analyze the phylogenetic tree for the stability among the clades (19).

3.11. Optimization Studies

The different parameters probably contributing to the increase in xylanase production like medium pH (7-12 with an increment of 1.0 pH unit), inoculum size (0.5%, 1%, 2%, 5% and 10%), incubation temperature (40-80 °C with 5°C increment), agitation rate (50 rpm to 250 rpm with an increment of 50 rpm) best carbon and nitrogen source were evaluated. All the optimization studies were performed using the conventional one-factor-at-a-time approach wherein only one factor was varied at a time keeping all others at a constant level.

3.12. Statistical Analysis

All the observations are mean of three experimental replicates. The descriptive statistics of the observations are represented as mean ± standard deviation. In order to find the relationship between the clearance zone diameter and the enzyme activity, Bivariate correlation was to determine Karl Pearson's coefficient of correlation (r). Linear regression analysis was performed to determine the correlation between the independent variable (zone diameter with that of dependent variable (enzyme activity). The *p*-value < 0.05 was considered statistically significant. The SPSS (version 16.0) statistical program was used for the analysis.

4. Results

4.1. Isolation and Screening

Isolation of xylanase producing bacteria from various probable samples (soil, dung, decaying wood and decomposed lignocellulosic material) were collected from Bhilai-Durg region of Chhattisgarh state, India. In total 177 bacterial isolates were collected, mostly from soil samples of forest and dense plantation areas. On qualitative screening on xylan agar medium using Congo red assay method, 24 bacterial isolates were selected on the basis of the clearance zone formation for the final screening process. All the 24 isolates produced a significant amount of extracellular xylanase when grown in liquid xylan medium under submerged conditions (**Table 1**). In order to find out if any correlation existed between the zone diameter and the enzyme activity, correlation and regression analysis was done. Bivariate correlation analysis was performed by Pearson correlation using quantitative variables, the results of which are summarized in **Table 2**.

The value of $r = 0.131$ ($p < 0.05$) indicates that there is a downhill or negative relationship between zone diameter and the corresponding xylanase activity. The scatter plot (**Fig. 1**) of enzyme activity with respect to

zone diameter reveals no correlation between the factors. The significance (2-tailed) value of 0.551 indicates that there is no statistically significant correlation between the clearance zone diameter and the corresponding enzyme activity. The regression value ($R = 0.126$) indicates a very low degree of correlation between zone diameter and xylanase activity. On the basis of secondary and final screening process; two bacterial isolates designated as ISL-03 and ISL-58 were selected for further identification studies.

4.2. Identification of Potent Cultures

The bacterial isolates exhibiting significant hydrolytic zone and maximum xylanolytic activity indicated by xylanase assay in the final screening procedure were obtained. This study reports the isolation of two novel thermoalkalophilic bacterial isolates possessing

xylanolytic ability. Both the strains show aerobic growth, round, cream coloured colonies when grown on nutrient agar medium at 37°C and are flat with no elevation. Both ISL-03 and ISL-58 show gram positive nature. ISL-03 and ISL-58 both are endospore formers but differ in spore position. Both are motile but ISL-58 is sluggishly motile. Both are catalase positive and can grow at pH 9.0. The data related to morphological, phenotypic and biochemical characters of ISL-03 and ISL-58 indicate that the two xylanolytic bacterial strains are the members of genus *Bacillus* (Table 3).

4.3. Phylogenetic Analysis

The evolutionary relationship of strain ISL-03 and ISL-58 with the closely related taxa was inferred using the Neighbour-joining method using MEGA5. The partial nucleotide sequence of the 16S rRNA gene

Table 1. Summary of secondary and final screening for xylanolytic bacteria. Two novel thermoalkalophilic bacterial isolates possessing xylanolytic ability are indicated as bold.

| S/N | Isolate identity | Qualitative assay | | Quantitative assay | | |
|-----|------------------|--------------------|--|---|--|--|
| | | Zone Diameter (cm) | Xylanase Activity (IU.mg ⁻¹) | Cellulase Activity (IU.mg ⁻¹) | Total Protein Content (mg.mL ⁻¹) | Specific Activity (IU.mg ⁻¹) |
| 1 | ISL-1 | 1.3 | 16.82 ± 1.034 | 4.88±1.53 | 2.78±0.25 | 6.050 |
| 2 | ISL-3 | 1.6 | 36.61±3.866 | 1.48±0.67 | 2.0±0.36 | 18.30 |
| 3 | ISL-6 | 1.9 | 8.85±1.221 | 3.18±0.40 | 1.83±0.38 | 4.83 |
| 4 | ISL-9 | 1.4 | 12.53±1.189 | 2.33±0.28 | 2.21±0.22 | 5.66 |
| 5 | ISL-10 | 1.2 | 21.45±1.808 | 3.35±0.30 | 2.43±0.20 | 8.82 |
| 6 | ISL-13 | 1.4 | 7.15±0.563 | 4.65±0.73 | 1.35±0.30 | 5.29 |
| 7 | ISL-16 | 1.1 | 13.84±3.668 | 3.85±0.35 | 2.05±0.08 | 6.75 |
| 8 | ISL-17 | 1.5 | 24.16±3.157 | 2.81±0.40 | 2.40±0.37 | 10.06 |
| 9 | ISL-19 | 1.9 | 14.02±2.190 | 2.2±0.40 | 2.18±0.16 | 6.43 |
| 10 | ISL23 | 1.3 | 22.47±1.028 | 2.41±0.76 | 1.61±0.17 | 13.95 |
| 11 | ISL25 | 1.4 | 8.44±1.752 | 3.15±0.13 | 1.43±0.36 | 5.90 |
| 12 | ISL-30 | 1.7 | 23.56±2.449 | 2.33±0.38 | 2.38±0.14 | 13.68 |
| 13 | ISL-35 | 1.2 | 19.35±2.177 | 1.43±0.40 | 2.48±0.17 | 7.80 |
| 14 | ISL-38 | 1.6 | 13.13±2.610 | 3.05±1.23 | 1.81±0.20 | 7.25 |
| 15 | ISL-41 | 1.2 | 15.76±0.525 | 4.48±1.11 | 2.71±0.10 | 5.81 |
| 16 | ISL-43 | 1.5 | 16.91±1.144 | 3.55±0.56 | 3.33±0.14 | 5.07 |
| 17 | ISL-44 | 1.6 | 10.87±1.488 | 2.56±0.55 | 3.01±0.17 | 3.61 |
| 18 | ISL-48 | 1.4 | 16.15±1.203 | 1.66±0.57 | 2.70±0.26 | 5.98 |
| 19 | ISL-50 | 1.0 | 18.25±1.145 | 1.16±0.28 | 2.65±0.20 | 3.11 |
| 20 | ISL-52 | 1.4 | 16.19±1.691 | 2.08±0.14 | 2.83±0.28 | 5.72 |
| 21 | ISL-55 | 1.2 | 14.15±1.693 | 1.15±0.37 | 2.73±0.24 | 5.18 |
| 22 | ISL-56 | 1.5 | 21.28±1.593 | 1.38±0.20 | 2.93±0.35 | 7.26 |
| 23 | ISL-58 | 1.8 | 33.96±2.059 | 1.11±0.10 | 2.25±0.27 | 15.09 |
| 24 | ISL-65 | 1.6 | 16.53±1.927 | 2.40±0.37 | 2.23±0.20 | 7.41 |

Table 2. ANOVA results for Correlation and Regression analysis.

| Model | SS | df | MS | F | Sig (p) |
|------------|----------|----|--------|-------|--------------------|
| Regression | 18.482 | 1 | 18.482 | 0.352 | 0.559 ^A |
| Residual | 1154.901 | 23 | 52.496 | - | - |

of ISL-03 strain displayed 99.75% sequence similarity with the available 16S rRNA gene sequences of the type strains *B. stratosphericus* 41KF2a^T (AJ831841), *B. altitudinis* 41KF2b^T (ASJC01000029) and *B. aerophilus* 28K^T (AJ831844); 99.66% similarity with *B. xiamenensis* HYC-10^T (AMSH010000114) and 99.24% similarity with non type strain *B. safensis* FO-36b^T (ASJD01000027). However, ISL-58 revealed 99.86 to 99.79 % sequence similarity with *B.aerophilus*, *B. stratosphericus*, *B. altitudinis* and *B. xiamenensis*. According to the Neighbor-joining phylogentic tree, strain ISL-03 and ISL-58 form a clade with *B. xiamenensis*, *B.altitudinis*, *B. stratosphericus* and *B. aerophilus*. Although *B. aerophilus*, *B. stratosphericus*, *B. altitudinis*, ISL-03 and ISL-58 show 100% sequence similarity with one another but the morphological properties and phenotypic characteristics between the two novel isolates and with the closely related *Bacillus sp.* differ considerably, thus strongly recommending the novelty of the isolated strains. Therefore, ISL-03 and ISL-58 are being designated as *Bacillus sp.* MCC2728 (**Fig. 2**) and *Bacillus sp.* MCC2727 (**Fig. 3**) respectively. The 16S rRNA sequences of the two bacterial isolates have been submitted to GenBank. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA

gene sequences of strains *Bacillus sp.* MCC2728 and *Bacillus sp.* MCC2727 are KP742971 and KT444621 respectively.

4.4. Optimization of Various Parameters

4.4.1. pH

The study on the effect of initial medium pH on the production of xylanase by *Bacillus sp.* MCC2727 and *Bacillus sp.* MCC2728 was done in the range of pH 7-12. All the medium pH evaluated seemed to produce detectable amounts of enzyme activity. However, maximum titer of xylanase was attained by both *Bacillus sp.* MCC2728 (39.51 ± 1.04 IU.mL⁻¹) and *Bacillus sp.* MCC2727 (36.52 ± 0.50 IU.mL⁻¹) at pH 9.0 after 72 and 60 hours of incubation period respectively (**Fig. 4A**). Xylanase productivity was maximum and consistent at pH 9.0, while inconsistent and lower productivity was obtained at higher medium pH (pH 10, 11 and 12).

4.4.2. Incubation Temperature

One of the important environmental factors which significantly affects the level of extracellular enzyme production is temperature. Optimum temperature for xylanase production by *Bacillus sp.* MCC2727 and *Bacillus sp.* MCC2728 was evaluated at varying temperatures in the range of 40-80 °C with 5 °C increment. Maximum enzyme productivity was observed at 55 °C for *Bacillus sp.* MCC2728 and at 50 °C for *Bacillus sp.* MCC2727. Both *Bacillus sp.* MCC2727 and *Bacillus sp.* MCC2728 recorded negligible amounts of xylanase when incubated at 75 °C and no enzyme activity was detected at 80 °C (**Fig. 4B**).

4.4.3. Inoculum Size

The influence of inoculum size on xylanase production by *Bacillus sp.* MCC2727 and *Bacillus sp.* MCC2728 was assessed by altering the amount of inoculum added to the production medium. Although detectable amounts of xylanase production was observed with lower inoculum sizes but maximum enzyme activity was only recorded at 5% inoculum (**Fig. 4C**) and enzyme activity dropped drastically at 10% v/v inoculum size.

4.4.4. Shaking Conditions

The influence of agitation on xylanase production was investigated at different rotating speeds (**Fig. 4D**). Maximum xylanase production was obtained at 150 rpm by both *Bacillus sp.* MCC2727 and *Bacillus sp.* MCC2728. Lower yields of xylanase were recorded at lower agitation speeds of 50 rpm and 100 rpm.

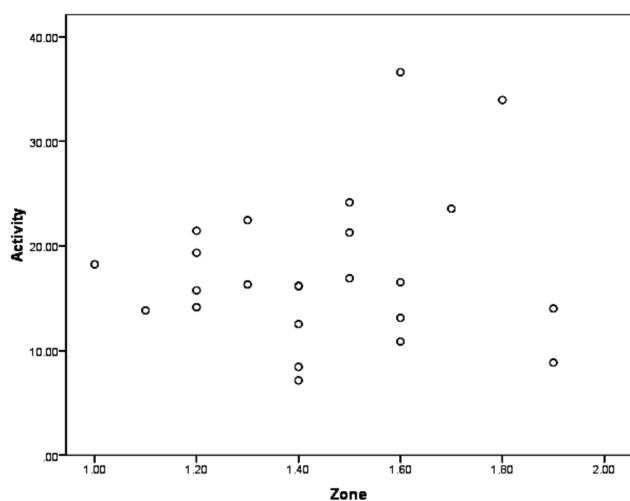


Figure 1. Scatter plot between Zone diameter and Xylanase activity.

Table 3. Phenotypic and biochemical characteristics that differentiate ISL-03 (*Bacillus sp.* MCC2728) and ISL-58 (*Bacillus sp.* MCC2727).

| Characteristic | Isolate Identity | | | | |
|------------------------------|---------------------|--------------------------------|--------------------|--------------------|--------------------|
| | 1 | 2 | 3 | 4 | 5 |
| Size | 2-3 mm | 3-4 mm | 2-3 mm | 5-8 mm | 3-5 mm |
| Shape | cream | Cream | white | white | white |
| Margin | Wavy | Entire | regular | irregular | irregular |
| Consistency | Translucent | Opaque | NA | NA | NA |
| Elevation | Flat | Flat | convex | raised | raised |
| Gram Nature | Gram positive rods | Gram positive thin rods | Gram positive rods | Gram positive rods | Gram positive rods |
| Motility | Motile | Sluggishly motile | motile | motile | motile |
| Spore position | Central round spore | Sub terminal ellipsoidal spore | NA | NA | NA |
| Catalase | + | + | + | + | + |
| Growth at: | | | | | |
| pH 5.0 | - | - | + | - | - |
| pH 9.0 | + | + | - | + | + |
| 37°C | + | + | + | + | + |
| 50°C | + | + | + | - | - |
| Ribose | + | + | NA | + | + |
| Fructose | + | + | NA | + | + |
| Glucose | + | + | - | + | + |
| Rhamnose | - | - | + | + | + |
| Arabinose | - | - | + | + | + |
| Galactose | - | - | NA | + | + |
| Casein Hydrolysis | + | + | - | NA | NA |
| DNAase | + | + | NA | NA | NA |
| Starch | - | - | + | + | - |
| Gelatin | - | - | + | NA | NA |
| Lactose | - | - | NA | + | + |
| Xylose | - | - | - | + | + |
| Maltose | - | - | NA | + | + |
| Raffinose | - | + | - | + | + |
| Trehalose | + | + | + | + | - |
| Melibiose | - | - | NA | + | + |
| Sucrose | + | + | + | + | + |
| Mannose | + | + | NA | + | + |
| Inulin | - | - | + | + | + |
| Sodium Gluconate | - | - | NA | NA | NA |
| Glycerol | + | - | + | + | + |
| Salicin | + | + | NA | NA | NA |
| Dulcitol | - | - | + | - | + |
| Inositol | - | - | + | - | + |
| Sorbitol | - | + | + | + | - |
| Mannitol | + | + | + | + | + |
| Adonitol | - | - | NA | NA | NA |
| Arabitol | - | - | NA | NA | NA |
| Erythritol | - | - | NA | NA | NA |
| α -Methyl-D-Glucoside | - | - | NA | NA | NA |
| Xylitol | WP | - | NA | + | - |
| ONPG | - | - | + | + | + |
| Aesculin hydrolysis | + | + | + | + | + |
| Citrate Utilization | + | + | - | + | - |
| Malonate Utilization | - | - | NA | NA | NA |
| Sorbose | - | - | + | + | + |
| Voges-Proskuer | - | - | - | + | + |
| Nitrate Reduction | - | - | - | + | + |
| Arginine | - | + | - | + | - |
| Cellobiose | + | - | + | - | + |
| Melezitose | - | - | NA | NA | NA |
| α -Methyl-D-Mannoside | - | - | NA | NA | NA |

Strains: 1, *Bacillus sp.* MCC2728 (ISL-03); 2, *Bacillus sp.* MCC2727 (ISL-58); 3, *Bacillus altitudinis* 41KF2b⁺; 4, *Bacillus aerophilus* 28K⁺; 5, *Bacillus stratosphericus* 41KF2a⁺. +, Positive; -, Negative; WP, Weakly positive; NA, Data not available. All the data of 3-5 have been derived from Shivaji *et al.* 2006 (31).

the maximum xylanase production. Yeast extract and peptone individually served as efficient nitrogen sources for xylanase production in case of *Bacillus sp.* MCC2728. However the combined effect of yeast extract and peptone resulted in a noteworthy increase in xylanase activity (Table 5) but *Bacillus sp.* MCC2727 preferred yeast extract over other nitrogen sources. Inorganic forms of nitrogen proved inefficient in inducing xylanase production.

5. Discussion

5.1. Isolation and Screening

The isolation and screening of thermostable alkalophilic xylanase producing bacteria was performed in three stages from the various collected samples. Forest soil

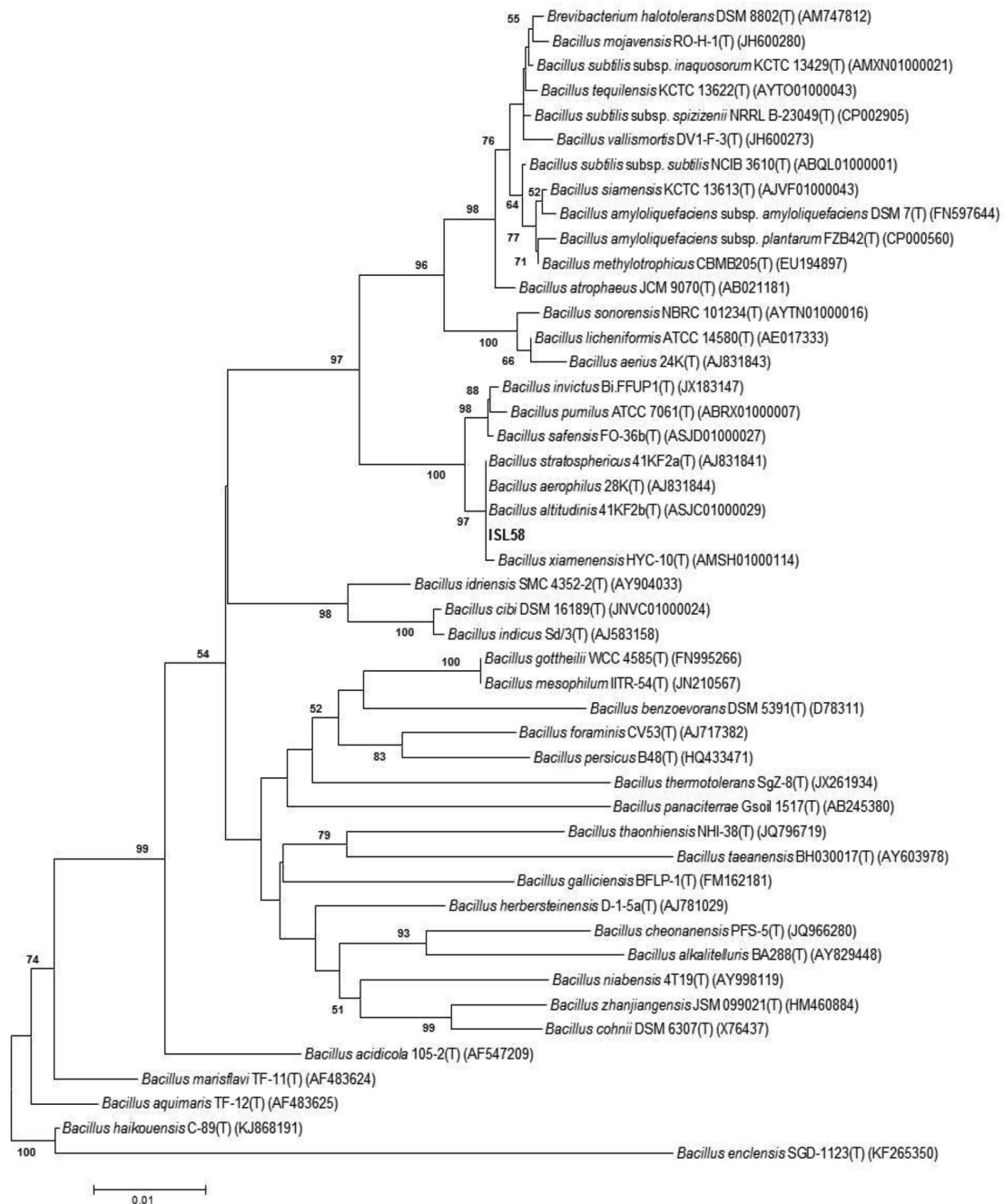


Figure 3. Phylogenetic tree of ISL-58 (*Bacillus sp.* MCC2727).

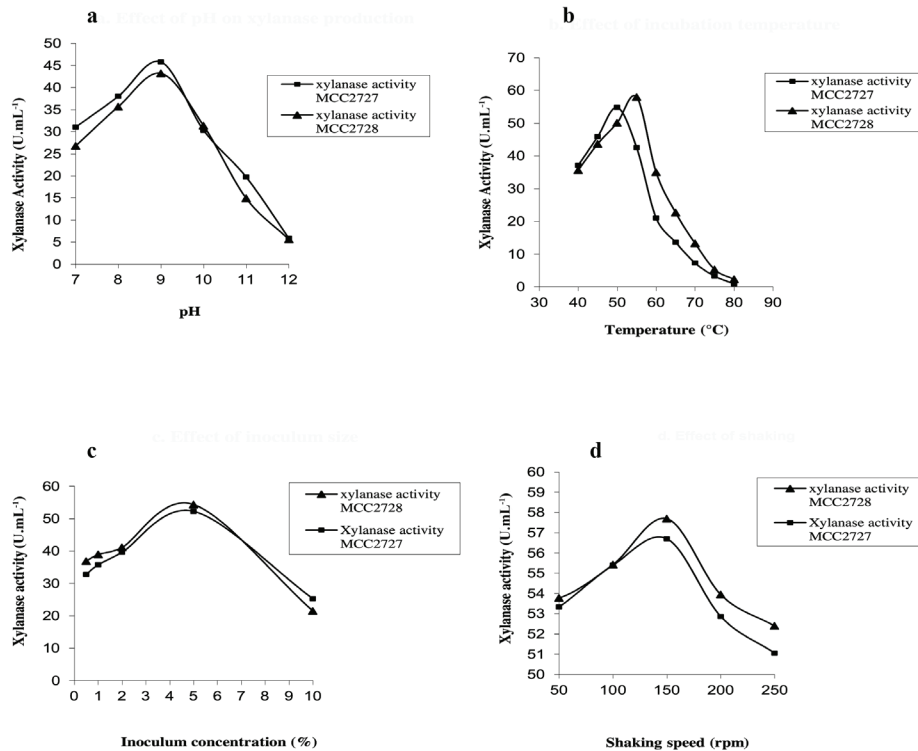


Figure 4. Optimization of growth parameters. a) Effect of pH on xylanase production. b) Effect of incubation temperature. c) Effect of inoculum size. d) Effect of shaking.

samples produced the maximum number of xylanase producing bacteria. It can be argued that soil is the best and common source for isolating various industrially important organisms. Moreover, the biological system of forest soil includes organic constituents and minerals which makes it a high microbial diversity hub (20). The studies on soil profiles indicate both fungal and

bacterial diversity is abundant in forest top soils as they are rich in decomposing litter materials (21). The main objective of this study was to isolate xylanolytic bacteria and undoubtedly forest soils are rich in foliage which surely encourages the growth and survival of xylanase producing organisms. Cordeiro *et al.* (2002) successfully isolated a thermophilic bacterial isolate from local soil (22). Also xylanolytic bacteria like *Bacillus cereus* (23); new species of *Bacillus sp.* similar to *Bacillus arsenicisolanatis* DSM 15340 (24) was isolated from soil. Keeping in view, that no single method can reveal all the microorganisms present in a sample, therefore, screening of xylanase producing bacteria was attempted in three different stages. As a part of enrichment technique in the preliminary screening, isolation was performed on wheat bran agar medium. Primary screening on this medium helped to specifically screen potential organisms from a broad range of organisms present in soil. The critical step of primary screening on wheat bran agar medium helped eliminate bulk of unwanted isolates. Nagar *et al.* (2012) were successful in isolating and screening out 70 xylanolytic bacterial isolates from 14 samples of decaying wood, soil and compost using wheat bran agar medium (25).

Wheat bran is rich in xylan; however the presence of starch and protein will also favor the growth of

Table 4. Optimization of the best carbon source.

| Carbon source | Xylanase Activity (IU.mL ⁻¹) | |
|------------------|--|-----------------------------|
| | <i>Bacillus sp.</i> MCC2728 | <i>Bacillus sp.</i> MCC2728 |
| Glucose | 1 ± 0.15 | 0.95 ± 0.72 |
| Xylose | 46.56 ± 0.82 | 36.22 ± 0.53 |
| CMC | 1.19 ± 0.12 | 1.12 ± 0.56 |
| Arabinose | 2.04 ± 0.09 | 1.25 ± 0.56 |
| Xylan | 37.84 ± 0.87 | 44.62 ± 0.25 |
| Galactose | 1.88 ± 0.62 | 1.50 ± 0.30 |
| Sucrose | 1.02 ± 0.30 | 1.20 ± 0.65 |
| Wheat bran | 78.13 ± 0.49 | 79.22 ± 0.70 |
| Rice bran | 32.03 ± 0.50 | 33.45 ± 0.42 |
| Straw | 30.50 ± 0.32 | 30.25 ± 0.54 |
| Cotton seed meal | 6.65 ± 0.56 | 8.55 ± 0.63 |
| Gram meal | 0.19 ± 0.07 | 1.95 ± 0.37 |
| Mannose | 0.59 ± 0.30 | 0.62 ± 0.46 |

Table 5. Optimization of Nitrogen Source.

| Nitrogen source | Xylanase Activity (IU.mL ⁻¹) | |
|--------------------------------|--|-----------------------------|
| | <i>Bacillus sp. MCC2728</i> | <i>Bacillus sp. MCC2728</i> |
| Urea | 12.35 ± 0.60 | 10.35 ± 0.56 |
| Peptone | 34.50 ± 0.64 | 56.25 ± 0.23 |
| Beef extract | 30.45 ± 0.50 | 26.02 ± 0.54 |
| Yeast extract | 60.80 ± 0.72 | 83.50 ± 0.21 |
| Casein | 3.21 ± 0.23 | 2.56 ± 0.41 |
| Yeast extract + peptone | 86.05 ± 0.25 | 55.01 ± 0.35 |
| Tryptone | 23.05 ± 0.56 | 19.35 ± 0.52 |
| Sodium nitrate | 11.23 ± 0.42 | 8.62 ± 0.46 |
| Ammonium nitrate | 3.52 ± 0.28 | 3.21 ± 0.55 |
| Ammonium di hydrogen Phosphate | 13.24 ± 0.51 | 12.03 ± 0.32 |

amylase or proteinase producing bacteria along with xylanase producers. Therefore, secondary screening was performed on xylan agar medium with xylan as sole carbon source. This helped to weed out non-xylanolytic bacterial isolates. The identification of xylanase producing bacteria was possible with Congo red assay method.

Twenty four isolates were screened out by this method. However, selection of potent isolates simply on the basis of their zone diameter would be unreliable and biased as some probably high potential enzyme producing bacterial isolates may not produce any extracellular enzyme on solid media (26). Thus, correlation and regression analysis which was initiated to analyze the existence of any relationship between zone diameter and enzyme activity in this study confirmed that there was statistically no significant correlation between the two variables [$r = 0.131$, p (2 tailed) = 0.551, $n = 24$]. The zone diameter accounts for only 1.6% of the variation in enzyme activity and 98.4% of variation in activity depends on other factors ($R^2 = 0.016$). It can be concluded from regression analysis ($p = 0.559$) that there is no significantly supported relationship between zone diameter and enzyme activity or put simply the zone diameter does not statistically affect xylanase activity. The regression equation can be constructed as follows: Activity (Y) = 12.169 + 3.743*Zone (X). Also studies by Teather and wood (1982), suggest that clear zone by an organism is not a correct indicator of it being the highest producer (27). Thus, screening should be done using both solid agar as well as liquid medium (28). Therefore, to rule out such biased selection all the selected isolates were grown in liquid xylan medium and xylanase enzyme was quantitatively evaluated. Two isolates ISL-03 (36.61 ± 3.866 U.mL⁻¹) and ISL-58 (33.96 ± 2.059 U.mL⁻¹) were maximum xylanase

producing bacterial isolates and were subjected to identification studies.

5.2. Identification and Phylogenetic Analysis

The morphological characterization of ISL-03 and ISL-58 revealed significant differences between the two isolates. However, both were Gram-positive endospore formers and catalase-positive. These features are in accordance with *Bacillus subtilis* (29); *Bacillus cereus* (23) and *Bacillus aerophilus* KGJ2 (30). One hundred percent sequence homology was observed between 16S rRNA gene sequences of both the isolated bacterial strains and with their closest neighbours. Literature survey reveals that although Shivaji *et al.* (2006) observed 100% sequence homology between *Bacillus aerius*, *Bacillus aerophilus* and *Bacillus stratosphericus* and also with their closest neighbours, but classified them into different species as he argued that strains exhibiting phenotypic and chemotaxonomic differences amongst themselves and from their related phylogenetic type strains in spite of having 100% sequence similarity imply that they are different species (31).

The indigenous, potent bacterial isolates designated as *Bacillus sp. MCC2727* and *Bacillus sp. MCC 2728* are interesting novel bacterial species with xylanolytic properties which can be exploited for their ability to convert biomass and agricultural wastes to industrially important products.

5.3. Optimization

Medium pH greatly influences growth of microorganisms and the transportation of nutrients across the membrane into the cell (32). The studies concerned with optimization of medium pH showed that both *Bacillus sp. MCC2727* and *Bacillus sp. MCC2728* produced maximum xylanase activity at pH 9.0. These

results are in accordance with that of *Staphylococcus* sp. (33) and *Enterobacter* sp. MTCC 5112 (34) which are reported to have produced maximum xylanases at pH 9.0. Further increase to pH 11 and 12 recorded 70% decline in xylanase production which might be due to the direct effect of extreme medium pH on cytoplasmic pH of microbial cells which in turn may have affected the growth and thus enzyme productivity. xylanase production was observed in a wide range of incubation temperatures but optimum temperature for *Bacillus* sp. MCC2728 was determined to be 55 °C and for *Bacillus* sp. MCC2727, it was 50 °C. The results are in accordance with those of Subramaniyan and Prema (2000) who reported maximum xylanase production by *Bacillus* sp. at 50°C (35). Also, Annamalai *et al.* (2009) has reported the optimum temperature for another *Bacillus* sp. to be at 55°C (29). So it may be argued that at these optimum temperatures, probably, the physical properties of the bacterial cell membranes might have changed thus influencing the increased extracellular xylanase production. However, negligible xylanase production at 70 °C and above may be attributed to either reduced solubility of oxygen in the medium at high temperatures or due to denaturation of enzymes (36).

Optimization of inoculum size is important to ensure maximum enzyme production as the initial bacterial count greatly affects the rate of consumption of limited nutrients in the medium. The suitable inoculum size for maximum xylanase production by *Bacillus* sp. MCC2727 and *Bacillus* sp. MCC2728 was observed to be at 5% v/v, which may be due to availability of suitable growth conditions contributed by provision of sufficient amount of nutrients and oxygen supply thereby enhancing xylanase production. Literature survey reveals varied optimum inoculum sizes for different bacterial isolates. Kumar *et al.* 2014 (37) reported 1% v/v as the best inoculum size for *Bacillus pumilus* VLK-1 while 1-2% v/v was reported to be optimum for *Bacillus* sp. isolated by Sanghi *et al.* 2009 (38) and Nagar *et al.* 2012 (25). High inoculum volumes (10% v/v) proved to be unfavorable probably due to tremendous increase in microbial growth resulting in increased viscosity of the medium (39). Also, large inoculum volumes may result in excessive imbalance in nutrient contents in the medium. Successful process development on a small scale largely depends on shaking conditions of the production medium. Studies on effect of shaking in maximizing the xylanase production revealed increased xylanase production by both the isolates compared to static conditions. This can be explained by the fact that agitation of medium helps adequate mixing of medium

components and also proper heat and mass transfer. The effect of shaking speeds on xylanase production showed that maximum xylanase titer was obtained at 150 rpm by both *Bacillus* sp. MCC2727 and *Bacillus* sp. MCC2728. It can be argued that this optimum shaking speed probably ensured adequate amounts of dissolved oxygen and helped maintain homogenous physical and chemical conditions in the culture medium (40). Medium formulation containing appropriate carbon and nitrogen source is one of the most crucial factors for any process development. Among the wide range of carbon sources tested, the complex lignocellulosic material; wheat bran proved to be the best carbon source for both the bacterial isolates probably because it is rich in both xylan and proteins which can be suitably used as carbon and energy sources (41). Wheat bran consisting of 20-30% xylan is an abundantly available agriculture waste residue strongly suggesting it to be a suitable candidate for use as an inexpensive substrate for xylanase production at industrial scale. These findings are in accordance with the reports of Bajaj *et al.* 2010 (42); Geetha and Gunasekaran, 2010 (43) and Gupta and Kar, 2010 (44). The findings on optimization of nitrogen sources suggests that organic nitrogen sources are more efficient in xylanase induction probably due to the fact that they contain most of the required essential amino acids for proper growth and xylanase production. Among the tested sources, a blend of yeast extract and peptone facilitated maximum xylanase production in *Bacillus* sp. MCC2728 while yeast extract as the sole nitrogen source was capable of producing optimum xylanase levels in *Bacillus* sp. MCC2727. These findings are in line with those of Giridhar and Chandra, 2010 (45) who recorded maximum xylanase levels with yeast extract and tryptone. The observation of yeast extract as the best nitrogen source for *Bacillus* sp. MCC2727 coincides with the reports of Kumar *et al.* 2014 (37) and Subramaniyan *et al.* 2001(11). Finally it can be concluded that the two novel bacterial isolates which have been isolated from the forest regions of Bhilai-Durg of Chhattisgarh state; India are potent enough to be used in the industrial production of various value added products. Moreover, the ability of these isolates to utilize inexpensive waste agricultural materials for xylanase production can be exploited for both biodegradation and other biotechnological purposes, keeping in view of the huge annual turnover of forest biomass and agriculture waste residues in this region.

Acknowledgment

The authors thank Dr. Shubha Thakur for her help and assistance in statistical analysis of the data.

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