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A Cost Effective Method for Screening and Isolation of Xylan Degrading Bacteria Using Agro Waste Material

Sushil Nagar, Anuradha Mittal and Vijay Kumar Gupta
Department of Biochemistry, Kurukshetra University, Kurukshetra, 136 119, India

Corresponding Author: Sushil Nagar, Department of Biochemistry, Kurukshetra University, Kurukshetra-136119, India Tel: +91 9813448824

ABSTRACT

Bacteria are well known for their ability to secrete extracellular enzymes into the environment. Xylanolytic enzymes are a group of enzymes that hydrolyze xylan and arabinoxylan polymers into smaller sugars. With this objective, a screening medium containing wheat bran, an agro-residue, with high economic competitiveness was developed. In this study, 70 xylanase producing bacteria were isolated from 14 samples of decaying wood, soil, compost etc., collected from different places in India on the basis of their zone of hydrolysis. Among these isolates, five strains (SV-34S, SV-43S, SV-85S, SV-190 and SV-205) were selected on the basis of their hyper production of xylanase. In the selected strains, xylanase activity in submerged and solid state fermentation ranged from 197-341 IU mL^{-1} and 1405-5302 IU g^{-1}, respectively under un-optimized conditions. Xylanase from the isolated strains was cellulase-free as it exhibited either undetectable or negligible cellulase activity. The method employed for isolation and production of xylanolytic bacteria using wheat bran is cost effective compared to the commercially available xylan in the medium.

Key words: Xylan, xylanase, submerged fermentation, wheat bran, Congo red dye

INTRODUCTION

Xylan, a major hemicellulosic polysaccharide found in the plant cell wall (Collins *et al*., 2005), represents up to 30-35% of the total dry weight of land plants (Joseleau *et al*., 1992). It is a heteropolysaccharide made up of a backbone of 1, 4-linked $-D-xylopyranosyl residues which can be substituted to varying degrees with glucopyranosyl, "-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaryl side chain groups (Kulkarni *et al*., 1999). Several hydrolytic enzymes are required for the complete hydrolysis of xylan of which xylanases are most important. Xylanases are produced by diverse genera and species of micro-organisms. These have been studied mostly from bacteria (Anuradha *et al*., 2007; Sanghi *et al*., 2008), fungi (Okafor *et al*., 2007) and actinomycetes (Ninawe *et al*., 2008). Among bacterial xylanases, members of the genus *Bacillus* have been extensively studied. The production has been carried out under both submerged fermentation (Smf) as well as Solid State Fermentation (SSF) conditions. Smf involves fermentation by micro-organisms in the liquid medium, which have specific nutrient components for the production of enzymes and with a constant shaking condition throughout the process. In SSF, the fermentation is carried out on moist solid substrate in the absence (or near absence) of free water (Pandey, 2003).

Xylanolytic enzymes from micro-organisms have attracted attention for the last few decades, particularly because of their biotechnological potential in various industrial processes such as food,

feed and pulp and paper industries (Beg *et al*., 2001). In baking, they improve elasticity and strength of the dough thereby increasing loaf volumes and texture of bread (Maat *et al*., 1992). In feed industry, the incorporation of xylanase into the rye-based diet of broiler chickens results in the reduction in intestinal viscosity, thereby improving both weight gain of chicks and their feed conversion efficiency (Bedford and Classen, 1992). Xylanases are used for the conversion of xylan in wastewater released from agricultural and food industries into xylose. They are also used for clarifying juices and for liquefying fruits and vegetables, for degumming of fibers and deinking of waste newspapers (Beg *et al*., 2001). Currently, the most effective application of xylanases is in the pre-bleaching of Kraft pulp to minimize use of harsh chemicals in the subsequent treatment stages of pulp bleaching (Bajpai, 1999). The pre-treatment of pulp with xylanase may provide a cost-effective method for pulp bleaching resulting in a decrease of chlorine consumption. This in turn, reduces the discharge of toxic organochlorine compounds and consequently, lowers environmental pollution impact. Xylanase cleaves and solubilizes reprecipitated xylan and lignin located on the surface of cellulose microfibrils (Khandeparkar and Bhosle, 2007).

Cellulase-free xylanases are preferred for bleaching of paper pulp to ensure minimal damage to fibers. Since, both pulping and bleaching are performed at high temperature and alkaline pH, the paper industry needs xylanases that are thermostable and preferably, active at neutral and alkaline pH (Srinivasan and Rele, 1999). Initially, xylanase from fungal sources was used as pre-bleaching agent but later, due to the presence of cellulase, low optimum pH and less stability of enzyme, fungal enzymes found limited acceptability on a commercial scale. Thus, it would be advantageous to produce xylanases from bacterial sources for industrial application.

There are many reports in the literature on isolation of xylan degrading microorganisms using pure xylan, which is costly and hence, increases the cost of enzyme production. So, the objective of this study was to develop an economical medium for the isolation of cellulase free xylanolytic bacteria using wheat bran instead of pure xylan.

MATERIALS AND METHODS

Isolation media: During the isolation of xylanase producing micro-organisms three types of media were used such as Nutrient Agar (NA), Wheat Bran Agar (WBA) and Xylan Agar (XA). The compositions of these media are shown in Table 1.

Sample collection and processing: Samples of decaying woody materials, sea sand, stagnant water, soil, near hot spring and compost collected from different places of India were used for

Table 1: Composition of various culture media used for isolation and screening of xylanolytic bacteria

Components	NA (g)	WBA (g)	XA (g)
Peptone	0.5	0.5	0.50
Beef extract	0.3	0.3	-
Yeast extract	-	-	0.20
$MgSO_4$	-	-	0.05
NaCl	-	-	0.05
$CaCl_2$	-	-	0.01
Wheat bran	-	1.0	-
Xylan	-	-	0.50
Agar-agar	2.0	2.0	2.00
pH	7.0	7.0	7.00

The compositions are for 100 mL of media

isolation of xylanase producing micro organisms. One gram of sample was suspended in 10 mL sterile distilled water and serial dilutions were made. The diluted samples (70-80 µL) were spread on NA plates.

Qualitative method: Xylanolytic bacteria were qualitatively identified with the help of Congo red dye (Teather and Wood, 1982). The selected purified xylanolytic colonies were grown on both WBA and XA media. The colonies were then flooded with 1% aqueous Congo red dye for at least 1 h followed by destaining with 1 M NaCl. The plates were examined for the appearance of yellow zone of hydrolysis around the colonies.

Viable bacterial cell counts: Viable cell counting was done using nylon membrane (47 mm, 0.2 µm) disk filter. The materials viz. normal saline (0.9% NaCl), test tubes (18×150 mm with cotton plugs), micropipette tips and forceps were autoclaved (121°C, 15 psi) for 20 min. An aliquot (0.5 mL) of serially diluted ($10G^{6}$, $10G^{7}$ and $10G^{8}$) culture was passed into the disc filter using filtration unit assembly with vacuum. For each dilution a separate disk membrane was used. The membrane was placed on NA plate with the help of forceps and incubated at 37ºC for 24 h. The colony grown membranes were floated in 1% Congo red dye solution for better reproducibility and proper counting of colonies on membranes. The colonies grown on the membrane were counted to calculated the viable bacterial cell count. After 20 min, the membranes were dried, heat fixed at 50°C in a hot air oven and preserved. The growth curve for each strain was plotted by estimating the CFU at different time intervals of 12, 18, 24, 30 and 36 h.

Growth rate and generation time: Nutrient broth was inoculated with all five cultures and CFU was calculated after different time intervals. The growth rate and generation time (doubling time) were estimated using the following Eq. 1 and 2, respectively:

$$\text{Growth rate (k)} = \frac{\log X_t - \log X_0}{\log 2 \times t} \quad (1)$$

$$\text{Generation time (tg)} = \frac{1}{k} \quad (2)$$

where, X_t is CFU mLG^{1} at higher level; X_0 is CFU mLG^{1} at lower level; t is time interval between two points.

Production of xylanase in SMF: Xylanase production through Smf was carried out using modified Horikoshi medium containing (g LG^{1}): wheat bran 20.0; peptone 5.0; yeast extract 5.0; KNO_3 5.0; KH_2PO_4 1.0 and $MgSO_4$ 0.1 at pH 7.0. The flasks were autoclaved (121°C, 15 psi) for 20 min and cooled. These were then inoculated with 0.5% (w/v) overnight grown nutrient broth cultures and incubated at 37°C for 48 h under shaking at 200 rpm. The contents of the flasks were centrifuged at 10,000 rpm for 20 min at 4°C and the clear cell free supernatant was used for xylanase assay.

Production of xylanase in SSF: Enzyme production through SSF was carried in Erlenmeyer flasks (500 mL) containing wheat bran and distilled water in a ratio of 1:2. The flasks were autoclaved (121°C, 15 psi) for 15 min; cooled and inoculated with 10% (v/w) of overnight grown

inoculum in nutrient broth and incubated at 37ºC for 72 h. The flasks were gently tapped intermittently to mix the contents. The contents of the flasks were extracted with distilled water, centrifuged at 10,000 rpm for 20 min at 4°C and the clear cell free supernatant was used for xylanase assay.

Xylanase assay: Xylanase activity was assayed according to the method of Bailey *et al*. (1992) by measuring the amount of reducing sugars (xylose equivalent) liberated from xylan using 3, 5-dinitrosalicylic acid (Miller, 1959). The reaction mixture containing 490 µL of 1% birch wood xylan (Sigma) as substrate and 10 µL of appropriately diluted enzyme extract was incubated at 55°C for 5 min. The reaction was then terminated by adding 1.5 mL of 3, 5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of enzyme. The contents were placed in a boiling water bath for 10 min followed by cooling in ice cold water. The absorbance of the resulting colour was measured against the control at 540 nm in a spectrophotometer. One unit (IU) of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar as xylose equivalent per minute under the specified assay conditions.

Cellulase assay: Cellulase activity was determined according to the method of Ghose (1984). The 2.0% carboxymethyl cellulose (Sigma) and Whatman No. 1 filter paper strip (1×6 cm) was used for carboxymethyl cellulase (CMCase) reaction mixture for carboxymethyl cellulase (CMCase) and filter paper activity (FPase), respectively. A control was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of enzyme. The absorbance of the resulting colour was measured against the control at 540 nm in a spectrophotometer. One unit (IU) of cellulase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar as glucose equivalent per minute under the specified assay conditions.

Identification: The two strains SV-85S and SV-205 were identified by the Institute of Microbial Technology, Chandigarh, India as *Bacillus pumilus* with accession No. 9861 and 9862. The remaining three cultures were identified as *Bacillus* sp. on the basis of their physiological and morphological characteristics in our laboratory. The characteristics of all five strains are shown in Table 2.

Identification using HiCrome *Bacillus* agar: Dissolve 4.9 g of HiCrome *Bacillus* agar in 100 mL of distilled water in Erlenmeyer flask and boil to completely dissolve the constituents of the medium. The flask is not to be autoclaved as directed by the manufacturer. The medium was cooled to a temperature of 50-55°C, poured in petri plates and left for solidification. The selected cultures were streaked on HiCrome *Bacillus* agar medium plates and incubated at 37°C for 24 h.

RESULTS AND DISCUSSION

Screening and isolation of xylanolytic bacteria: Xylanase producing micro-organisms are likely to be found at places where decaying hemicellulosic material is present. In this study, 14 samples were collected from 5 different places of India i.e. 3 from Ambala Cantt, 4 from Saha, 3 from Kurukshetra, 2 from Leh Ladhakh and 2 from Chennai for isolating xylanolytic bacteria Table 3. On NA plates, 140 strains were obtained of which 70 strains produced zones of hydrolysis on Wheat Bran Agar (WBA). The zones of hydrolysis on WBA and XA plates produced by two bacterial isolates (SV-85S and SV-205) are shown in Fig. 1. Congo red dye is known to interact only

Table 2: Morphological and physiological characteristics of the five selected strains of bacteria

	Strain				
Characteristics	SV-34S	SV-43S	SV-85S	SV-190	SV-205
Cell shape	Rods	Rods	Rods	Rods	Rods
Arrangement	Short chains	Long chains	Short chains	Short chains	Short chains
Spore	+	+	+	+	+
Position	Central	Terminal	Central	Central	Central
Motility	+	+	+	+	+
Gram staining	+	+	+	+	+
Colony colour	White	Pale yellow	Cream	Cream	Cream
Colony shape	Round	Oval	Round	Round	Round
Growth temp. (°C)	10-50	20-40	10-55	20-45	10-60
Growth pH	5.0-10.0	5.0-9.5	5.0-10.5	5.0-9.0	5.0-11.0
Growth at NaCl (%)	1-10	3-10	2-10	2-9	2-10
Catalase test	+	+	+	+	+
Growth under anaerobic conditions	+	+	+	-	+
Identified as	*Bacillus* sp.	*Bacillus* sp.	*Bacillus pumilus*	*Bacillus* sp.	*Bacillus pumilus*

+: Positive growth, -: Negative growth

Table 3: Sample collection and location for xylanolytic strains

Sample place	Type of material	Samples collected	No. of isolates
Ambala Cantt	Soil, compost	3	14
Saha (distt. Ambala)	Stagnant water, soil	4	21
Kurukshetra	Soil, decaying wood	3	12
Leh Ladhakh	Near hot Spring samples	2	11
Chennai sea	Decaying wood and sea sand	2	8

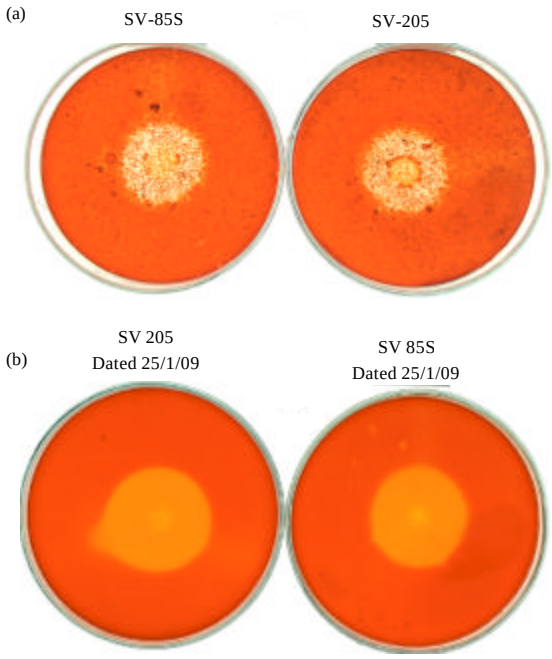

Fig. 1(a-b): The zones of hydrolysis produced by SV-85S and SV-205 on the following media, (a) Wheat bran agar and (b) Xylan agar plates

with intact (1,3- and 1,4-) $-D-glucans. The appearance of zone after staining with Congo red dye indicates that xylanase secreted by bacterial culture hydrolyzes the xylan backbone resulting in a mixture of xylo-oligosaccharides of various degrees of polymerization and composition. As a consequence, the average molecular mass of the substrate will gradually decrease as the enzymatic reaction proceeds. The formation of zones on WBA is due to high xylan content of wheat bran and hence, degraded by xylanase. The bacteria producing zone of hydrolysis were maintained on NA medium.

Xylanolytic bacteria have been isolated from different kind of samples. *Bacillus circulans* D1 was isolated from decaying wood (Bocchini *et al*., 2002), *Alternaria* sp. ND-16 from the soil of apple garden (Li *et al*., 2009), *Geobacillus thermoleovorans* from a pulp sample collected near to pulp and paper industry (Sharma *et al*., 2007) and *Bacillus* sp. GRE7 from an Ethiopian hyper thermal spring soil (Kiddinamoorthy *et al*., 2008).

The earlier studies emphasized the need for cost effective enzyme production rather than screening of xylan degrading micro-organisms. Pure xylan is commonly used in the agar medium for qualitative detection of xylanolytic micro-organisms. Mohana *et al*. (2008) reported the isolation of xylanase producing *Burkholderia* sp. DMAX form soil collected from decaying agricultural waste using xylan rich medium (in g L^{-1}: xylan, 2.0; peptone, 5.0; yeast extract, 1.0; NaCl, 5.0; K_2HO_4, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.1 and Na_2CO_3, 10.0). Sudan and Bajaj (2007) isolated xylan degrading micro-organisms from different samples using xylan agar (in g L^{-1}: $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 3.0; CH_3COONH_4, 6.0; oat spelt xylan 5.0 and agar 20.0 at a pH 8). Khurana *et al*. (2007) isolated *Streptomyces violaceoruber* from effluent of paper and pulp industry and maintained on Horikoshi agar medium containing (%w/v): glucose, 0.5; peptone, 0.5; yeast extract, 0.5; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.01; agar 20.0. Bernier *et al*. (1983) isolated 25 xylanase producing *Bacillus* strains by growing them in Casamino broth at pH 5.0 and 7.0 in the presence of 1% larch xylan. The use of pure xylan is, however, uneconomical. The hemicellulosic material such as wheat bran provides an alternative cost-effective substrate for screening.

Production of xylanase in SMF: The bacterial strains showing zones of hydrolysis on WBA were maintained on Nutrient Agar (NA) slants and tested for xylanase production in submerged fermentation. Based on the xylanase titre 20 strains were selected, which exhibited activity of more than 100 IU mL^{-1} as shown in Fig. 2, where x-axis is representing the strain and y-axis xylanase

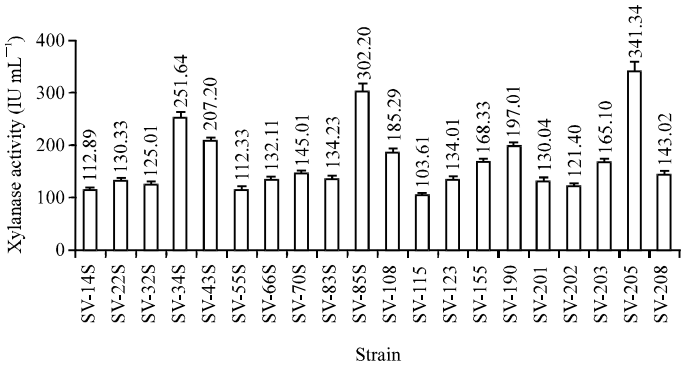

Fig. 2: Submerged fermentation of xylanase by different isolated strains. The flasks contained 50 mL of production medium, inoculated with 1% of inoculum and incubated at 37°C for 48 h along with shaking at 200 rpm

activity in IU mL^{-1}. The lowest xylanase activity was shown by SV-115 (103.01 IU mL^{-1}). All the activity was obtained under un-optimized conditions and represented the mean of three replicates. Among the above 20 bacterial strains, the best five strains (SV-34S, SV-43S, SV-85S, SV-190 and SV-205) showing highest enzyme activity (ranging from 251-341 IU mL^{-1}) were selected for further experiments. The xylanase production by SV-85S strain in Smf was optimized leading to a very high enzyme titre (2995 IU mL^{-1}), which was 9.91-fold higher than the activity before optimization (Nagar *et al*., 2010).

Production of xylanase in SSF: Solid state fermentation by the above five selected bacterial strains was also carried out using wheat bran as substrate and the results are given in Table 4. The xylanase production under unoptimized conditions was lowest (1405 IU g^{-1}) for SV-43S and highest (5302 IU g^{-1}) for SV-85S. The enzyme produced under solid state is very much cost effective as it is produced with easily available agro residues such as wheat bran. In this case enzyme was produced at room temperature with distilled water and without the addition of any mineral salt solutions, leading to marked decrease in the cost of production. The enzyme production by SV-85S under unoptimized conditions was higher than that of *Bacillus* sp. GRE7 (Kiddinamoorthy *et al*., 2008) and *Bacillus* sp. JB-99 (Virupakshi *et al*., 2005) using wheat bran as substrate under optimized conditions.

CMCase and FPase activity: The cellulase activity (CMCase and FPase) in the culture filtrates of above five selected bacterial strains was found to be negligible or undetectable. The CMCase activity in culture filtrates of SV-34S, SV-85S and SV-205 was undetectable, whereas in SV-43S and SV-190 it was negligible (Table 4). However, the FPase activity was undetectable in all the five strains. The cellulase free xylanase is important in pulp bleaching causing reduction in the consumption of toxic chlorinated compounds (Sanghi *et al*., 2009; Srinivasan and Rele, 1999). Cellulase may adversely affect the quality of paper pulp by destroying the structure of cellulose. Xylanase has been isolated from various sources but limited reports are available for cellulase free enzyme (Battan *et al*., 2007; Manimaran *et al*., 2009; Nagar *et al*., 2010; Sanghi *et al*., 2009; Sharma *et al*., 2007).

Identification of the xylanolytic bacteria: All the selected isolates were examined for their cell shape, colony appearance, spore formation, motility, Gram reaction and pigmentation after incubation at 37°C for 24 h. The results obtained from these tests are listed in Table 2. Under light microscopic study, bacterial strains SV-34S, SV-43S and SV-190 produced a mucilaginous resin due to which they were found in a group, where as SV-85S and SV-205 were scattered. All the five selected colonies were applied on chromomeric media i.e., HiCrome *Bacillus* agar (HiMedia),

Table 4: Qualitative analysis of xylanase, quantitative analysis of xylanase and cellulase activity by the selected bacterial isolates

Strain designation	Zone of diameter (cm)		Xylanase activity		CMCase activity	FPase activity
	XA	WBA	SMF (IU mL^{-1})	SSF (IU g^{-1})	(IU mL^{-1})	(IU mL^{-1})
SV-34S	2.9	1.9	251.64	3108.3	Nil	Nil
SV-43S	2.8	2.2	207.20	1405.2	0.02	Nil
SV-85S	3.0	2.4	302.20	5302.0	Nil	Nil
SV-190	2.7	2.1	197.01	1727.0	0.05	Nil
SV-205	4.1	3.0	341.34	4510.0	Nil	Nil

XA: Xylan agar, WBA: Wheat bran agar, Smf: submerged fermentation, SSF: Solid state fermentation

containing peptic digest of animal tissues and meat extract along with chromogenic mixture which provides nitrogenous compounds. Mannitol serves as the fermentable carbohydrate, fermentation of which can be detected by phenol red. Mannitol fermenting organisms like *B. megaterium* yield yellow coloured colonies and *B. subtilis/pumilus* gives light green to green coloured colonies. The growth of *B. pumilus was* luxuriant, where as the growth of *B. subtilis* was poor-good. *Bacillus coagulans* and *E. coli* yield pink coloured colonies on this medium. All the five strains yielded green to light green colours, indicating that the strain are either *B. subtilis* or *pumilus* (Fig. 3).

Viable cell counting and growth curve: The CFU was estimated by viable cell counting using nylon disk membranes. The strain SV-34S, SV-43S, SV-85S, SV-190 and SV-205 showed 84, 79, 105, 67 and 92 CFU/0.5 mL, respectively, at a dilution of 10^6 (Fig. 4). The staining of xylanolytic bacterial colonies with Congo red for enhancing their visualization for photography has not been reported earlier. The growth curves for selected five cultures were also plotted on the basis of CFUs estimated at different time intervals of 12, 18, 24, 30 and 36 h. A graph was plotted between time (h) at x-axis against Log CFU at y-axis (Fig. 5) and the peak time of 24 h was estimated for SV-34S, SV-43S, SV-205 and 18 h for SV-85S, SV-190. The generation time (doubling time) was calculated using the peak CFU number for each strain. Table 5 represents the number of CFUs,

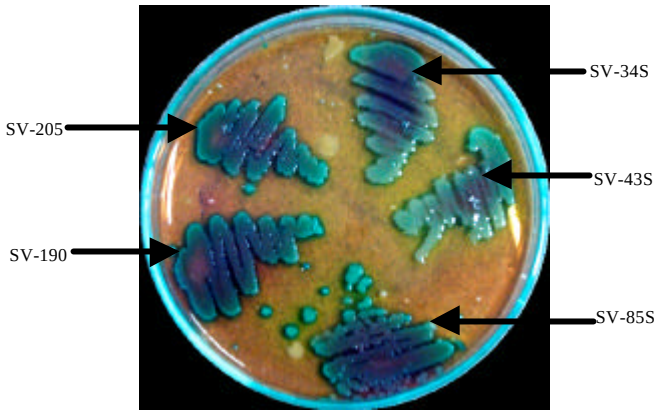

Fig. 3: Growth of *Bacillus* strains on HiCrome *Bacillus* agar

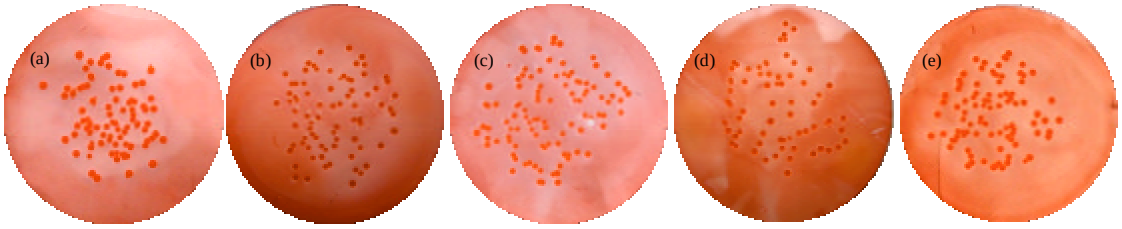

Fig. 4(a-e): Cell counts on nylon disk membrane (47 mm, 0.2-µm) produced from serially diluted 0.5 mL (a) SV-34S, (b) SV-43S, (c) SV-85S, (d) SV-190 and (e) SV-205 strain

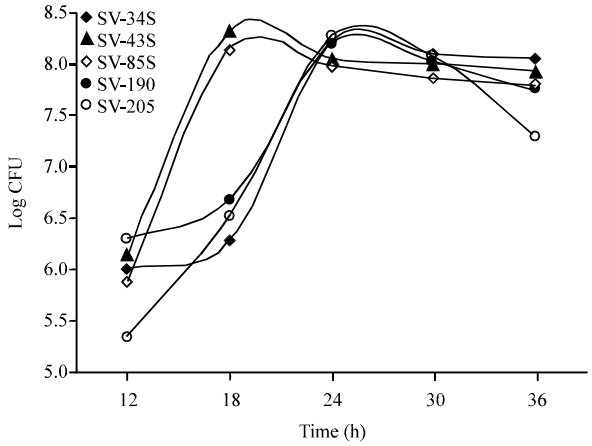

Fig. 5: Growth curves using viable counts of SV-34S, SV-43S, SV-85S, SV-190 and SV-205

Table 5: The viable cell counts, growth rate and generation time of the selected strains

Strain	Lower (CFU mL^{-1})	Higher (CFU mL^{-1})	Growth rate (k)	Generation time (min)
SV-34S	19×10^5	16.8×10^7	1.078	55.67
SV-43S	47×10^5	15.8×10^7	0.845	70.98
SV-85S	14×10^5	21.0×10^7	1.205	49.80
SV-190	76×10^4	13.4×10^7	1.244	48.24
SV-205	33×10^5	18.4×10^7	0.967	62.05

growth rate and generation time for the selected strains. The strain SV-85S and SV-190 showed a highest growth of 1.205 and 1.244 with a lowest generation time of 49.80 and 48.24 min.

CONCLUSION

Wheat bran could be used as an alternative substrate for routine qualitative screening of xylanolytic microorganisms in comparison with pure xylan. The selected five strains (SV-34S, SV-43S, SV-85S, SV-190 and SV-205) isolated in the present study were alkali stable and moderately thermostable. These strains produced high titres of cellulase free xylanase under un-optimized conditions. The growth curves were plotted for these strains on the basis of their colony forming units estimated after a particular time period by viable cell counting, using nylon disk membranes. The staining of xylanolytic bacterial colonies with Congo red has been shown to enhance their visualization by photography.

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