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Isolation of microorganisms simultaneously producing xylanase, pectinase and cellulase enzymes using cost effective substrates

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Abstract: Screening of cellulase, xylanase and pectinase producing microorganisms was done by using agrowaste like wheat bran and orange peel. Screening of enzyme producing microorganisms using commercial substrate is an expensive process. So to reduce the cost of isolation, various agricultural residues were used. Wheat bran was used for the isolation of xylanolytic and cellulolytic microorganisms and citrus peel was used for screening of pectinolytic microorganisms. A qualitative study of xylanase, pectinase and cellulase activities was detected by flooding plates containing respective substrates with 0.5% (w/v) Congo red for 15 min followed by repeated washing with 1 M NaCl. Quantitatively bacterial isolates were screened for enzymes activity in submerged fermentation by using modified Horikoshi medium. Enzyme produced under SmF was then assayed by measuring the amount of reducing sugar released by using the 3,5 Dinitrosalicylic acid. The use of cost effective substrates for the isolation of enzymes producing microorganisms is good process and will ultimately lower the cost of screening and isolation.

Keywords: Screening, Isolation, pectinase, cellulase, xylanase

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Enzymes are distinct biological polymers that catalyze the chemical reactions and convert substrates to particular products (Haq et al. 2006). Xylanases, cellulase and pectinases have gained a unique place in the biotechnological sector due to their potential application in paper-pulp industry (Kirk and Jefferies, 1996), food and feed industry (Bhat, 2000), textile industry (Csiszár et al. 2001) and Biofuel production (Goldschmidt, 2008). Cellulases hydrolyse the β-1,4 glucosidic bonds in cellulose (Bayer et al. 1998). Pectinase degrade of the long and complex molecules called pectin (Kashyap et al. 2001). Xylanases constitute one of the most important industrial enzymes that depolymerizes xylan molecule into xylose units (Garg et al. 2011). The production of enzymes generally depends on variety of growth parameters like inoculum size, pH value, temperature, inducers, medium additives, aeration, growth and time (Immanuel et al. 2006) and also the enzyme activities depend on the presence of various metal ions as activators and inhibitors (Muhammad et al. 2012). Enzymes have various applications different industries. The major industrial applications of enzymes are in textile industry for 'biopolishing' of fabrics and producing stonewashed look of denims, in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998), in food, leather, paper/pulp industries, in the fermentation of biomass for the biofuel production, in ruminant nutrition for improving digestibility, in fruit juices processing and another emerging application is de-inking of paper (Sakthivel et al. 2010). These industries required enzymes which can be highly stable and active at extreme pH and temperature.

The technology should be cost effective for the commercial viability. In this concern, use of agricultural residues for the production of xylanase, cellulase and pectinase have been reported by several workers (Ahlawat et al. 2007; Cui et al. 2008; Azeri et al. 2010). The earlier studies concentrate on low cost enzyme production rather than cost effective screening. Literature has shown the use of xylan (Nair et al. 2008; Yasinok et al. 2008; Gupta et al. 2009), pectin (Boccas et al. 1994; Mellon and Cotty, 2004; Ahlawat et al. 2007; Janani et al. 2011), and cellulose (Baharuddin et al. 2010) for screening purposes. The use of these purified

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substrates would be too expensive for qualitative estimation of enzyme activity during screening. Attempts have been made to replace the expensive commercial substrates, especially xylan, pectin and cellulose, with agricultural residues i.e., Wheat-bran and citrus-peel for screening of microorganisms. This new method may be potentially applicable in large-scale microbial screening purposes.

MATERIAL AND METHODOLOGY

Qualitative screening of microorganisms

Soil samples of decaying fruit and vegetable waste, woody materials, and sugar mill were collected from different places of India, were used for the isolation of xylanase, cellulase and pectinase producing bacteria. Each soil samples of 1 g were suspended in 50ml distilled water with 1% wheat bran and 1% citrus peel powder for the enrichment of microorganisms. The flasks were incubated in an orbital shaker incubator at 37°C with shaking at 120 rpm for 48 hrs. After incubation mixtures made in previous step were allowed to settle down then dilutions up to 10^{-6} were prepared. In primary screening 100 µl from dilution 10^{-4}, 10^{-5} and 10^{-6} was taken and spread evenly over the surface of sterilized nutrient agar wheat bran media containing (g/l): Peptone 5.0, Beef extract 3.0, Agar 15.0 and wheat bran 10.0. The plates were incubated at 37 ℃ for 24 h. The colonies found on the plates were spotted onto fresh nutrient agar wheat bran plates for qualitative test and nutrient agar plates for the maintenance of culture. The plates were incubated under same condition. Enzyme producing strains were selected by flooding nutrient agar wheat bran plates with 0.5% (w/v) Congo red for 15 min. Then destaining was done by repeated washing with 1M NaCl solution for zone analysis (Kaur et al. 2011).

In secondary screening the cultures isolated in the previous step were spotted onto citrus peel agar containing (g/l): Peptone 5.0, Beef extract 3.0, Agar 15.0 and citrus peel 10.0. The plates were incubated at 37 ℃ for 24 h. After 24 hrs of incubation, plates were stained with congo red solution (0.5%) for 15 min followed by repeated washing with 1 M NaCl for zone analysis (Kaur et al. 2011). Colonies that showed areas of clear zones were selected for further screening in liquid medium where wheat bran was the main carbon source. In final step, the bacteria showing clear zone in secondary screening were further inoculated on the medium containing both wheat bran and citrus peel. The composition of medium is Peptone 5.0, Beef extract 3.0, Agar 15.0, Wheat-bran 10.0 and Citrus-peel 10.0. The plates were incubated under same condition. After 24 h of incubation the plates were stained with congo red solution (0.5%) for 15 min. Then destaining was done by repeated washing with 1M NaCl solution for zone analysis. Positive and better zone producing strain was chosen and continued for further studies. Colonies showing areas of clear zones were further evaluated by growing the cultures on 0.25% of commercial birch wood xylan, 0.25% of cellulose and 0.25% pectin instead of 1% wheat-bran and 1% Citrus-peel. The remaining protocol was exactly same and the zone formation was observed. Commercial substrates had been reported by many scientists for the isolation of enzymes (Bai et al. 2012; Ghani et al. 2013; Baharuddin et al. 2010; Roy and Rowshanul, 2009; Irfan et al. 2012; Shaikh et al. 2013).

Quantitative screening of microorganisms

The bacterial isolates were further screened for enzyme activity in submerged fermentation in 250 ml Erlenmeyer flasks. The microbial cultures were grown in modified Horikoshi medium (Peptone, 0.5%; yeast extract, 0.5%; KNO_3, 0.5%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.01%; Wheat bran, 2.0%) inoculated with 2% of 24 h old inoculum at 37°C under shaking conditions in orbital shaker incubator at 150 rpm for 48 h. Crude enzymes were extracted from the fermented culture filtrate by centrifugation at 10,000 rpm for 25 min at 4°C and the clear supernatant (crude extract) was used for enzyme assay using 3,5-dinitrosalicylic acid .

Birchwood xylan, 1%; polygalacturonic acid, 0.5%; and carboxymethyl cellulose, 1% were used as substrates for assaying the activity of xylanase, pectinase, and cellulase, respectively. The reaction mixture for xylanase and cellulase assay contained 490 µl of respective substrate (prepared in phosphate buffer of pH 7) and 10 µl of appropriately diluted enzyme. Pectinase assay contained 400 µl of substrate (prepared in HCl Tris base buffer pH 9) and 100 µl of appropriately diluted enzyme. Mixture was incubated at 55°C for 10 min. The reaction was terminated by adding 1.5 ml of 3, 5- dinitrosalicylic acid reagent (Miller, 1959). Controls for the enzyme assay were run simultaneously. The contents were boiled for 15 min and after cooling, the colour developed was read at 540 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar (equivalent to xylose, galacturonic acid and glucose for xylanase, pectinase, and cellulase, respectively) per minute under standard the assay conditions.

RESULTS AND DISCUSSION

Xylanase, cellulase and pectinase 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.

The objectives of this study were to develop an economical medium for isolation of xylanolytic, cellulolytic and pectinolytic bacteria using agrowaste instead of pure commercial substrate. In this investigation, bacterial strains producing Xylanase, cellulase and pectinase enzyme simultaneously has been isolated using cheap agro-residues as substrates. Wheat bran is a complete nutritious feed for microorganisms, having all the ingredients and remains loose even under moist conditions providing a large

surface area (Babu and Satyanarayana, 1996). Citrus peel is an enriched source of pectin. It contains 30% of pectin (Huang and Luyen, 1989).

Isolation and screening for xylanase, cellulase and pectinase producing bacteria.

Xylanase, cellulase and pectinase producing bacteria are likely to be found at places where decaying cellulosic, woody and citrus fruit waste material is present. In this study, soil samples were collected from different locations for isolating xylanolytic, cellulolytic and pectinolytic bacteria. Varghese et al. (2013) and Raju et al. (2013) isolated pectinase and cellulase producing bacteria respectively from fruit and vegetable waste dump soil.

Qualitatively the bacteria were screened on the basis of zone of hydrolysis around the bacterial growth. In primary screening, the colonies forming a clear zone, after Congo red staining of nutrient agar wheat bran plates indicate the colonies to be the xylanase and cellulase producers (Figure 1). Efficiency of this method was further confirmed by growing the isolates on nutrient agar medium containing 0.25 % birch wood xylan and 0.25% cellulose individually to confirm the production of xylanase and cellulase (Figure 2, 3). The isolates producing no zone of clearance on nutrient-agar plate containing wheat bran did not show any zone of clearance on nutrient-agar plate containing birch wood xylan and cellulose (Table 1). Nutrient agar medium containing xylan and cellulose for the screening of xylanase and cellulase producing strains respectively through the formation of zone of hydrolysis have been reported by many workers (Cordeiro et al. 2002; Nair et al. 2008; Yasinok et al. 2008; Gupta et al. 2009; Baharuddin et al. 2010).

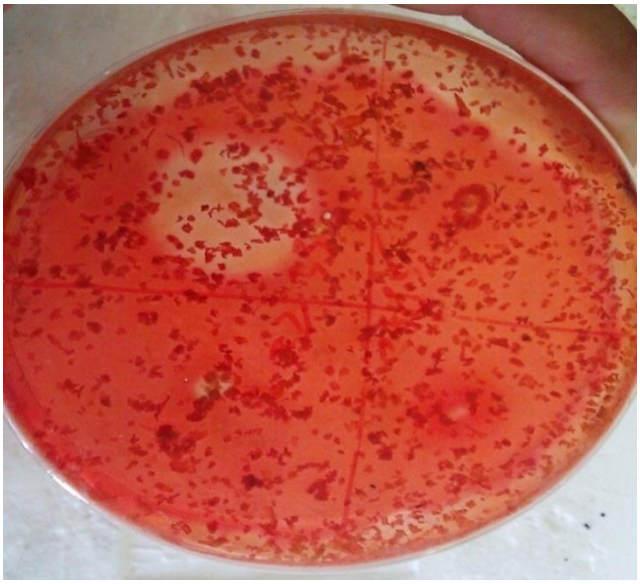

Figure 1 The zone of hydrolysis produced by bacterial strains on nutrient agar medium containing wheat bran

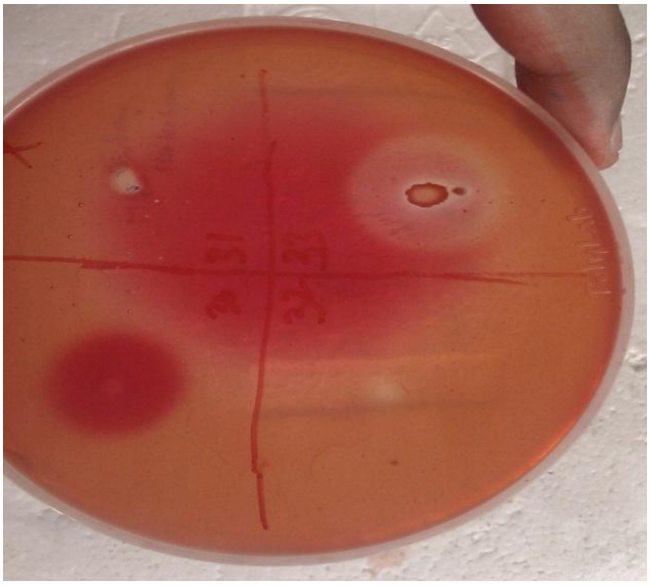

Figure 2 The zone of hydrolysis produced by bacterial strains on nutrient agar medium containing xylan

Total 33 bacterial isolates were obtained of which 14 isolates produced zone of hydrolysis on medium containing wheat bran, 10 isolates shows zone of hydrolysis on commercial xylan and 12 isolates produced zone on medium containing cellulose (Table 1).

In secondary screening, the isolates were transferred onto nutrient-agar medium containing citrus peel, colonies forming a clear zone of hydrolysis on citrus peel medium were indicated to be the pectinase producers. Cultures showing clear zone on nutrient-agar plate containing citrus-peel were further tested on commercial pectin (Fig. 4).

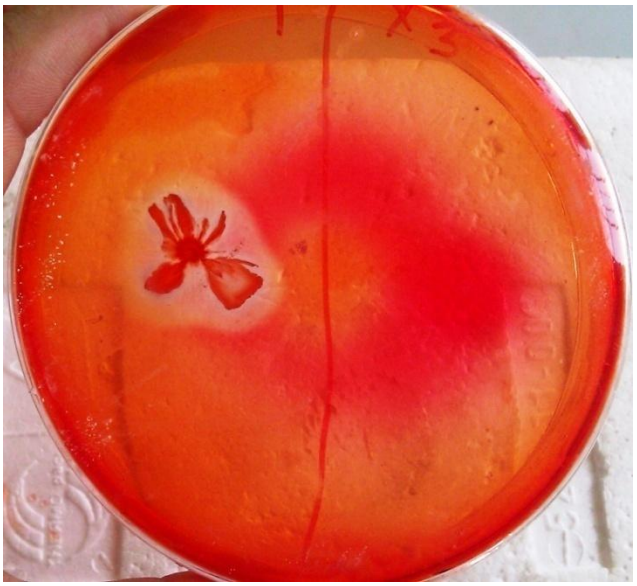

Figure 3 The zone of hydrolysis produced by bacterial strain on nutrient agar medium containing cellulose

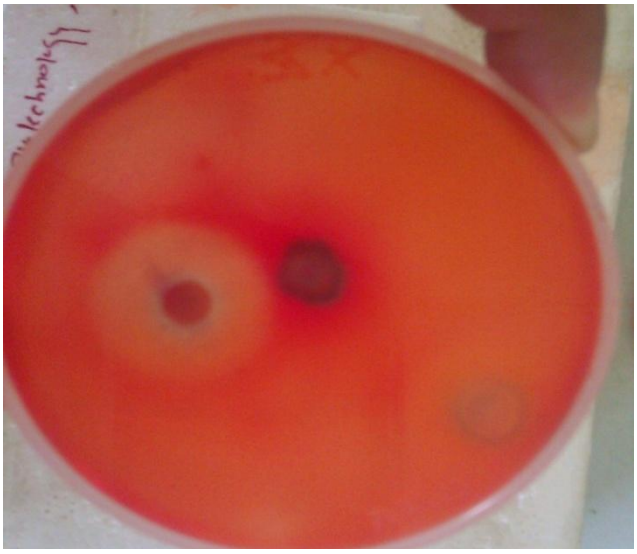

Figure 4 The zone of hydrolysis produced by bacterial strain on nutrient agar medium containing pectin

Isolates forming clear zone on citrus peel also showed clear zone on commercial pectin (Table 1). Commercial pectin has been used by many workers for the isolation of pectinase producing microorganisms (Boccas et al. 1994; Mellon and Cotty, 2004; Ahlawat et al. 2007). Out of 33 isolates, 10 isolates shows zone of hydrolysis on citrus peel as well as commercial pectin (Table 1).

In the last the isolates showing the positive results were spotted on the medium containing wheat bran and citrus peel simultaneously. Clear zone on nutrient medium containing both substrate shows that the isolates are producing all the three enzymes. Congo red dye is known to interact only with intact (1,3- and 1,4-) β-D-glucans. The appearance of zone after staining with Congo red dye indicates that xylanase, cellulase and pectinase secreted by bacterial culture hydrolyzes the xylan, cellulose and pectin respectively. As a consequence, the average molecular mass of the substrate will gradually decrease as the enzymatic reaction proceeds. The bacteria producing zone of hydrolysis were maintained on NA medium. Xylanase, cellulase and pectinase bacteria have been isolated from different kind of samples. Das and coworkers (2011) reported xylanolytic , cellulytic and pectinolytic activity of different bacteria like *Bacillus* sp. L6, *Bacillus pumilus* strain EK-17, *Bacillus* sp. YACS30, *Bacillus pumilus* strain IKMB13-518F, *Agrobacterium* sp., *Bacillus pumilus* strain, *Microbacterium* sp. PVC8, *Bacillus pumilus* strain IK-MB12-518F isolated from the jute retting water bodies.

Quantitatively the bacterial isolates were screened for the production of xylanase, cellulase and pectinase in SmF. Out of 33 isolates 6 isolates were producing zones on all medium containing wheat bran, xylan, cellulose, pectin and citrus peel plates were grown in modified Horikoshi medium and incubated at 37°C for

Table 1 Qualitative analysis of the bacterial isolate for xylanase, cellulase and pectinase production

Isolate	Media Type				
	WBA	XA	CA	CPA	PA
GSM-1	+++	+++	++	++	++
GSM-2	++	++	+	+	++
GSM-3	-	-	-	-	-
GSM-4-	-	-	-	-	-
GSM-5	+++	++	+	++	++
GSM-6	+	-	+	+	+
GSM-7	-	-	-	-	-
GSM-8	-	-	-	-	-
GSM-9	-	-	-	+	++
GSM-10	-	-	-	+	+
GSM-11	+	+	-	-	-
GSM-12	-	-	-	-	-
GSM-13	-	-	-	-	-
GSM-14	+	-	+	+	++
GSM-15	-	-	-	-	-
GSM-16	+	+	-	-	-
GSM-17	-	-	-	-	-
GSM-18	++	++	+	-	-
GSM-19	+	-	+	-	-
GSM-20	+	-	+	-	-
GSM-21	-	-	-	-	-
GSM-22	-	-	-	-	-
GSM-23	+	+	++	+	+
GSM-24	-	-	-	-	-
GSM-25	-	-	-	-	-
GSM-26	-	-	-	-	-
GSM-27	+	+	+	++	+++
GSM-28	-	-	-	-	-
GSM-29	-	-	-	-	-
GSM-30	+++	+++	++	+++	+++
GSM-31	-	-	-	-	-
GSM-32	-	-	-	-	-
GSM-33	+++	+++	++	-	-

WBA: Wheat Bran Agar; XA: Xylan Agar; CA: Cellulose agar; CPA: Citrus peel Agar; PA: Pectin agar

48 h under shaking conditions at 120 rpm. The culture filtrate in each flask was centrifuged at 10,000 rpm for 25 min at 4^0C and the clear supernatant was used for the estimation of xylanase, cellulase and pectinase activity. Based on the xylanase, Cellulase, pectinase assay strain was selected, which exhibited highest activity under un-optimized conditions. Among the above 6 bacterial strains, the best strain (GSM-30) showing highest enzyme activity were selected for further experiments. A record of the quantitative analysis of xylanase, cellulase and pectinase production under SmF for 6 selected strains is given in Fig 5. The isolates GSM-30 showed maximum activity of xylanase activity 37.33 IU/mL, cellulase activity 0.603 IU/mL and pectinase activity 2.11 IU/ml respectively. On the basis of qualitative and quantitative results, isolated strain GSM-30 was the highest simultaneous producer of all three enzymes.

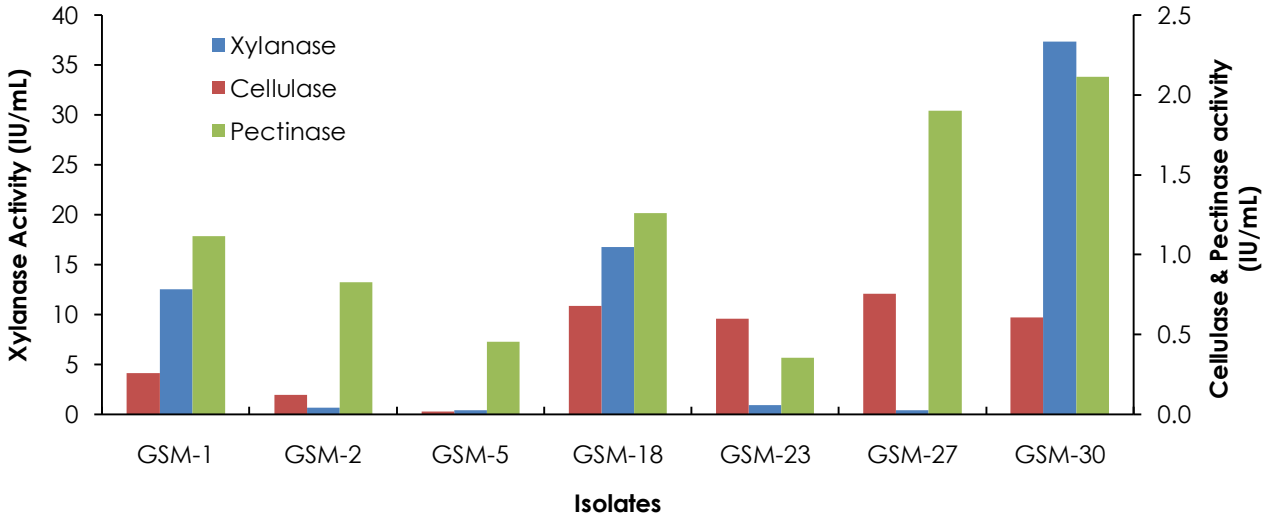

Figure 5 Xylanase production in SmF by bacterial strains isolated from different samples under unoptimized conditions. The flasks contained 50 mL of initial production medium, inoculated with 1% of inoculum and incubated at 37°C for 48h along with shaking at 120 rpm

Researchers have isolated various bacteria from different environmental sources which produces different enzymes. Hatami et al. (2008) isolated aerobic cellulolytic bacteria from forest and farming soils and determined their ability to decompose cellulose. *Cellulomonas* sp. ASN2, a cellulolytic bacteria isolated by Irfan and coworkers (2012) from soil. Yin et al. (2010) isolated *Cellulomonas* sp. YJ5 from soil samples and cellulase activity was determined after 48 hours of incubation. Das et al. (2010) isolated eight bacterial strains from cow dung samples and they found that maximum amount of cellulase producing bacterial isolate was *Bacillus* sp. Ghani and colleagues (2013) isolated five strains of *Bacillus licheniformis* producing alpha-amylase, glucoamylase, protease and pectinase activities from vegetative field. In another study, Otajevwo and Auyi (2010) isolated *Pseudomonas* sp. and *Serratia* sp. from soil samples, which have greater ability to produce cellulase enzyme.

Conclusion

From the results of this study it has been revealed that the isolation of microorganism simultaneously producing xylanase, cellulase and pectinase, using agriculture substrate is cost effective and attractive screening procedure. The result on agro residues as well as on commercial substrate is comparable. Since, the agro-wastes are used for the isolation of the microorganism, so agricultural waste will be used for the production of these enzymes which reduces the cost of production of all the three enzymes simultaneously.

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