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RESEARCH ARTICLE

Efficient retting of bast fiber yielding stems by extracellular enzyme conglomerate and oxalic acid produced by a newly isolated *Penicillium sp*.

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Abstract

In a screening program aimed at isolating a fungus capable of retting jute (*Corchorus olitorius*) and mesta (*Hibiscus sabdariffa*) ribbons within a short time, a fungus producing multiple extracellular enzymes such as polygalacturonase (83.28 IU/g), pectinolytic activity (42 IU/g), xylanase (117.96 IU/g), carboxymethylcellulase (9.81 IU/g) and oxalic acid (8.73mM) on wheat bran was isolated. The fungus was identified as *Penicillium oxalicum* on the basis of morphological characteristics. A significant feature is that xylanase activity increased >20 fold in solid state fermentation on wheat bran from that in submerged fermentation in minimal medium with birch wood xylan. Compatible pH and temperature optima of polygalacturonase and xylanase facilitated their joint action on the substrate. Thermostability of polygalacuronase , better than other strains of *P.oxalicum* aided in its functional capability. The boiled culture extract of *P.*oxalicum was found to remove significant amount of pectin from different portions of jute and mesta stem ribbons within 40h showing calcium chelating effect of oxalic acid. The culture extract possessing the functional enzymes and oxalic acid was able to remove 65-100% pectin from different portions of jute ribbons and 100% pectin from all portions of mesta ribbons releasing well separated fibres within 40h. The culture extract of the fungus on the sixth day of fermentation was able to ret whole jute and mesta ribbons within 40h producing well separated fibers.

INTRODUCTION

Jute industry is one of the major industries in West Bengal, India. The demand for fine jute fiber is increasing day by day for its use in apparels, chiefly as blends with cotton or other fibers. The quality of jute fiber depends largely on retting. Scarcity of adequate water is the main cause of improper retting. This warrants post-retting improvement of fiber at a cheap cost.

The middle lamella of parenchymatous cells around fiber bundles in jute stems mainly contain pectin. Dissolution of this pectin along with other polysaccharides like xylan and carboxymethyl- cellulose is needed for release and proper separation of fibers. A combination of pectinase and xylanase and a small amount of carboxymethylcellulase therefore, has potentiality for application in various areas of textile processing like bast fibre degumming and improvement of fiber characteristics like fineness and softness (Majumdar et al.,1991; Sharma and Sumere, 1992; Kapoor, 2001; Sharma and Satyanarayana, 2005; Tahir et al., 2011). Sreenath et al. (1996) reported that Jute/Cotton blended fabrics could be smoothened with enzyme combination of pectinase and xylanase. The galacturonic acid residues of pectin aggregate with Ca^{++} that crosslink pectin chains to each other. The presence of calcium reduces

the action of endopolygalacturonase. Treatment with chelators like EDTA and oxalic acid can improve enzyme performance (Majumdar et al., 1991 ; Henriksson et al.,1997) .

A number of fungi have been reported as high producers of multiple enzymes of pectinase and xylanase group. Amongst the mesophilic filamentous fungi *Apergillus niger* is noteworthy for their production of similar multiple enzymes. For example, *Aspergillus niger* A-20 was reported to produce active multienzyme systems containing pectinase, cellulase and xylanase after 5 days in shake culture on orange peel. Insignificant level of amylase and lipase were also produced (Ismail, 1996). Another strain of *A. Niger* BTL was also found to produce xylanase, polygalacturonase and pectin lyase on by products of citrus processing industry. In the same study, *Fusarium oxysporum* F3 and *Penicillium decumbens* were found to produce relatively lower levels of the enzyme (Mamma et al., 2008). Umsza-Guez et al. (2011) reported multiple enzyme production by *Aspergillus awamori* on tomato pomace. *Penicillium viridicatum* RFC3 (Silva, 2005) was also reported to produce pectinase, xylanase and carboxy methyl cellulase. Ikotun (1984) reported production of cellwall-degrading enzymes by *Penicillium oxalicum* Curie Thom isolated from infected yum tubers. Twenty seven thermophilic and thermotolerant fungal strains were isolated from soil decaying organic matter and sugarcane piles. The highest cellulose and xylanase producers were identified as *Aspergillus fumigatus* M 7.1 and *Myceliophthora thermophila* M 7.7 (Moretti, 2012).

In the present study an attempt has been made to isolate a fungus with extracellular pectinase, xylanase, very low concentration of carboxymethylcellulase and with significant quantity of oxalic acid. The degummining ability of the fungal strain will be useful in cleaning and softening of bast fibers in the industry.

Materials and methods

Substrates used: Citrus Pectin(pure, methoxy content-Min 7%) from SRL, India; Birchwood Xylan from Sigma-Aldrich; Carboxymethylcellulose sodium salt, pure from Merck; Wheat Bran (Mesh Size- 2mm.), fresh plant stems of jute (*Corchorus olitorius*) and mesta (*Hibiscus sabdariffa*) devoid of the central woody xylem core, also called the ribbons. The plants were obtained from Central Research Institute of Jute and Allied Fibres, West Bengal,India.

Isolation of fungus: Soil was collected from a jute growing field in North 24 Parganas, West Bengal. Ten gram of soil sample was taken in 500 ml Erlenmeyer flask containing 90 ml of sterile distilled water. The suspension was mixed in a vortex mixer. After the coarse particles settled down, the suspension was decanted in a sterile flask. Following this serial dilution of the suspension was made. Selected dilutions were plated on Omeliansky's basal agar medium (Rao,1999) containing $(NH_4)_2SO_4$,1.0g; K_2HPO_4,1.0g; $MgSO_4.7H_2O$,0.5g; $CaCO_3$,2.0g; NaCl, trace; yeast extract,5g in 1000 ml distilled water supplemented with 1% citrus pectin and solidified with 2% agar. The plates were incubated for 5 days at 30°C. The fungus showing rapid growth and exhibiting the largest zone of clearance was isolated, purified and finally maintained by growing on slants of the same medium.

Identification of the fungus : The fungus was identified on the basis of morphological characters using compound microscope.

Enzyme production at different times of incubation: The isolated fungus was grown in Omeliansky basal liquid medium supplemented with 1% citrus pectin, 1% birchwood xylan and 1% carboxymethycellulose (CM-cellulose) separately. Cultivation was done in batches of 100 ml each in 500 ml Erlenmeyer flask and continued for 15 days at 30°C under shaking condition (100 rev/min). At time intervals, portion of cultures were filtered, centrifuged in a cold centrifuge (4°C and at 12,000 rev/min) and the supernatants were collected for enzyme assay.

Enzyme assay: Pectinase, xylanase and carboxymethylcellulase activities were determined by measuring the release of reducing sugars from citrus pectin, birch wood xylan and CM- cellulose respectively using the 3,5-dinitrosalicylic acid (DNS) reagent assay method (Miller,1959). The reaction mixture containing 0.8 ml of 1% substrate in 0.2 M acetate buffer (w/v), pH 5.0 and 0.2 ml of crude enzyme solution was incubated at 40°C for 10 min (Soares et al., 1999). One unit of pectinase (polygalacturonase), xylanase and CM-cellulase activities were defined as the amount of enzyme which released one µmol of galactouronic acid, one µmol of xylose and one µ mol of glucose respectively per min under the stated assay condition.

Pectin liquifying activity was measured by the viscometric method. One ml of suitably diluted sample was mixed with 17 ml of 20g/l citrus pectin solution in 0.1M acetate buffer, pH 4.6 at 40°C. Reduction of the viscosity of the mixture was monitored using Ostwald viscometer. One unit of pectin liquefying activity was defined as the amount of the enzyme that reduced viscosity of the solution by 50% per min under the stated condition.

Pectate lyase activity was determined by incubating 1 ml of enzyme with 2.5 ml of 0.5% citrus pectin solution in 0.2 M acetate buffer, pH 5.0 and 1 mM calcium chloride or 50 mM Tris-HCl buffer, pH 8.0 and 1mM calcium chloride for 10 min at 30°C. The absorbance at 235 nm was noted throughout the time period and compared with that of the control (without enzyme).

Xylan liquifying activity was measured by the same viscometric method using birch wood xylan as the substrate and 0.1M acetate buffer, pH 5.0 as the buffer. One unit of endo-xylanase activity was defined as the amount of the enzyme that reduced viscosity of the xylan solution by 50% per min under the stated assay condition.

Characterization of polygalacturonase and xylanase: The submerged cultures of the fungus in Omeliansky's basal medium containing 1% of the substrate (pectin / xylan) was harvested on the 8^{th} and 14^{th} day for characterization of polygalacturonase and xylanase respectively.

Determination of optimum pH: Polygalacturonase and xylanase activities were determined at 40°C in different pH using appropriate buffers; 0.2M sodium acetate buffer (pH 3.0 – pH 5.0), 0.2M citrate- phosphate buffer (pH 5.5 – pH 7.0), 0.2M Tris-HCl buffer(pH –7.5) and glycine – NaOH buffer (pH 8.5 – 11).

Determination of optimum temperature: The optimum temperature of each enzyme was determined by incubation of the reaction mixture at temperatures between 30°C and 70°C at optimum pH.

Determination of pH and temperature stability of polygalacturonase and xylanase: For detemination of pH stability, each enzyme solution is mixed (1:1) in the above mentioned buffers of different pH (pH 3.0 – 10.5) and kept at 25°C for 24h. After 24h, the residual enzyme activity was determined at optimum pH and temperature for the enzymes.

For the determination of temperature stability, each enzyme was incubated at different temperatures between 30°C and 70°C for 3h at pH 4.6. An aliquot was withdrawn at hourly interval, cooled and the residual enzyme activity was assayed at the optimum pH and temperature for the enzymes.

Solid state fermentation: Solid state fermentation was carried out in 500 ml Erlenmeyer flask each containing 10 g of commercial wheat bran. The flasks were sterilized at 120°C for 40 min and subsequently inoculated with aliquots of spore suspension (approx. 10^7 spores /g dry substrate). The spores were harvested from 7 day old Omeliamsky - pectin/xylan agar slant cultures, with distilled water. The final moisture content of the medium was approximately 67%. The cultivation of the fungus in wheat bran was carried out at 30°C for 15 days. At 24 h intervals, 10g of fermented wheat bran culture present in one Erlenmeyer flask was mixed with 60 ml distilled water (6g of fermented material/ml), stirred for 30 min with a glass rod, filtered and centrifuged. The supernatant was used as the crude enzyme source and assayed as described before.

Estimation of oxalic acid in the wheat bran culture filtrate of the fungus: Oxalic acid was extracted from the wheat bran culture extracted and was estimated as per the standard titrimetric method using $KMnO_4$ (Amadioha, 1993).

Study on the degumming ability of *Penicillium oxalicum* JFF3: Jute and mesta stem ribbon pieces (5cm in length) cut from the top, middle and root portion were pressed with blotting paper to remove excess water and weighed. One set was treated with 6 day old wheat bran culture extract of the isolated fungus. Another set was treated with the boiled culture extract. In each case, the pH was adjusted to 4.6 with 2M acetate buffer. The final molarity of the acetate buffer was kept at 0.2M. The ribbon:culture extract ratio was kept at 1:4. For the control set, jute ribbon pieces were immersed in 0.2M acetate buffer, pH4.6. The experimental and control sets were incubated at 40^0C. The whole jute and mesta ribbons were treated with wheat bran culture extract as described above and incubated at 40^oC in a shaker incubator (100 rev/min) for 40h. The ribbons were then washed with water and air-dried.

Estimation of pectin: Pectin was estimated by the gravimetric method following extraction by 0.25% ammonium oxalate pH 4.6 as described by Nazaruddin et al. (2013).

Results and Discussion:

Identification of the fungal strain

The isolated fungus was identified on the basis of morphological characteristics observed under compound microscope. The fungus was identified as *Penicillium oxalicum* by consulting suitable literature. As per laboratory number protocol, the fungus will be referred to as *Penicillium oxalicum* JFF3.

Growth and utilization of carbon source by *Pencillium oxalicum* JFF3 in liquid Omeliansky medium: The fungus liquefied pectin completely and xylan partially within 5days when grown in liquid Omeliansky medium supplemented with 1% (w/v) citrus pectin and 1% (w/v) birchwood xylan respectively. The fungus, however, did not show visible growth on Omeliansky medium supplemented with 1% CM-cellulose.

Enzyme production by *P. oxalicum* JFF3 in Omeliansky basal medium with specific carbon source: The culture filtrate of *P. oxalicum* JFF3 when grown in Omeliansky liquid basal medium supplemented with 1% (w/v) pectin or 1% (w/v) xylan separately, showed significant polygalacturonase but comparatively low xylanase activities. Polygalacturonase production reached a peak first on the 3rd day (9.022 IU/ml) and then on the 8th day (12.27 IU/ml) of cultivation (Fig.1). Xylanase production showed increase in activity upto 14 days of cultivation.

The production was low, being 0.96 IU/ml after 14 days (Fig.2). Since the fungus did not show any visible growth in Omeliansky's basal medium supplemented with 1% (w/v) CM-cellulose; assay of carboxymethyl cellulase was not done.

Partial characterization of the enzymes: The optimum pH (4.6) for polygalacturonase and xylanase of *P. oxalicum* JFF3 (Fig.3) was found to be comparable to acidic polygalacturonases and xylanases of different *Penicillium* species, which generally ranges between 4 to 5 and occasionally 6 (Li et al., 2007; Liao et al., 2012). Though the optimum temperature (40^0C) for polygalacturonase activity of *P. oxalicum JFF3* (Fig.4) was found similar to the optimum temperature of *Penicillium* sp.EGC5 (Martin et al., 2004), it was found to be low in comparison to polygalacturonases of most other *Penicillium* species including *P. oxalicum* (CGMCC0907) where the optimum temperatures were reported to be 50^0C or higher (Silva,2007; Okafor et al., 2010; Banu et al., 2010). The optimum temperature (50^0C) for xylanase activity of *P. oxalicum* JFF3 is similar to most other xylanases, particularly xylanases of *P. oxalicum* ZH-30 (Li et al. ,2007) or *P. oxalicum* GZ2 (Liao et al., 2007).

Same optimum pH (4.6) for both xylanase and pectinase of *P. oxalicum* JFF3 will be advantageous for its use in processes where the activities of both the enzymes are required simultaneously as in the case of bast fibre degumming. Similarly the activity temperature of both the enzymes of *P. oxalicum* JFF3 is compatible since pectinase is active at a broad range of temperature from 30°C to 60°C and the optimum temperature for xylanase is at 50°C. The enzymes polygalacturonase and xylanase of the fungus was able to retain above 80% of activity at 50°C and around 60% of activity at 60^0C when held for 3h (Table 1). In comparison polygalacturonase of another strain of *P. oxalicum* was able to retain only 25% of activity when held at 60^0C for 1h. (Neagu et al., 2012). Li et al. (2007) reported that xylanase of *P.oxalicum* ZH-30 was able to retain only 18.6% and 0.90% of activity when held for 3h at 50^0C and 60^0C respectively. Maximum pH stability of both polygalacturonase and xylanase (100% activity after 24h) was at pH 7.5.In the case of polygalacturonase 50-52% of initial activity were retained in the acidic range (pH 3.6-6.5) when held for 24h. In the case of xylanase, pH stability was better with 67-74% of initial activity being restored in the pH ranges 3.6-6.5. The pH stability was better in the alkaline range than in the acidic range in both the cases (Table 2)

Multiple enzyme activity of *P. oxalicum* JFF3 in solid state fermentation: Enzyme activities of *P. oxalicum* JFF3 particularly that of xylanase increased manyfold in solid state fermentation using wheat bran as the sole nutrient source. Polygalacturonase activity, when studied as a function of days of fermentation in wheat bran showed two peaks, one on the 3rd day (45.6 IU/g) and another on the 6th day of fermentation (83.28 IU/g). Xylanase activity increased gradually reaching its peak on the 4th day of fermentation (124.92 IU/g). On the 6th day of fermentation activity dropped a little to 117.96 IU/g. (Fig.5). Solid state fermentation on wheat bran is compatible to industrial usage since wheat bran is cheap and is easily available in large quantities throughout the year. The two peaks of polygalacturonase activity in both submerged and solid state cultivation indicated a sequential production of isoenzymes by the fungi. This has been reported by several authors earlier (Martin et al., 2004; Silva et al., 2007). There was a striking increase in xylanase activity (>20 fold in terms of activity per ml of culture extract in both the cases) when the cultural condition was changed from submerged fermentation with 1% birch wood xylan as the inducer to solid state fermentation in wheat bran. In comparison, increase in polygalacturonase production from submerged fermentation with 1% citrus pectin to solid state fermentation in wheat bran was comparatively much less (< 2 fold). This may be because arabinoxylan is the major polysaccharide other than starch and cellulose in wheat bran (Chilkunda et al., 2004). Wheat bran was reported to be a good medium for exopolygalacturonase production (Neagu et al., 2012) and the best medium for xylanase production (Li et al., 2007) in other strains of *P.oxalicum*. In the case of *Penicillium oxalicum* JFF3 the extent of increase of xylanase production in wheat bran from that in submerged fermentation with 1% birchwood xylan was much higher compared to the earlier report of Li et al. (2007). In the latter case, however, oat spelt xylan was used instead of birch wood xylan. Majumdar et al. (1991) showed that, pectin liquifying activity and carboxymethyl cellulase activity are also required for enzymatic retting of jute ribbons. Since on the 6th day of fermentation in wheat bran, exopolygalacturonase (release of reducing sugars from citrus pectin solution) activity was highest and xylanase activity was also high; pectin and xylan liquifying activity, pectate lyase and carboxymethyl cellulase activities were also determined on that day. Pectin liquifying activity was found to be 42 IU/g. Interestingly some amount of carboxymethylcellulase and negligible amount of xylan liquifying activiity were also found (Table.3). Production of multiple enzymes by fungi using solid state fermentation has been reported by previous workers. Norazlina (2013) reported production of xylanase by *Trichoderma sp* via solid state fermentation using sugarcane bagasse (1%). Singh et al. (2015) has developed an improved novel protocol for the isolation of xylano-pectino cellulytic positive microorganisms using practically low cost substrates like 2% citrus peel, 2% wheat bran and 2% waste paper.

Kinetics of polygalacturonase activity: When the activity of endopolygalacturonase (reduction of viscosity) and the activity of exopolygalacturonase (release of reducing sugar) were noted against time, it was found that reduction of viscosity to 50% of initial viscosity occurred within 42 sec., when less than 15% reducing sugar was released from citrus pectin. According to Rombout and Pilnik (1980), an enzyme presents an endo character, when the initial viscosity is reduced by half and less than 15% glycosidic bonds are hydrolyzed. Since, the enzymes are not purified, it can only be said that the activity of endopolygalacturonase was predominant. Based on the same logic, it can also be said that the activity of exoxylanase is predominant , because xylanolytic activity is negligible.

Oxalic acid production: Oxalic acid production as a function of time of cultivation showed a sigmoid curve reaching 1.1 mg/ml (8.73 mM) oxalic acid on the 6^{th} day of cultivation (Fig. 6) .

Retting of jute ribbons by the wheat bran culture extract of *P.oxalicum* : In the case of 5cm long jute / mesta ribbon pieces, there was considerable reduction in weight and removal of pectin along with significant softening of the ribbons upon treatment with boiled culture extract of *P. oxalicum* JFF3.This was due to the effect of the presence of oxalic acid in the boiled culture extract. Oxalic acid has high affinity for Ca^{++} ions and chelates Ca^{++} ions which crosslinks the pectic chains in the middle lamella, thus breaking the pectin mesh. In the case of treatment with culture extract (not boiled), there was reduction in weight and removal of pectin of very high order along with complete maceration of the ribbons to well separated fibers showing synergistic activity of oxalic acid and the pectinolytic activity of the culture extract. Henriksson et al. (1997) showed that, addition of 50 mM oxalic acid to the retting bath of flax reduces the amount of Flaxzyme, according to the Fried test by a factor of 50. In this case, oxalic acid is present within the culture extract along with the enzymes, which is obviously an advantage. The boiled culture extract of *P.oxalicum* was found to remove 45%, 31% , 20% and 49%, 45%, 30% pectin from the top, middle, root portion of jute and mesta ribbons respectively within 40h .The unboiled culture extract possessing the functional enzymes and oxalic acid was able to remove 100%, 80% and 65% pectin from the top, middle and root portion of the jute ribbons respectively and 100% pectin from all portions of mesta ribbons within 40h. This may imply that the high degumming ability of the culture extract of *P.oxalicum* JFF3 . Significant role played by multiple enzymes produced by fungi in bio retting and improvement of fiber quality has been reported earlier. Molina et al. (2001) have done screening and genetic improvement of pectinolytic fungi for degumming textile fibers. In the present investigation the newly isolated fungal strain could be useful in degumming, cleaning and softening of lignocellulosic bast fibers, in general whether they are present singly or in blends.

Table1. **Temperature stability of exopolygalacturonase and xylanase of *P. oxalicum* JFF3**

Temp (◦ C)	Enzyme activity (% restored)					
	Exopolygalacturonase*			Xylanase*		
	1h	2h	3h	1h	2h	3h
30	100	100	100	100	100	100
40	100	100	99	95.7	94.0	92.2
50	90.8	90.4	86.6	81.8	80.8	80.1
60	68.1	62.8	61.6	64.9	62.6	59.7

* optimum pH for enzyme activity : 4.6

Table 2. **pH stability of exopolygalacturonase and xylanase of *P. oxalicum* JFF3**

pH	Enzyme activity (%)restored after 24h	
	Exopolygalacturoase*	Xylanase+
3.6-6.5	50 - 52	67 - 74
7.5	100	100
8.5-10.5	53 - 85	67 - 88

* optimum temperature for exopolygalacturonase activity : 40◦C ; + : optimum temperature for xylanase : 50 ◦C

Table 3 : Enzyme profile of 6 day old wheat bran culture extract of *P. oxalicum* JFF3

Name of the enzyme	**Activity (IU /g)**
Exopolygalacturonase	83.30
Endopolygalacturonase	42
Pectate lyase	Nil
Xylanase	117.96
Endoxylanase	0.38
Carboxymethyl cellulase	9.81

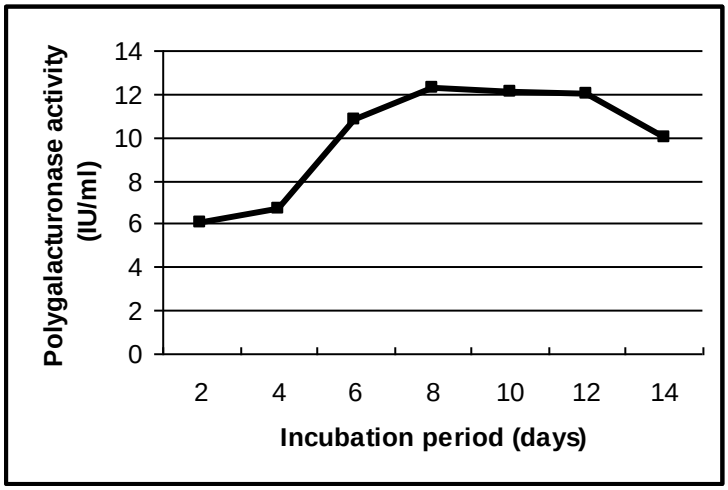

Fig 1: Polygalacturonase activity of *P. oxalicum* JFF3 as a function of days of incubation grown in Omeliansky's-pectin broth.

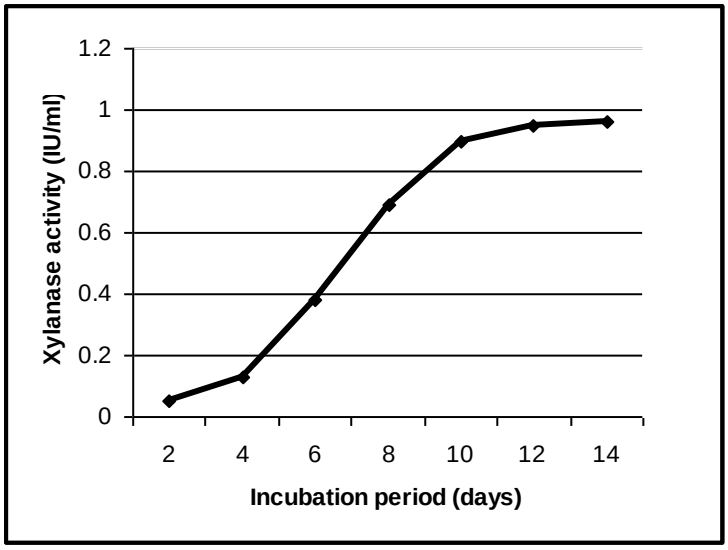

Fig.2. Xylanase activity of *P. oxalicum* JFF3 as a function of days of incubation grown in Omeliansky's-xylan broth.

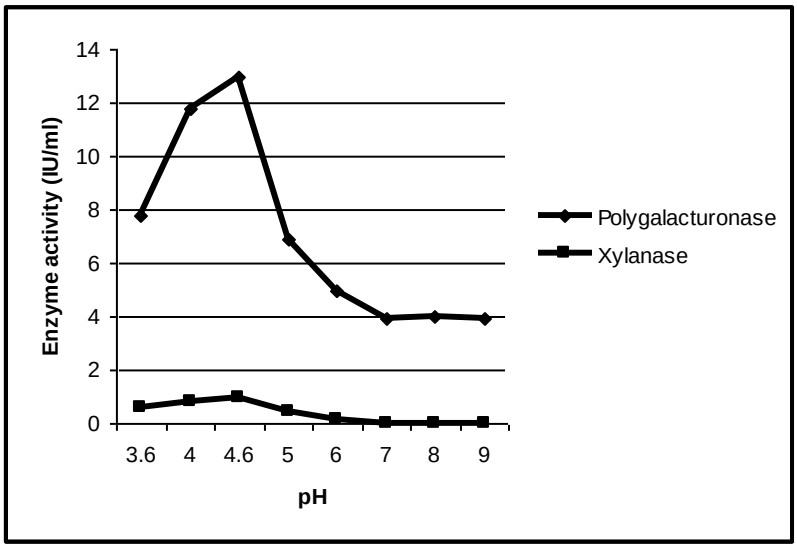

Fig.3: Effect of pH on the activity of polygalacturonase and xylanase.

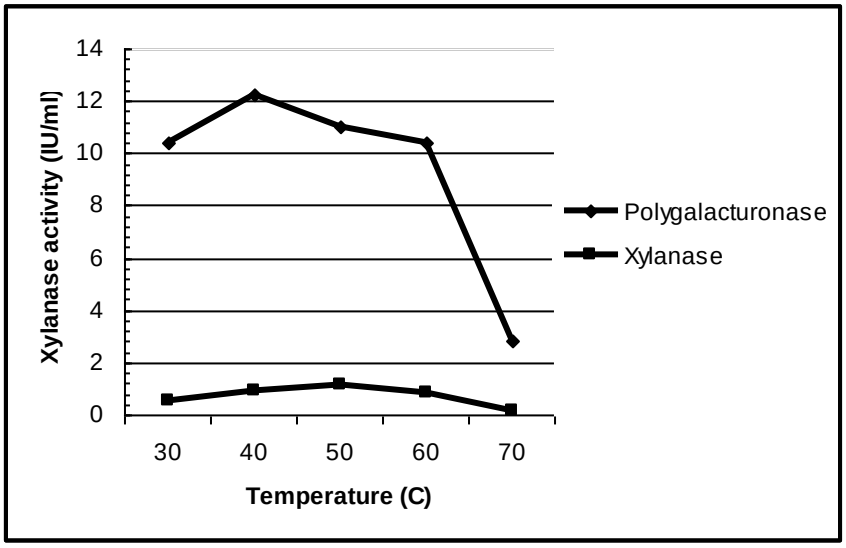

Fig.4 : Effect of temperature on the activity of polygalacturonase and xylanase

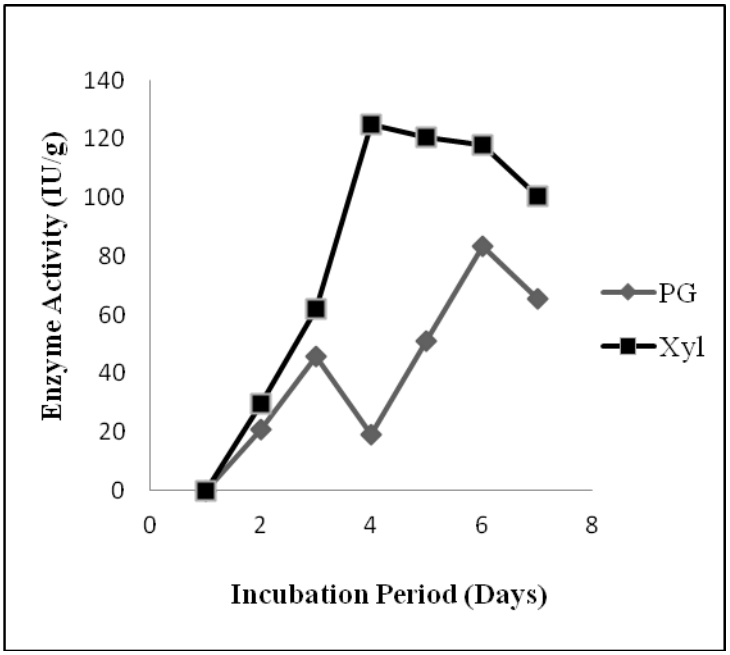

Fig. 5: Production of polygalacturonase (PG) and xylanase (Xyl) by *P. oxalicum* JFF3 in solid state fermentation on wheat bran.

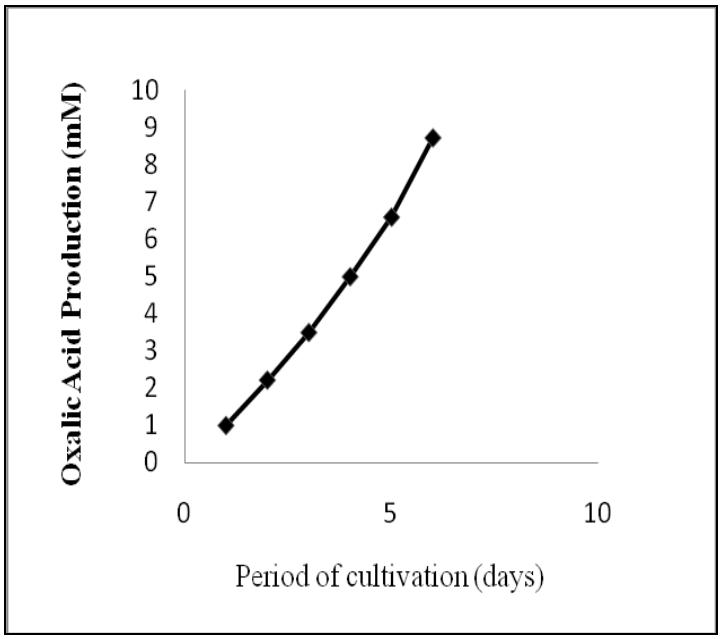

Fig. 6: Production of oxalic acid by *P. oxalicum* JFF3 in solid state fermentation on wheat bran.

CONCLUSION:

In conclusion, it may be said that the newly isolated *P. oxalicum* JFF3 is a potential source of pectinase, xylanase, carboxymethylcellulase and oxalic acid. The activity pH and temperature of xylanase and pectinase are compatible for their joint action on complex plant gummy substrates. The enzymes are moderately stable. This enzyme conglomerate along with oxalic was found to ret green jute / mesta ribbons within 40h releasing fibers. Optimization of cultural conditions and strain improvement will further increase its enzyme and productivity.

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