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RESEARCH ARTICLE

ISOLATION SCREENING AND IDENTIFICATION OF FUNGAL MANNANASE PRODUCER

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ABSTRACT

Isolation of local potential fungus capable of degrading mannan was explored from various sources of soil sample collected from agricultural areas. The isolates were screened based on the clearing zone method on selective agar media containing guar gum as substrate. A total of 40 isolates were screened and only 10 had activity ratio ranging from 1.6 mm to 3 mm which were used for further analysis. These isolates were incubated in shake flasks at 30 °C for 48 h having minimal media and 1% guar gum as substrate. The best enzyme producer was isolate AZF8 with mannanase enzyme acitivity of 108 U/ml. The isolate AZF8 was identified as Aspergillus nidulans and was further used for the production of mannanase from agroresidues. Different agroresidues were screened for mannanase production of which soy straw supported maximum mannanase production with enzyme activity of 80.18 U/ml.

Key words: Mannan, guar gum, Mannanase, Agroresidues.

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INTRODUCTION

β-mannanase is the enzyme that catalyses the endolytic hydrolysis of β-1,4mannosidic linkages in mannopolysaccharides such as β-1,4-mannan, glucomannan and galactomannan. Mannan and heteromannans are a part of the hemicellulose fraction in plant cell walls. (Dhawan, *et al.,* 2008) Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78) commonly known as mannanase, randomly hydrolyzes β -1,4-glycosidic linkage of mannan, glucomannan, galactomannan and galactoglucomannan, to yield manno oligosaccharides (Zhengaqiang Jiang *et al.,* 2006). Endo -β-mannanase is an endohydrolase, which is a member of glycosyl hydrolase family 5 and 26. As an endohydrolase it hydrolyses the internal bonds of a polysaccharide. Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants have been produced. A vast variety of bacteria, actinomycetes, yeasts and fungi are known to be mannan degraders (Talbot and Sygusch, 1990; Puchart *et al.,* 2004). Production of ß-mannanase by micro-organisms is more promising due to its low cost, high production rate and readily controlled conditions. Mannanases are generally produced in the presence of different mannan-rich substrates. These included locust bean gum (LBG), guar gum, konjac flour, and copra meal.

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and oil extraction processes for the hydrolysis of high molecular weight mannans (Daniela Alonso Bocchini Martins, *et al.,* 2011). Mannanases produce manno-oligosaccharides that are used as prebiotics in foodstuffs and pharmaceutical applications. The paper and pulp industry uses mannanases in combination with xylanases as biological prebleaching agents enhancing the extractability of lignin and reducing environmental pollution by avoiding the chemicals used (Khanongnuch, *et al.,* 1998). The breakdown of β 1,4-mannasidic linkages in a variety of mannan containing polysaccharide is of great importance in industrial processes for production of second generation biofuels, (Matt and Sweeney and Feng, 2012),which puts a premium on studies regarding the prospection and engineering of β mannanase. The main objective of this study was to isolate potential fungal mannanase producer and production of enzyme using cheap source of substrate. Mannanases have several industrial applications such as in food industries where they are used in coffee, fruit juices

MATERIALS AND METHODS

Chemicals and substrate: Guar gum powder, bovine serum albumin, and all other chemicals were obtained from HiMedia and were of analytical grade. Various agroresidues were collected from different agricultural areas of Aurangabad district, Jalna district, Buldhana district. Copra meal was prepared in laboratory using coconut from local market.

Source of microorganisms: 50 soil samples from farm areas, garbage dumping areas collected randomly from Aurangabad,

Jalna, Buldhana were used as source of microorganisms for screening, isolation and identification.

Treatment of copra: The copra was finely ground and sieved (1mm mesh) and then was boiled for 2 h with 2 volumes of distilled water. The cooled copra meal suspension was then placed at 4 ◦C overnight to allow the oil to solidify and finally be removed. The copra meal was further refined using solvent extraction using mixtures of different solvents for 24 h. One litre of solvent (methanol, ethanol, ether and n-hexane) was mixed with 100 g of ground copra in beaker and left overnight. The copra suspension was then filtered through What man No. 1 filter paper. The product was designated as defatted copra meal, after the residues were oven-dried and sieved. (Tse-Chun Lin *et al.,* 2004)

Isolation and enumeration: The sample, 1 g of solid sample was suspended in 10 ml of sterilized 0.85% normal saline (NaCl). The solution was mixed using vortex and one percent (v/v) of the solution was transferred into 20 ml of sterilized fungal isolation medium (potato dextrose media) with one percent copra meal. The microbial cells were grown under aerobic condition by shaking at 150 rpm for 24 hr at 40^0C (Khampheng Phothichitto *et al.,* 2006).

Preliminary screening: The culture broth from enumeration step were serially diluted and spread on potato dextrose agar plates containing guar gum instead of copra meal. The cells were allowed to grow at 30^0C for 3-7 days. The colonies with a clear zone of mannanase activity were observed after flooding the plate with grams iodine solution and the ratio of diameter of clear zone to colony was calculated. The positive isolates were selected and preserved on PDA slants at 40Cfor further studies.

Identification of isolate: Identification of the isolate was performed on basis of colony morphology and structural characteristics observed under light microscope. The fungal characteristics were described and identified based on both macroscopic characters (colony growth, colony surface) and microscopic characters (aseptate hyphae, branched hyphae, conidiophore, vesicle).

Enzyme production

The positive isolates were grown in a liquid medium consisting of basal medium (g/l): NH4NO3,0.3; KH2PO4, 2.32; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 7.54; $FeSO_4$, 0.01; $CaCl_2.2H_2O$, 0.05; PH 7.0 (Mona Mabrouk *et al.,* 2008) and replacing the gaur gum with different agroresidues apple peels, mango peels, banana peels, long bean peels, groundnut pods, green gram pods, sweet lime peels, orange peels, custard apple peels, potato peels, pineapple peels, cucumber peels, pomegranate peels ,peas peels, wheat bran, soystraw, corn cob, corn peels, kardi oil cake, gulmohar pods. The fungal spores were inoculated into 150 ml of growth medium in an Erlenmeyer flask and kept in an incubator shaker with an agitation rate of 150 rpm and incubated at 30 ± 2 °C for 72 to 96h.

Determination of enzyme activity

Mannanase was assayed by measuring the reducing sugars using dinitrosalicylic acid (DNS) method (Miller, 1959). The mannanase assay mixture contained 0.5 ml of 0.5% (w/v) guar gum (substrate), prepared in 50 mM phosphate buffer, pH 7 and 0.5ml of appropriately diluted culture broth. The reaction mixture was maintained at 40°C for 60 min. After incubation, 1 ml of DNS reagent was added and boiling took place from 5 – 15 min. The developed red brown color was measured at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1 _mol of mannose per minute under the assay conditions.

Protein determination: Soluble protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Preliminary screening of organisms

Fifty different soil samples were primarily screened for effective fungi. A total of 40 isolates showed clear zone of mannanase activity in isolation medium using guar gum as substrate at 30^0C (Table 1). Isolates AZF8 showed the highest activity ratio (diameter of clear zone/diameter of colony) of 3 in guar gum medium and also isolates AZF1, AZF2, AZF3, AZF14, AZF19, AZF20, ZF27, AZF30, AZF34 also showed good activity ratio as shown in table:1 and were subjected to secondary screening.

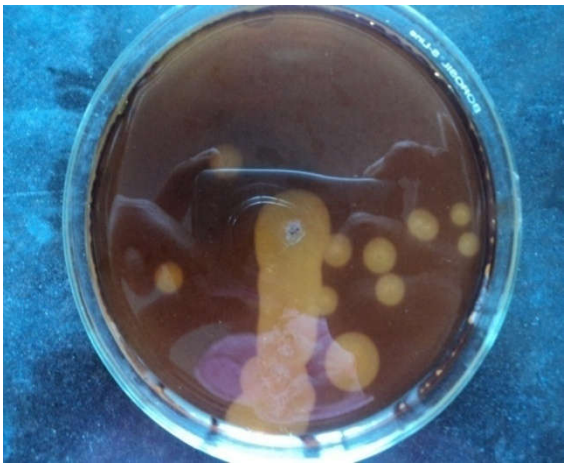

Fig. 1. Zone of hydrolysis shown by AZF 8 on minimal medium agar plate containing guar gum as sole source of carbon. The plate is flooded with grams iodine to make the zone visible

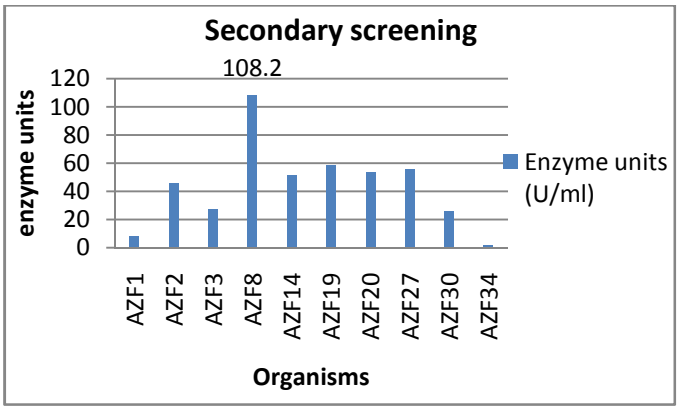

Fig.2. Isolate AZF 8 showed maximum enzyme activity of 108.2 U/ml.

Secondary screening: 10 fungi selected from primary screening were secondarily screened on basis of enzyme production.

Table 1. Activity ratio of 40 different isolates in which isolates AZF8 showed maximum activity ratio and AZF1, AZF2, AZF3, AZF14, AZF19, AZF20, AZF27, AZF30, AZF34 also showed good activity ratio

Sr.no.	Fungus code	Colony diameter (mm)	Zone diameter (mm)	Activity ratio (Zone of clearance/ colony diameter)
1.	AZF1	20	32	1.6
2.	AZF2	15	25	1.6
3.	AZF3	12	25	2.08
4.	AZF4	18	28	1.5
5.	AZF5	30	00	00
6.	AZF6	40	00	00
7.	AZF7	50	65	1.3
8.	AZF8	15	45	3
9.	AZF9	20	28	1.4
10.	AZF10	40	45	1.1
11.	AZF11	60	62	1.03
12.	AZF12	30	35	1.1
13.	AZF13	26	35	1.3
14.	AZF14	15	28	1.8
15.	AZF15	13	20	1.5
16.	AZF16	40	48	1.2
17.	AZF17	22	00	00
18.	AZF18	25	30	1.2
19.	AZF19	17	29	1.7
20.	AZF20	10	20	2
21.	AZF21	17	00	00
22.	AZF22	42	50	1.1
23.	AZF23	38	47	1.2
24.	AZF24	32	41	1.2
25.	AZF25	28	38	1.3
26.	AZF26	30	35	1.1
27.	AZF27	18	29	1.6
28.	AZF28	30	40	1.3
29.	AZF29	31	35	1.1
30.	AZF30	14	28	2
31.	AZF31	15	19	1.2
32.	AZF32	20	25	1.2
33.	AZF33	31	00	00
34.	AZF34	21	38	1.8
35.	AZF35	25	00	00
36.	AZF36	31	40	1.2
37.	AZF37	24	29	1.2
38.	AZF38	31	00	00
39.	AZF39	30	00	00
40.	AZF40	41	00	00

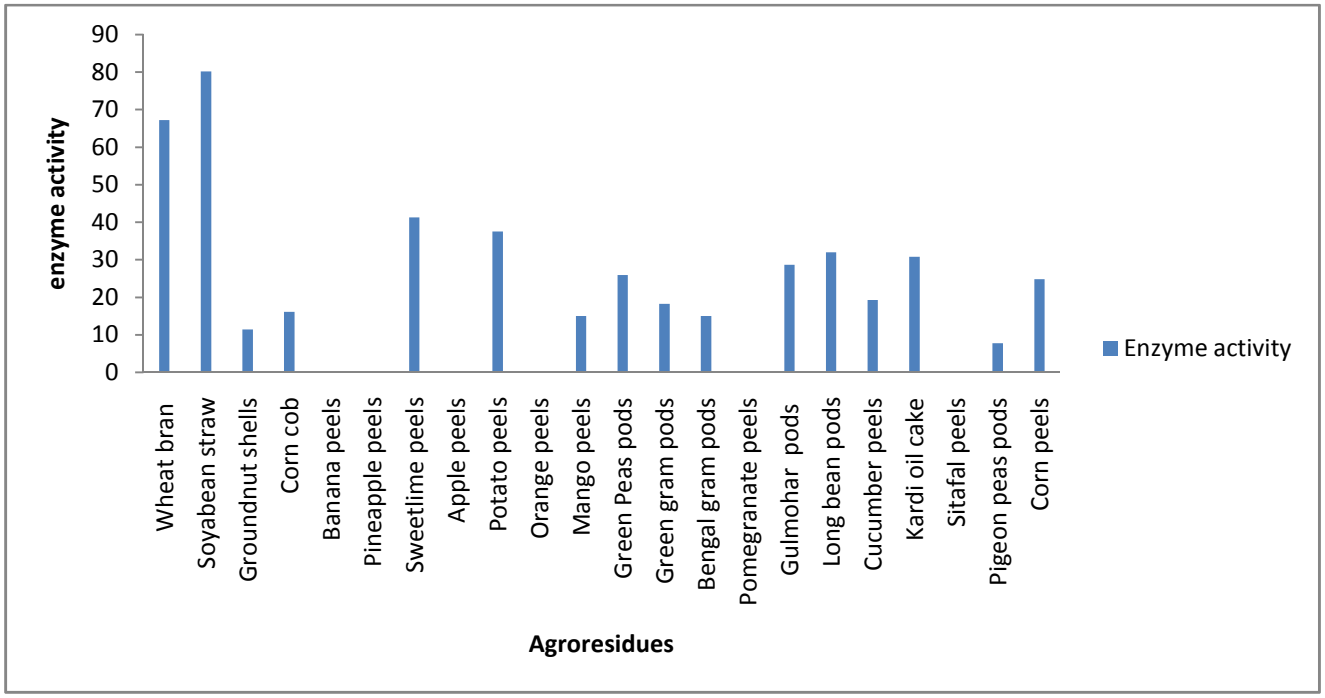

Fig.3. 23 different types of agroresidues collected from different agricultural areas were screened for production of β-mannanase, of which soystraw showed maximum enzyme production with enzyme activity

Isolate AZF 8 showed maximum enzyme activity of 108.2 U/ml, as shown in fig.2 which is much higher than reported by K. Khairul Asfamawi, *et al.* 2013 and Khampheng Phothichitto et al.2006.

Screening of agro residues: The selected fungi was used for the production of mannanase from agroresidues.23 different types of agro residues collected from different agricultural areas were screened for production of β-mannanase, of which soystraw showed maximum enzyme production with enzyme activity of 80.18 U/ml as shown in fig.3.

Identification of isolate:

Aspergillus nidulans (Eidam) Wint. Var. echinulatus Fennell and Raper, in Mycologia 47(47) : 79-80, Fig 4C (1955). Colonies on Czapek's solution agar growing well at room temperature (24^{o}C), attaining a diameter of 4 to 5 cm. in 10 days to 2 weeks; generally duplicating the species in appearance, but differing from it in developing a pronounced pinkish cinnamon cleistothecia; color due to the production of abundant cleistithecia and only limited conidial structures. Heads slightly larger than in typical representatives of the species; cleistothecia substantially larger, commonly 400 µ in diameter and occasionally up to 450 to 500 µ; asci eight spored; ascospores lenticular, red –orange in color, with two prominent pleated equatorial creasts about 1.0 µ wide and with convex surfaces conspicuously echinulate rather than smooth.

Conclusion

40 isolates were screened and 10 isolates were selected showing the activity ratio ranging from 1.6 mm to 3 mm. These isolates were quantitatively screened for mannanase enzyme production in liquid minimal medium with 1% guar gum as substrate and incubated in shake flasks at 30 °C for 72 h. Isolate AZF8 showed maximum enzyme production with mannanase enzyme acitivity of 108 U/ml. The isolate AZF8 was identified microscopically as Aspergillus nidulans and was further used for the production of mannanase from agroresidues. Different agroresidues collected from agricultural land were screened for mannanase production of which soy staw showed maximum mannanase production with enzyme activity of 80.18 U/ml.

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