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Production, purification and characterization of a thermostable β-Mannanase from *Aspergillus niger* AD-01: potential application in aquaculture

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Abstract

The potential for microbial β-mannanase enzyme as a feed additive has attracted substantial interest from feed manufacturers as a means to improve animal performance. This necessitates production of β-mannanase from novel strains with desired characteristics for feed applications. A fungal strain isolated from garden soil was found to be capable of producing extracellular β-mannanase enzyme. The strain was identified as *Aspergillus niger* and nucleotide sequence has been submitted in NCBI database under accession number MN239884.Optimum enzyme production in terms of specific activity 12.49 U/mg of total protein was obtained at 30°C, pH=5.0, using 2% locust bean gum as carbon source and yeast extract as nitrogen source, after 5 days of incubation.Fungal crude enzyme was purified by 6 fold with 24% yield and specific activity of 78.07U/mg by two step purification i.e. ammonium sulfate precipitation and gel-filtration chromatography. Thermo stability studies revealed retention of 50% β-mannanase activity upto 40°C.Moreover enzyme remained stable the pH range of 4-8.β-mannanase activity remained stable in the presence of 7 different metals, however activity declined sharply in the presence of Hg^{2+} and Ba^{2+}. Inhibition by Hg^{2+} suggests that enzyme contains an essential sulfhydryl group. No loss of enzyme activity was observed after incubating the enzyme with 1 M or 2 M NaCl, proteinase K or trypsin at 37°C and pH 6.0 for 1 h. The weakly acidic, low temperature active, protease resistant profile of this particular type of β-mannanase enzyme makes it a promising candidate for use as a feed additive for agastric fish in aquaculture industry.

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Introduction

Hemicelluloses present alongside cellulose in the plant cell wall, are the second most abundant polysaccharides in nature. Unlike cellulose which is hard and crystalline, hemicellulose is amorphous and random(Kote *et al.*, 2009) .Hemicellulose is mainly composed of β-D-xylans and β-D-mannans (Wainø and Ingvorsen, 1999).Heteroxylans are mainly present in grasses, hardwoods and cereals whereas hetero β-D-mannan are abundant in softwoods. The biotechnological interest in the hydrolysis of hemicelluloses for the feedstock and paper and pulp industry has recently reignited interest in the enzymology of hemicellulose degradation(Arisan-Atac *et al.*, 1993).

β-Mannanases generally present in plants and microorganisms are a kind of hemicellulase enzymes which randomly hydrolyze glucomannan and galactomannan chains into mannooligosachrides (Kote *et al.*, 2009). These oligosaccharides can be further degraded by other hemicellulose degrading enzymes like α-D-galactosidase, β-D-mannosidase and β-D-glucosidase to produce galactose, mannose and glucose respectively(Burke and Cairney, 1997). The extent of hydrolysis is determined by distribution of substituents and degree of substitution(Reese and Shibata, 1965).

In recent years there has been increasing interest in use of enzyme products to increase the performance and health of feed stock. In Asia the main feed ingredients are soybean meal and corn meal, both of which contain high amounts of mannan and galactomannan (Sornlake *et al.*, 2013). Approximately 13-20g/kg of mannan is present in soybean meal(Mok *et al.*, 2013; Yiğit *et al.*, 2014).Studies on poultry, fish and pigs have shown that mannan acts a strong antinutritive agent by increasing viscosity of digesta which in turn adversely affects animal performance(Cheng *et al.*, 2016; Yamka *et al.*, 2005).However, the adverse effects of undigested mannan can be relieved by supplementation of mannan degrading enzyme in the feed(Cheng *et al.*, 2016; Jackson *et al.*, 1999; Odetallah *et al.*, 2002; Pettey *et al.*, 2002; Wu *et al.*, 2005; Zou *et al.*, 2006)
In the burgeoning aquaculture industry, as it transitions towards using more plant-based proteins, the use of exogenous enzymes that can work in the physiochemical conditions of the gastrointestinal tract and efficiently break down an antinutritive agent like mannan in soybean meal is of vital importance in improving nutrient utilization in fish.

Therefore, this study aimed to identify a potent β-mannanase producing strain that can produce β-mannanase as a fish feed supplement. A local strain of *Aspergillus niger* AD-01 was isolated and identified. Experiments were conducted to select the most appropriate physicochemical conditions leading to highest β-mannanase production. Then we explored the efficient purification and characterization process of β-mannanase enzyme. Lastly, we tested its potential as a feed supplement in simulated intestinal digestion conditions.

Materials and methods

Preliminary Screening and Identification of Fungi

To isolate β-mannanase producing fungal strains, soil samples from different garden areas of Punjab, Pakistan were collected. The samples were serially diluted in distilled water and plated on sterile Locust bean gum agar(Locust bean gum=1g, $NaNO_3$=1g, agar=15g, K_2HpO_4=1g, yeast extract=1g, $MgSO_4.7H_2O$=0.5g in 1 liter distilled water.0.080g of streptomycin was also added to eliminate bacterial contamination)surface and incubated for 3-4 days at 37±2°C.The morphological study of the isolates was carried out by making microscopic observations. The isolates were preserved on potato dextrose agar slants for further study.

Secondary Screening

The microorganisms that were obtained from preliminary screening were then cultured in Erlenmeyer flasks containing liquid media composed of LBG=2%, $MgSO_4.7H_2O$=0.05%, K_2HPO_4=0.1% at pH=5.5. The flasks were left to incubate at 30°C for 7 days on a rotary shaker. After 7 days, the culture broth was centrifuged at 12000Xg for 20min and

supernatant obtained was used for enzyme assay. Among the six isolates tested one was found to be a potent producer of β-mannanase enzyme. After morphological observation of this isolate, it was designated as *Aspergillus niger* AD-01.The isolate was maintained on potato dextrose agar slants for further production of β-mannanase enzyme.

Identification and Molecular characterization of selected β-mannanase producing fungal strain

The fungal strain that was the most potent producer of β-mannanase enzyme was observed phenotypically and morphologically. For molecular identification of the strain, DNA was extracted by using QIAamp® DNA Mini Kit. PCR amplification and sequencing of 18s rRNA was carried out by Macrogen DNA sequencing services USA. Mega 6 and NCBI-BLAST was used to examine the sequenced data. For phylogenetic analysis, comparison of the sequence was made with already available sequences in NCBI database.After molecular characterization DNA sequence was submitted in NCBI database.

Enzyme assay

For determination of β-mannanase activity 1%(W/V) locust bean gum(LBG) was used as substrate. An aliquot of 100μL of enzyme sample was mixed with 900μL of substrate solution in sodium phosphate buffer (pH6.0) for 20 minutes at 50°C.To stop the reaction 3,5-dinitrosalicylic acid was added and the reaction mixture was subsequently boiled at 90°C for 5 minutes in water bath. The reducing sugars released were measured at 540nm, using mannose as standard. Under standard assay conditions, the amount of enzyme that produced 1μmole reducing sugars per minute was expressed as one unit of β-mannanase activity.

Optimization of physicochemical conditions for β-mannanase production

To achieve maximum production of β-mannanase, different physicochemical conditions were optimized. The governing parameters were modified in a stepwise manner. First of all, the effect of carbon source (commercial mannans such as LBG, guar gum,Konjac powder and simple sugars as sole carbon source such mannose, glucose, xylose and galactose) on the production of β-mannanase enzyme was studied. Then the effect of temperature(20-60°C), pH(4.0-8.0) and addition of different nitrogen sources was studied. After optimization of all these parameters the effect of incubation time for production of β-mannanase enzyme was studied.

Purification of β-Mannanase

Ammonium sulfate [$(NH_4)_2SO_4$] Fractionation and Dialysis

500ml of crude β-mannanase was taken and calculated amount of solid ammonium sulphate was slowly added to achieve various saturation levels(40-80%) with thorough shaking on magnetic stirrer at 4°C.The mixture was stored overnight at 4°C and followed by centrifugation at 13000xg for 15min.Collected precipitates were suspended in 50ml of 50mM sodium phosphate buffer pH 6.0.

The solution was dialyzed against the same buffer for 15 hours at 4°C.The buffer was changed three times. After dialysis β-mannanase activity and protein content of each fraction was determined.

Gel Filtration chromatography on Sephadex G-75

Precipitated protein was applied to Gel filtration column(2.5,45.0cm) pre-equilibrated with 50mM phosphate buffer(pH=6).5 ml of concentrated enzyme sample was loaded on top of the column. The enzyme was eluted using 50mM phosphate buffer(pH=6) at flow rate of 30ml/h and fractions with maximum specific activity were collected for further analysis.

Estimation of molecular mass

For estimation of molecular mass of β-mannanase enzyme, sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE) according to Laemmeli (1970) was used.

Characterization of Purified β-mannanase enzyme

Characterization and stability studies of purified β-mannanase were carried out to gain an insight into the nature and possible applications of the enzyme.

Determination of optimal pH and stability of β-mannanase

The effect of pH on the activity of β-mannanase enzyme was studied by incubating purified β-mannanase enzyme in sodium phosphate buffer with pH ranging from(4.0-8.0) at 30°C.The pH stability of the β-mannanase enzyme was determined by preincubating purified β-mannanase enzyme in sodium phosphate buffer (pH ranging from 4.0-8.0) at 30°C for one hour. The residual activity was immediately determined by following DNS procedure.

Determination of Optimal temperature and thermostability of β-mannanase

The effect of temperature on the activity of β-mannanase enzyme was determined by assaying the activity of purified enzyme at different temperatures ranging from 20-60°C at pH 6.0.The thermal stability of purified enzyme was determined by incubating β-mannanase enzyme without substrate at temperatures 20-60°C for one hour at pH=6.0. The residual activity was immediately determined by DNS method.

Effect of different metal ions and inhibitors on β-mannanase activity

For determination of the effect of different metal ions(Cu^{2+},Mn^{2+},Mg^{2+},Ca^{2+},Co^{2+},Zn^{2+},Ni^{2+},Hg^{2+},Ba^{2+}) and inhibtors (EDTA,N-bromosuccinimide,β-mercaptoethanol, 1, 10-phenanthroline) onβ-mannanase activity, the purified enzyme was incubated at 30°C for 60 minutes in the presence of 1mM of each ion or inhibitor in the reaction mixture using 50mM sodium phosphate buffer pH6.0. Then the residual activity was promptly measured by DNS method.

Substrate specificity

For determination of substrate specificities of purified β-mannanase from *Aspergillus niger* AD-01, enzyme activities were assayed by DNS method towards soybean meal, coconut pulp, peanut shells, coffee beans, sugarcane bagasse, rice bran or 0.5%(w/v) of polysaccharides including locust bean gum(LBG), carboxy methyl cellulose(CMC) and oat spelt xylan.

Effect of Trypsin and Proteinase K

Resistance of β-mannanase to proteinases was determined by incubating β-mannanase enzyme for one hour at 37°C with trypsin (250 U mg−1, pH 7.0) or proteinase K (30 U mg−1, pH 7.0), at a ratio of 0.1:1 [mannanase to proteinase (w/w)]and the residual enzyme activity was measured in sodium phosphate buffer(pH6.0)at 50°C.

Potential assessment of β-mannanase enzyme as a feed additive for agastric fish

Simulated intestinal fluid containing 6.8mg/ml KH_2PO_4 and 10mg/ml trypsin at pH6.8, was prepared to determine the ability of β-mannanase to hydrolyze mannan in vitro. The reaction system (0.5U/ml) contained 2.5 U of β-mannanase enzyme,5.0 ml of SIF and 2.0%(W/V)wheat bran, soybean meal, rapeseed meal or sun flower meal. The mixture was left to incubate at 20°C for 4,8 and 12 hours at constant agitation. A similar experiment without the purified enzyme was done as a control experiment.

Statistical analysis

All experiments related to β-mannanase activity were performed in triplicate and expressed as mean ± SD (standard deviation). Graph pad prism software 5 was used to carry out the statistical analysis and to compare different treatment groups. One way or two way analysis of variance (ANOVA) was used.

Results and discussion

Screening of the High β-mannanase Activity Producing Strain

Six active β-mannanase producing fungal strains that formed colonies on mannan agar plate were selected for secondary screening. For this, these strains were grown in liquid media containing LBG as a sole carbon source. Out of six isolates, one fungal strain proved to be a potent producer of β-mannanase enzyme. This strain was selected for further study and it was designated as *Aspergillus niger* AD-01.

Identification and molecular characterization of selected β-mannanase producing fungal strain

The selected strain was sub-cultured on LBG agar

medium. At first the colonies were white, covered with feathery white aerial mycelia but as the culture matured, the colonies turned black and powdery. The back of the mature colony was buff colored. Analysis of 18S rRNA sequence of the fungal isolate showed a homology of 98% with *Aspergillus niger* strains in NCBI database. The nucleotide sequence of the strain AD-01 was submitted in NCBI under accession number MN239884.

Table 1. Effect of different carbon sources on production of β-mannanase enzyme by *Aspergillus niger*-AD01

Carbon Source(2% w/v)	Maximum β-Mannanase activity (U/ml)
Locust bean gum(LBG)	3.245±0.46
Guar gum	2.234±0.62
Konjac Powder	1.831±0.15
Glucose	0.003±0.05
Xylose	0.004±0.00
Mannose	0.001±0.00
Galactose	±0.002±0.02

Optimization of culture conditions for enhanced β-mannanase production

Different parameters were optimized for maximum production of β-mannanase enzyme: carbon source; pH; temperature; nitrogen source and time of incubation.

Among different carbon sources tested, maximum production of β-mannanase enzyme was achieved when locust bean gum (LBG) was used as sole carbon source (Table.1).This proves that LBG is the best inducer of β-mannanase production from *Aspergillus niger* AD-01. 2% LBG produced maximum production of enzyme (3.245U/ml) followed by guar gum (2.234U/ml). similar results were reported by other investigators while using commercial mannans for production of β-mannanase(Ademark *et al.*, 1998; McCutchen *et al.*, 1996; Odetallah *et al.*, 2002).

Table 2. Effect of different nitrogen sources on production of β-mannanase enzyme using 2% locust bean gum.

Nitrogen Source(0.5% in the medium)	Maximum β-Mannanase activity (U/ml)
Ammonium Sulfate	4.243±0.87
Ammonium nitrate	3.165±1.30
Urea	2.851±0.99
Yeast extract	5.628±1.03
Sodium nitrate	3.678±1.45
Potassium nitrate	4.031±0.87
Peptone	4.819±1.40

The increased production of β-mannanase from LBG was possibly due to increased mannan content in LBG as compared to other sources. In konjac glucomannan the ratio of glucose to mannose is 1.6:1(Kato and Matsuda, 1969),in guar gum the ratio of mannose to galactose is 2:1 while the ratio of mannose to galactose in LBG is 4:1(Lawrence, 1973).This shows that LBG has the highest mannan content which might be the reason that maximum production of β-mannanase was achieved when LBGm was used. Simple sugars like glucose, mannose, xylose and galactose did not induce β-mannanase production which might be due to catabolite repression, as reported for *Aspergillus sp.*(de Vries and Visser, 2001; Haltrich *et al.*, 1996). Therefore, the production of enzyme was not observed.

Table 3. Different steps of purification and percentage yield of β-mannanase enzyme from *Aspergillus niger* AD-01.

Purification Stage	β-Mannanase activity(U/ml)	Total Protein(mg/ml)	Specific Activity(U/mg)	Purification fold	Percentage Yield
Crude enzyme extract	8.12	0.65	12.49	1	100
$(NH4)_2SO_4$ precipitates	19.97	0.54	36.98	2.96	83.07
Purified enzyme	12.18	0.156	78.07	6.25	24

The effect of different nitrogen sources on the production of β-mannanase enzyme by *Aspergillus niger* AD-01 was also investigated. A greatly enhanced production of β-mannanase enzyme was observed when organic sources of nitrogen were used, instead of inorganic ones. The increased production of the enzyme in the presence of organic sources could be explained by the fact that organic sources contain vitamins and carbohydrates in addition to amino acids. In the present study maximum production of β-mannanase enzyme was obtained when yeast extract was used as nitrogen source followed by peptone (See, Table.2).

This agrees well with the results reported by other studies (Chantorn *et al.*, 2013; Kote *et al.*, 2009).

Table 4. Effect of different metal ions (10mM) and chemical reagents on the activity of purified enzyme.

Reagent	Relative Activity (%)
None	100±6.5
EDTA	118±12.5
SDS	0.0
Zn^{2+}	81.2±4.5
Co^{2+}	85.3±6.7
Ba^{2+}	13.5±5.4
Pb^{2+}	21.8±8.3
Mg^{2+}	140.6±9.8
Mn^{2+}	35.6±1.6
Cu^{2+}	110.5±1.8
Ca^{2+}	107.4±3.4
K^{2+}	92.6±2.5
β-Mercaptoethanol	160.5±1.5
Ni^{2+}	154.6±2.5
Hg^{2+}	15.7±3.5

[a] Relative β-mannanase activity was expressed as a percentage of the control reaction without any additive.

In pH optimization studies, the effect of initial pH of the culture media on the production of β-mannanase enzyme was studied in the range of 3-8.In the present study, maximum production of β-mannanase enzyme was achieved when the pH of the production medium was 5. Similarly, Kote & Patil (2009) obtained optimum production of β-mannanase enzyme from *Aspergillus niger* gr when the pH of the fermentation medium was 5. Mohammad *et al.* (2011) obtained maximum production of β-mannanase enzyme at pH 5.5.

To study the effect of temperature on the production of β-mannanase enzyme, different fermentation

temperatures ranging from 20-60°C were tested. Maximum production of β-mannanase enzyme was achieved when the temperature of the production medium was 30°C.Other investigators also observed maximum production of β-mannanase enzyme at 30°C(Chantorn *et al.*, 2013; Youssef *et al.*, 2006). Kote *et al.*(2009) however observed maximum production of β-mannanase enzyme at 37°C.Rashid *et al.*(2011) obtained maximum production of β-mannanase enzyme by *Aspergillus terreus* at 31°C.

Table 5. Effect of different substrates on β-mannanase activity.

Substrate	Concentration	Relative β-Mannanase activity(%)[a]
Locustbean Gum(LBG)	0.5	100±0.4
CMC	0.5	0
Oat Spelt Xylan	0.5	0
Soy bean meal	2	35.8±0.8
Coffee Shells	2	21.3±3.2
Coconut pulp	2	38.2±2.7
Peanut shells	2	13.6±2.3
Sugar cane bagasse	2	19.8±3.2
Rice Bran	2	26.6±2.6

After optimizing all the other parameters, the effect of time of incubation on the production of β-mannanase enzyme was investigated. For this, the production of β-mannanase enzyme was carried out under optimized conditions for 9 days. Maximum production of the enzyme was achieved at day 5.This is similar to the results reported in other studies(Kote *et al.*, 2009).After day 5 the production of β-mannanase enzyme was greatly reduced which could be due to depletion of nutrients in the fermentation medium.

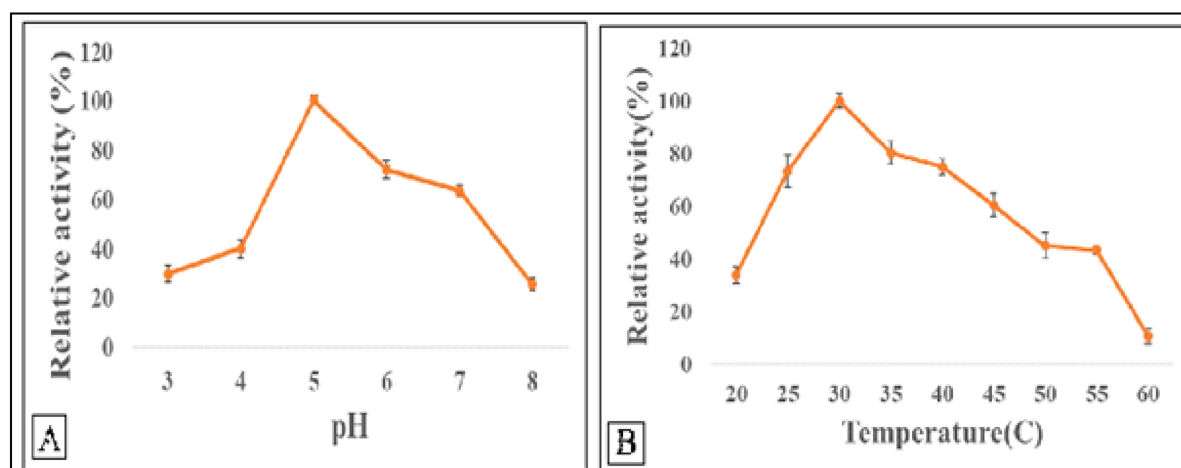

Fig. 1. Effect of pH(A) and temperature(B) on the production of β-mannanase enzyme by *Aspergillus niger* AD-01.

The enzyme production increased from 4.160 U/mg of protein in the nonoptimized media to 12.49U/mg of protein in the optimized medium.

So there was 3 fold increase in the production of β-mannanase enzyme by optimizing the media composition.

Purification of β-mannanase

Crude β-mannanase enzyme produced on optimized culture parameters showed a specific activity of 12.49U/mg of total protein. The enzyme was purified by subjecting 500ml of fermentation liquid to ammonium sulphate precipitation and gel filtration chromatography. Different steps of purification like %

yield, specific activity and purification fold are summarized in table (3).After multiple purification steps, β-mannanase enzyme was purified by 6 fold with 24% yield and the specific activity of β-mannanase, using LBG as sole carbon source reached 78.07U/mg. The purified β-mannanase was shown to be homogenous as examined by SDS-PAGE.

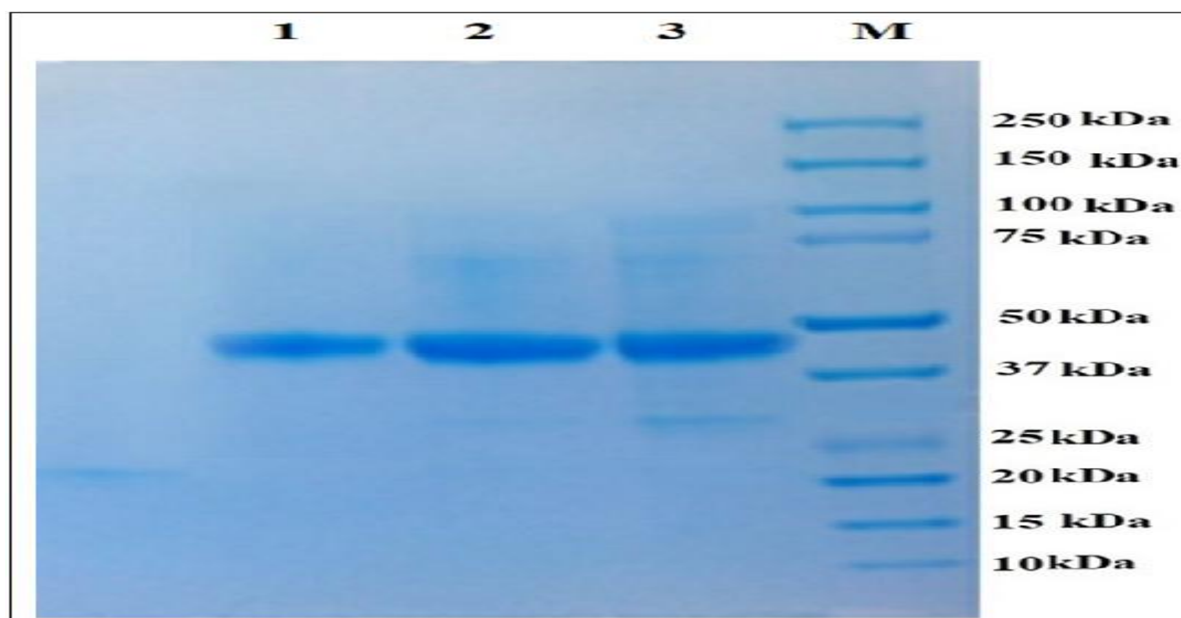

Fig. 2. SDS-PAGE analysis of purified β-mannanase enzyme. Lane M: protein molecular weight standard; Lane 1,2,3: purified β-mannanase.

Apparent Molecular mass of β-mannanase

The apparent molecular mass of β-mannanase enzyme was found to be ~45kDa (Figure . 2),which is less than most of the reported molecular masses of β-mannanase enzyme from *Aspergillus* species.The molecular mass of β-mannanase isolated from *Aspergillus oryzae* has been reported to be 110kDa,from *Aspergillus niger* gr 66kDa, from *Aspergillus terreus* FBCC1369 to be 49kDa,and from *Aspergillus sulphureus* 48kDa(Chen *et al.*, 2007; Naganagouda *et al.*, 2009; Regalado *et al.*, 2000; Soni *et al.*, 2016) .Due to low molecular mass of β-mannanase enzyme isolated from *Aspergillus niger* AD-01,it has more ability to depolymerize mannan because it can penetrate the lignocellulosic systems more efficiently.

Characterization of β-mannanase

Biochemical characterization and stability studies were carried out in order to have a brief insight into the nature and possible applications of the isolated β-mannanase enzyme.The effect of temperature, pH, metal ions, solvents, proteinases and NaCl on the residual activity of β-mannanase enzyme was investigated.

Optimal temperature and thermostability of β-mannanase

The purified β-mannanase was maximally active at 40°C (Figure .3A) and showed >50% of the maximum activity at temperature 20°C and at 10°C the activity of ~ 20%.The optimal temperature of β-mannanase purified from *A. awamori*, *A. tamarri*, *A. niger*, *A. oryzae*, *A. sulphureus*, *A. fumigatus*, *A. terrus*, and *A. aculeatus* are 80, 60,50, 40, 50, 60, 55 and 70°C respectively(Ademark *et al.*, 1998; Chen *et al.*, 2007; Christgau *et al.*, 1994; Civas *et al.*, 1984; Huang *et al.*, 2007; Regalado *et al.*, 2000).

Compared with the optimal temperatures obtained for β-mannanase purified from different *Aspergillus* species mentioned above, β-mannanase of AD-01 showed a pronounced activity at lower temperatures. Figure. 3(B) shows the thermostability profile of β-

mannanase enzyme. It is clear from Figure.3 (B) that the enzyme is very stable at 37°C after 60 min incubation. Approximately 50% of activity was retained at 40°C for 30min. The half-life of the enzyme was 30min at 40°C and only 10% of activity was retained after 10 minutes incubation at 50°C.

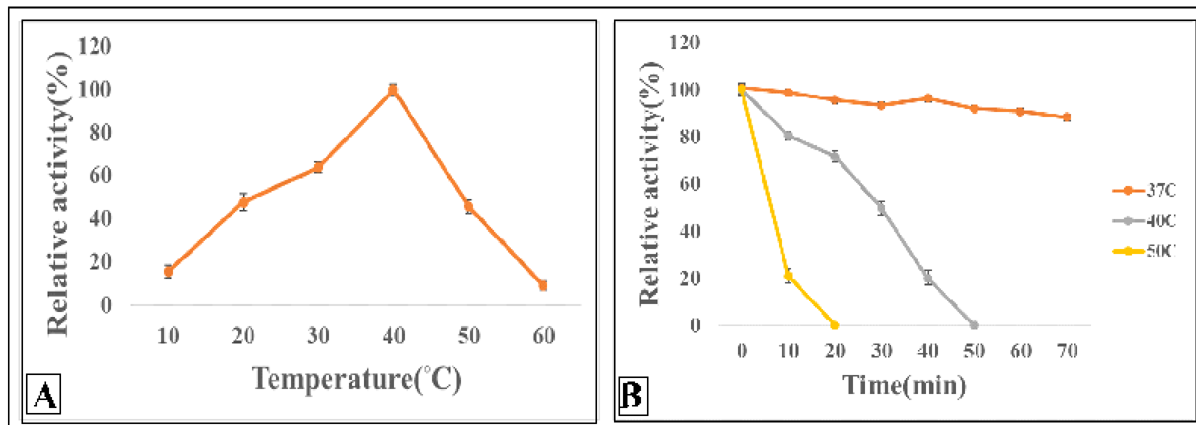

Fig. 3. The effect of temperature on the activity (A) and stability (B) of β-mannanase enzyme.

Optimal pH and stability of β-mannanase

β-mannanase enzyme purified from *Aspergillus niger* AD-01 has been found to be maximally active at pH 6.0 and retains more than 25% of its maximum activity between pH 5.0 and 8.0 (Figure.4A). From the literature it is evident that most of the β-mannanases from *Aspergillus* species have optimum pH that lies in the acidic range. β-Mannanase enzyme purified A.*oryzae, A.awamori, A.niger, A.tamarii, A.fumigatus, A.aculeatus, A.terrus and A. sulphureus* had an optimum pH of 6.0, 3.0, 3.0, 4.5, 4.5, 5.0, 7.5, 2.4 respectively(Chen *et al.*, 2007; Christgau *et al.*, 1994; Civas *et al.*, 1984; Huang *et al.*, 2007; Puchart *et al.*, 2004; Regalado *et al.*, 2000).

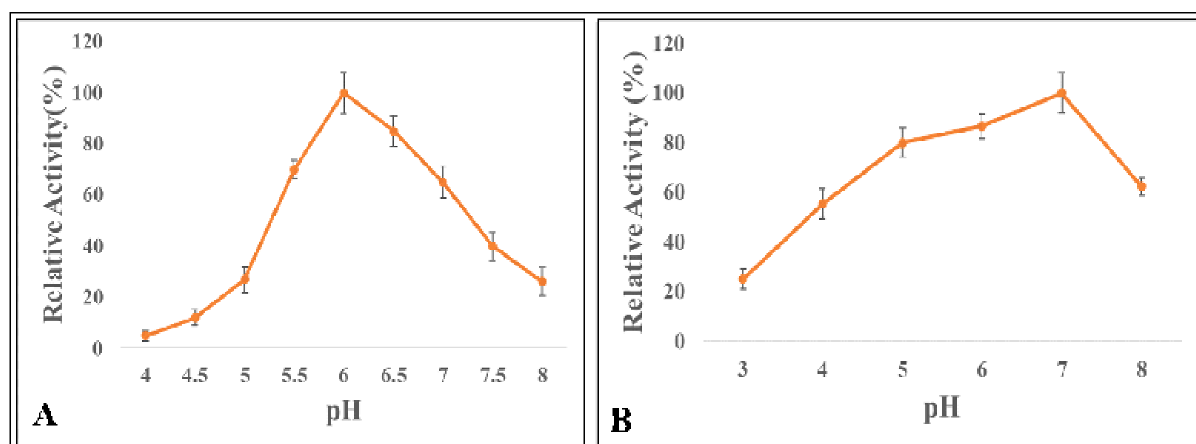

Fig. 4. The effect of pH on the activity (A) and stability(B) of β-mannanase enzyme.

The purified β-mannanase was found to have pH stability. It retained ~60% of its activity after incubating it in different buffers with pH ranging from 4-8 at 37°C for one hour(Figure.4B) β-mannanase isolated from *S.rolfsii* has been reported to be stable in buffers with pH between 3.0 and 6.0 .Setati *et al.* (2001) has reported that β-mannanase isolated from *S.cerevisiae* is stable between pH 4.0 and 6.0.β-Mannanase isolated from *Aspergillus niger* AD-01 shows good enzyme activity between pH range of 5-8.

Therefore the purified enzyme can be considered as a weekly acidic or neutral enzyme, making it suitable to be used a feed supplement for agastric fish which has a digestive system with a pH value of 6.8–7.3.

The Effect of Inhibitors and Metal Ions on β-mannanase Stability

Table 4. Shows the effects of different metal ions and inhibitors on β-mannanase activity.β-mannanase activity was found to be considerably enhanced by presence of β-mercaptoethanol and strongly inhibited by SDS.EDTA which envelops metal ions extensively, however did not inhibit β-mannanase activity which shows that the purified β-mannanase does not require metal ions for its activity.

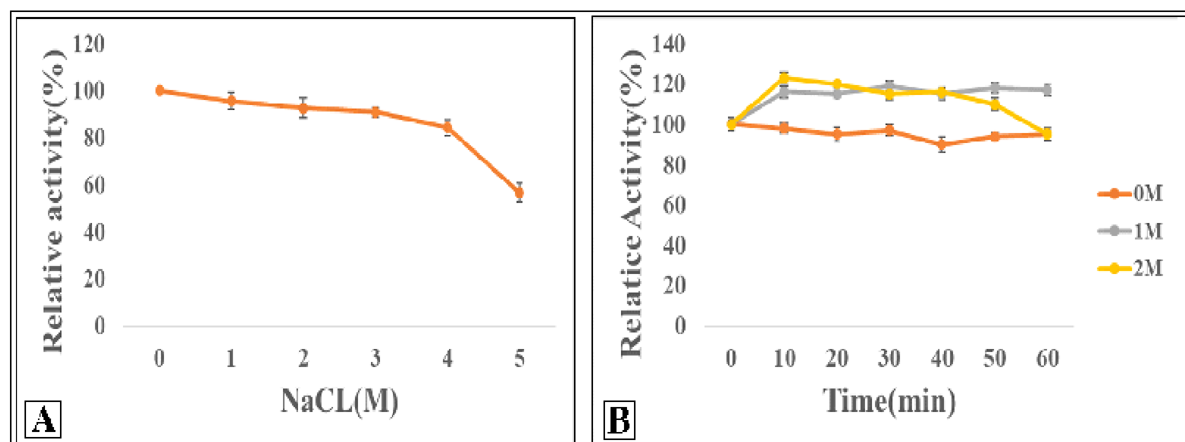

Fig. 5. The effect of NaCl on the activity(A) and stability(B) of β-mannanase enzyme.

Among the metal ions tested β-mannanase activity was partially inhibited by Zn^{2+} and Co^{2+}(retaining80-85%) and strongly inhibited by Ba^{2+} Pb^{2+} and Hg^{2+}(retaining <30% activity). Inhibition of purified enzyme by Hg^{2+} suggests that β-mannanase from AD-01 contains an essential sulfhydryl group. Similar to our results, β-mannanase from *Bacillus* sp. was strongly inhibited by Pb^{2+}(Cheng *et al.*, 2016) and β-mannanase from *Biospora* sp. was strongly inhibited by Hg^{2+} (Luo *et al.*, 2009) .This suggests that β-mannanase from *Aspergillus niger* AD-01 should not be contaminated by Pb^{2+} and Hg^{2+}.

Substrate Specificity of β-mannanase enzyme

The activity of purified enzyme was investigated against different substrates like LBG, CMC and oat spelt xylan with a concentration of 0.5%(w/v) and natural substrates like soybean meal, coffee shells, peanut shells, sugarcane bagasse and rice bran with a concentration of 2%(w/v).The results are presented in Table 5.It was observed that among the different tested substrates β-mannanase enzyme showed highest activity towards LBG while no activity towards oat spelt xylan or carboxy methyl cellulose. This indicated that β-mannanase from *Aspergillus niger* AD-01 was free from cellulase activity(towards CMC) and xylanase activity (towards oat spelt xylan).Similar results were obtained when activity of β-mannanase isolated from *philophora* sp. p13 and *Bispora* sp. MEY-1 was tested against LBG, CMC and oat spelt xylan(Luo *et al.*, 2009; Zhao *et al.*, 2010).β-mannanase isolated from *Penicillium Pinophilum* C1 also showed no activity towards CMC-Na, birchwood xylan or p-nitrophenyl-β-D-mannopyranoside(Cai *et al.*, 2011).Among the natural substrates tested the enzyme showed highest activity towards coconut pulp followed by soy bean meal. Similar results have been reported in another study with an *A.niger* strain that produced maximum production of the β-mannanase in the medium containing 2% (w/v) of coconut pulp among tested carbon sources (coconut pulp, soybean meal, date seeds, sucrose, mannose, fructose, glucose, carob pods, Rabbit feed)(Youssef *et al.*, 2006).

Salt Tolerance

β-mannanase enzyme exhibited good salt tolerance. It retained greater than 80% of the activity in the presence of 0-4M NaCl(Figure.5 A).

The purified β-mannanase enzyme when incubated with 1M or 2M NaCl at 37°C for one hour exhibited more than 100% of the initial activity (Figure. 5 B).

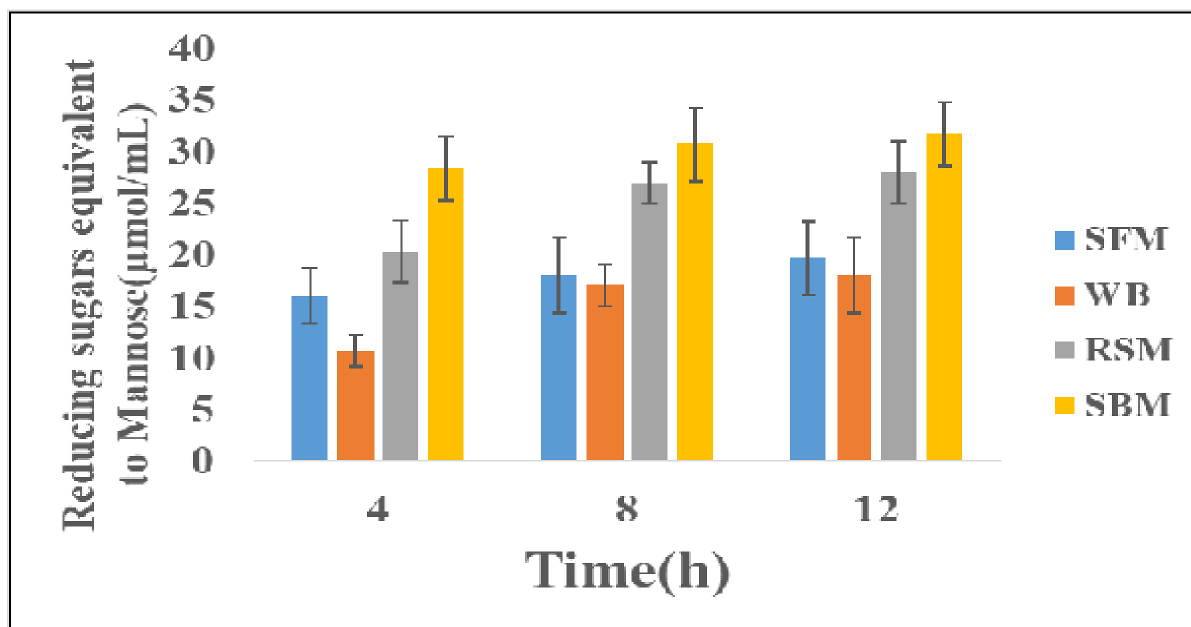

Fig. 6. Hydrolysis of 2.0 % (w/v) substrates by the purified β-mannanase enzyme in simulated intestinal fluid.The error bars represent the means±SD(n=3).

Proteinase resistance

Purified β-mannanase enzyme was resistant to proteinase. It showed no loss of activity after incubation for 60 min at 37°C with trypsin and proteinase K.

Potential assessment of β-Mannanase enzyme as a feed additive for agastric fish

During the simulated intestinal digestion phase in vitro, 16.0-19.6,10.6-18.0,20.3-28.0 and 28.3-31.6 µmol/ml reducing sugars were accumulated in 4-12 hours using 2.0%(w/v) Sun flower meal, wheat bran, rape seed meal and Soybean meal as simulated feeds respectively (Figure .6). Mannan increases the viscosity of feed which leads to lowering of FCR, thus exerting a powerful anti-nutritive effect(Cheng *et al.*, 2016; Yiğit *et al.*, 2014). The use of β-mannanase prevents viscosity of digesta because Mannan in the simulated feeds was degraded which is indicated by the release of reducing sugars.

Conclusion

Aspergillus niger AD-01 isolated from garden soil could produce β-mannanase enzyme.In optimization studies,the modification of culture parameters led to three fold increase in production of enzyme. β-mannanase was purified by 6 fold with yield of 24 % by two step process i.e. ammonium sulphate precipitation and gel filtration chromatography.The purified enzyme shows resistance against proteases, neutral pH adaptivity, thermostability and good activity at low temperature. The enzyme also shows good hydrolysis activity against soybean meal and wheat bran which are high in mannan content and are often used in fish feed. On the basis of these characteristics, β-mannanase from *Aspergillus niger* AD-01 is a good candidate to be used as a feed supplement for agastric fish such as common carp or grass carp. To meet the commercial demands, improvement of the specific activity by means of protein engineering will be our future objective.

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