Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio Tech. 53 (2012) 11932-11936

Elixir ISSN: 2229-712X

Production and partial characterization of β-Glucanase from Galactomyces sp. isolated from whey

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ARTICLE INFO

Article history:
Received: 10 October 2012;
Received in revised form:
20 November 2012;
Accepted: 7 December 2012;

Keywords
β-glucan,
Glucanase,
Induction,
Whey,
Yeast.

ABSTRACT

As per the results from the phylogenetic tree and BLAST analysis of the sequence data the isolated sp. identified as *Galactomyces* sp. of yeast. Isolated yeast sp. produced high levels of intracellular β-glucanase after incubation for 36 h at pH 7.0, temperature 27 °C in the presence of 8 % glucose. The optimum pH and temperature observed for the enzyme activity were 5.0 and 50ºC respectively. Addition of metal ion salts like $MnCl_2$, $COCl_2$ $CaCl_2$ cause activation of enzyme. Yeast cell cultures grown with nitrogen sources like $NaNo_3$ and yeast extract enhance β-glucanse activity.

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Introduction

Yeasts are the world's premier industrial microorganisms that have wide exploitation in the production of foods, beverages and pharmaceuticals. Alcoholic beverages are one of the major products in the world's market. Yeast cells can contaminate different dairy products because they have relatively low water activity (aw, 0.88), can easily grow at room temperature and utilize a variety of carbohydrate (11) like pentoses, hexoses, disaccharides and rarely polysaccharides (1). Yeast is able to produce several insustrially important enzymes like invertase and glucanase. β- glucanases are hydrolytic enzymes capable of causing lysis of cell walls. They are active on insoluble substrates, such as beta glucan components of fungal cell walls, laminarin and pachyman. They act both as exo or endo hydrolases.

β - glucanases have been found in higher plants as well as fungi and bacteria. Six different beta 1, 3-glucanases have been purified from *Saccharomyces cerevisiae*. Strains of *Bacillus* and *Streptomyces* sp. are reported to be highly active in producing extracellular beta-1, 3 -glucanases. The endo (1→6)-β-D-glucanase from *Acinetobacter* (7) hydrolyzes pustulan and lutean [(1→6)-β-D glucan]. Non specific beta glucanase i.e. beta glucosidase and beta-1, 3- glucanase enzymes have been purified from *Penicilium ochro-chloron* (6). The fungus *Trichoderma reesei* produces extracellular lytic enzymes such as β-1, 4 glucanase, β-1, 6 glucanase and β-1, 3 glucanase (13). In yeast, β-1, 3-glucanases are involved in morphogenetic events such as cell budding, conjugation and sporulation. The cell wall of yeast undergoes continuous rearrangement of beta glucans during growth period. The process involves making and breaking of bonds between wall polymers manipulated by beta glucanases through controlled hydrolysis. As a consequence, different glucanases are required at different stages during cell life cycle. For their higher specificity and selective hydrolysis of cell wall polymers, beta glucanases are employed in the preparation of protoplasts.

β- glucanases have been classified into several classes based upon the type of bond cleaved in β- glucan substrate as exo (1→3)-β-D glucanases, endo (1→3)-β-D glucanases and endo (1→6)-β-D-glucanases. Exoglucanases always act from the nonreducing end of polymeric chain removing sugar units successively whereas endo enzymes often cleave the polysaccharides by splitting of interior glycosidic bonds in a random fashion. Some of the exoglucanases also remove oligosaccharide units from the nonreducing end. (1→3)-β-D-glucanases may be specific or non-specific in nature according to the linkage cleaved and substrate attacked by them. Specific (1→3)-β-D-glucanases cleave only the (1→3)-β-D-glucan linkages present in the substrate like laminarin. These enzymes will not act upon (1→4) & (1→6)-β-D-glucans. The non-specific (1→3)-β-D-glucanases, apparently bind to (1→3)-β-D linkage; but may be split either (1→3) or (1→4)-β-D links. *Aspergillus fumigates* (5) produces endo (1→3)-β-D glucanase; the enzyme is a glycosylated 74 KDa protein.

Oerskovia xanthineolytica (16) produces endo (1→3)-β-D glucanase activity. *Penicillium italicum* is a filamentous fungus and produces (1→6)-β-D glucanase activity (12) when grown in a synthetic medium. Exo (1→3)-β-D glucanase was isolated from *Basidiomycete* and *Selerotinia libertian* (19) as well as from *Penicillium oxalicum.* Indcible β-glucanase has been purified from *Aureobasidium pullulans,* a dimorphic fungus (3). β-1,3-Glucanases in tomato roots were studied after arbuscular mycorrhizal (AM) symbiosis establishment and pathogenic infection by *Phytophthora parasitica* (10). A β-1, 3-glucanase (GLU-39) was isolated from a potato cultivar with a high level of field resistance (Solanum tuberosum L. cv Huinkul) (17).

β-glucanases have widescale industrial applications that are beneficial for food and environmental allergies, drug withdrawal, cell detoxification, colon cleaning, pain syndromes, Candida infections, gas, facial pain or paralysis and in textile industry as a fading agent. They are also applicable in the fermentation of biomass into bio fuels, although this process is relatively experimental at present.

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The present studies are intended for identification of the yeast sp. isolated from whey, optimization of cultural conditions for β-glucanase production and its characterization.

Materials and Methods

Microorganism:

The isolated pure culture of yeast sp. was routinely maintained on Sabouraud Dextrose Agar (SDA) (P^H 7.0).

Procedure for molecular identification for isolated yeast sp:

The following procedure regarding molecular identification was carried out by Agharkar Research Laboratory, Pune itself.

- The nearly ~350 bp rDNA fragments were successfully amplified using universal primers.
- The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit.
- The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency.

The following part was done in our laboratoty itself.

The sequence data was aligned with publicly available sequences those in the NCBI/GenBank database (www.ncbi.nlm.nih.gov/blast/) & analyzed to reach identity. A database search for closely related fungal species was aligned using the Clustalw program method of the BioEdit software. After analyses of their rDNA sequences, a phylogenetic tree was generated using the program Neighbor-joining (NJ) of Mega 4.0 program (15) by utilizing the sequence of related strains which were downloaded from GenBank.

Yeast propagation:

Yeast cells were propagated using Sabouraud dextrose (SD) broth (P^H 7.0) containing yeast extract (0.5%), peptone (0.5 %) and glucose (2 %, 4%, 6%, 8%). The inoculum was grown at 25 ± 2°C for 24 h and then transferred to major SD broth where fermentation was carried out at 25 ± 2°C for 48 hrs.

Harvesting:

Yeast cells were harvested from culture broth after 48 h of fermentation by centrifugation under cold conditions (+4°C) at 10,000 rpm for 10 min. Cells were washed repeatedly with chilled distilled water. These cells were used further for extraction of β-glucanase enzyme.

Enzyme extraction:

Isolated yeast cells were washed twice with distilled water and once with phosphate buffer (0.1 M, PH 7.0). The cell mass collected was suspended in phosphate buffer (0.1 M, PH-7.0) and crushed using mortor – pestle along with glass beads under cold conditions. The resultant homogenate was centrifuged at 10,000 rpm for 20 min at +4°C. The supernatant obtained was used as the source of intracellular β-glucanase enzyme (2). β glucanase assay was done with crude β-glucan substrate.

Preparation of β- glucan substrate: The β- glucan substrate was prepared by alkali digestion method (14).

Enzyme assay:

The enzyme assay was carried out using β- glucan substrate isolated from yeast cake. The reaction mixture containing 0.5 ml β- glucan substrate along with 0.5 ml acetate buffer (P^H-5.5) was kept at 45°C for 5-10 min. to attain the required temperature. The reaction was initiated by addition of 1ml of enzyme extract and incubation was carried out for 1 h. A suitable control tube was also prepared along with the sample tube. Then the reaction mixture from both sample and control tube was centrifuged to separate out unreacted β- glucan substrate. 1ml supernatant from both control and sample tube was incubated along with GOD-POD reagent at 37° C for 30 min. The glucose released in the reaction mixture was measured colorimetrically at 540 nm using glucose oxidase peroxidise method (9). The enzyme was partially purified by ammonium sulfate fractionation (4) and was further utilized to determine kinetic parameters.

Determination of optimum P^H

Assay tubes containing crude β-glucan substrate prepared in acetate buffer of varying pH from 2.5- 8 and 1ml enzyme extract were incubated at 45° C for 1 hr. The glucose released was measured at 530 nm against the blank.

Determination of optimum temperature

Crude β-glucan substrate prepared in acetate buffer (0.1 M, P^H-5.5) was incubated with 1ml enzyme at variable temperatures as from 20-70°C for 1 hr. The reaction was terminated by immersing the assay tubes in boiling water bath for 5 min. The reaction mixture was centrifuged and the amount of glucose released was measured at 530 nm against the blank.

Effect of substrate concentration:

The effect of substrate concentration on the activity of β-glucanase was studied by maintaining the assay system at pH 5.5 & temperature of 45° C using variable concentrations of crude β-glucan substrate. The reaction mixture contained varying substrate concentrations from 600 mg to 2000 mg. The enzyme reaction was initiated with the addition of 1 ml enzyme solution in each assay tube. After 1 h incubation period the glucose released in the reaction mixture was analysed by using GOD-POD reagent.

Effect of nitrogen sources:

Yeast culture was grown in media containing equimolar concentrations of different nitrogen salt sources ($NaNO_3$, NH_4Cl, KNO_3, Peptone, Yeast extract, Urea and $(NH_4)_2SO4$ for 48 h. After harvesting of yeast cells, the enzyme isolated from these cells was further utilized to carry out assay using β-glucan as a substrate at 45°C for 1hr.

Effect of metal ions

Effect of different metal ions on the activity of β- glucanase enzyme was studied. The assay tubes containing 0.5 ml crude β-glucan substrate and 0.5ml acetate buffer (PH-5.5) containing 10 mM concentrations of different metal ions (Fe, Na, Cu, Mg, Mn, Ca, Co) along with 1ml of enzyme were incubated at 45°C for 1hr. The enzyme activities remaining after incubation were determined under assay conditions.

Results

Result for Sample:

Isolated strain showed 98% sequence similarity with genus *Galactomyces* (8). The genus currently has 6 species as mentioned in the *Dictionary of Fungi*, 10th edn. edited by Paul Kirk et al. in 2008 (18).

Sequence analysis

The sequences of rDNA from 1371-MD2 has been assigned the GenBank accession number DQ286062.1. The rDNA sequence of 1371-MD2 was homology searched in Nucleotide Sequence Database of GenBank. Then Phylogenetic tree was constructed. Results of phylogenic tree are shown in Figure 3.

As per the results from the phylogenetic tree (Figure 3), BLAST analysis of the sequence data (DQ286062.1); showed 99% sequence identities with *Galactomyces geotrichum* isolates (GQ458022.1, DQ148946.1, JN227076.1), 98 % with *Galactomyces geotrichum* isolate (JN227080.1) and 98% with *Galactomyces sp*. isolate (DQ286062.1). According to the morphological observation and molecular identification of isolated strain, 1371-MD2 belongs to Galactomyces sp.

Figure 1. Phylogenetic tree derived from of the isolated sp. of yeast

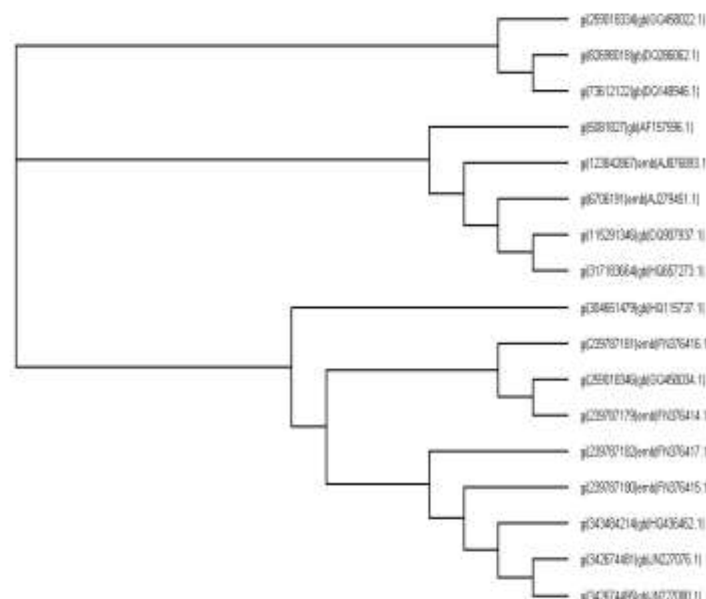
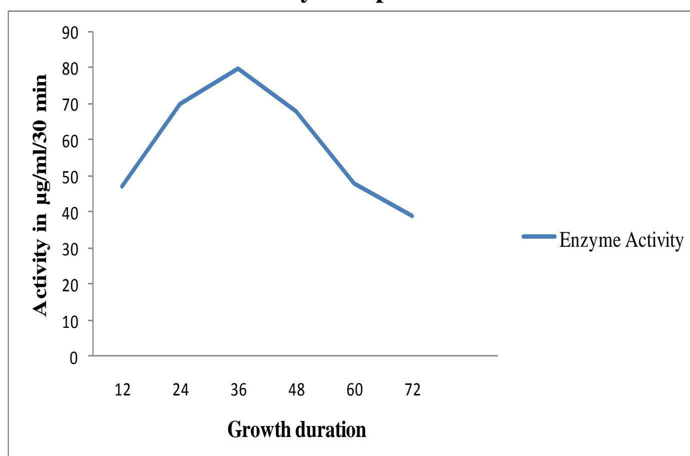
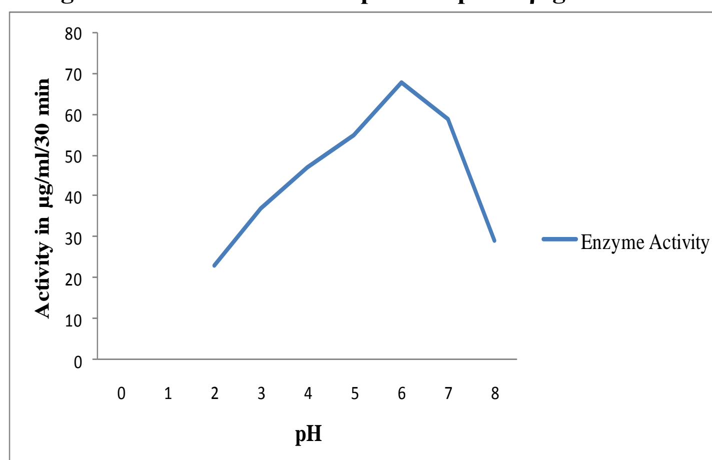


Figure 2. Time course of glucanase production by isolated yeast sp.



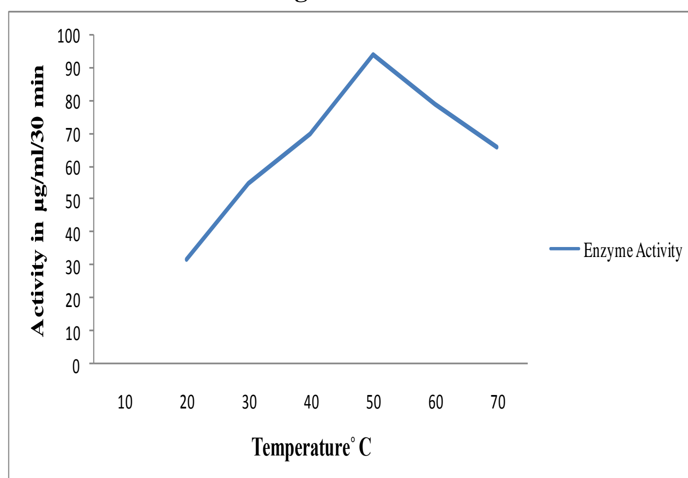
The activity of glucanase was estimated at various time intervals during growth of yeast culture. From figure 2 it indicates that production of β-glucanase was higher during 24-48 h of fermentation period. The production level of enzyme was found to be maximum for yeast cells grown for 36 h duration.

Figure 3. Determination of optimum pH of β-glucanase



From Fig. 3 it is observed that the enzyme shows optimum activity at pH 5.5. Negligible enzyme activities were observed towards acidic (pH 2.5) as well as basic pH range (pH 8.0 and above). Above P^H 2.5, the enzyme activity increased progressively. The maximum activity was obtained between P^H range of 4-7.

Figure 4. Determination of Optimum temperature of β-glucanase



From Fig. 4 it is denoted that the enzyme activity was minimum at temperatures of 20° C & 30° C and thereafter it increased upto 50° C. Above 50°C the activity was found to be decreased in exponential manner. Thus enzyme exhibits optimum temperature of 50° C and is thermostable upto 70° C. Thus accordingly, β- glucanase showed increase in its activity with increase in temperature from 20 to 50°C, thereafter it decreased.

The activity of β-glucanase was studied in response to varying β-glucan substrate concentration. The effect of substrate concentration on the activity of β-glucanase was analyzed by keeping temperature & P^H constant (temperature 50° C, P^H 5.5). The substrate concentration used was within the range of 600 - 2000 mg. From the Fig. 5 it indicates that the activity of enzyme goes on increasing with increasing substrate concentration from 600 mg to 1200 mg. Thereafter, the activity of enzyme remained constant with further increase in substrate concentration from 1200 mg to 2000 mg showing substrate saturation curve.

Figure 5. Effect of Substrate concentration on β-glucanase activity

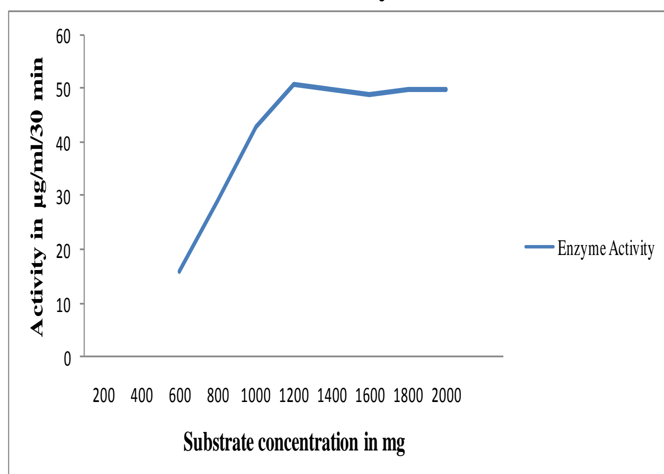
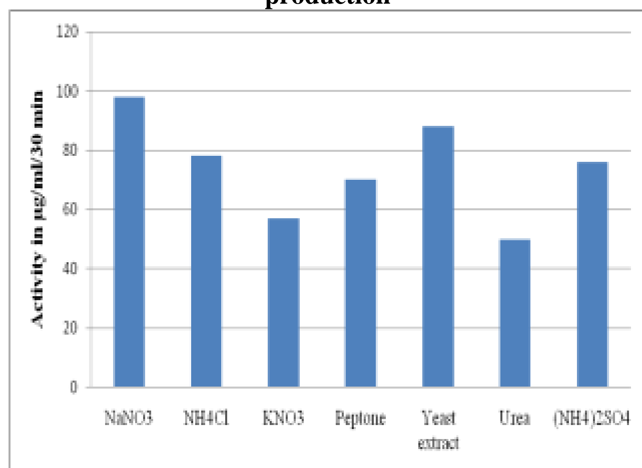


Figure 6. Effect of nitrogen sources on β-glucanase production



As observed from fig.6 it indicates that use of $NaNO_3$ as a source of nitrogen exhibits significant production of β-glucanase. Addition of yeast extract gave maximum enzyme production. Addition of salts like $(NH_4)_2SO_4$ also enhanced the level of enzyme production. Urea and KNO_3 did not show any substantial increase in β-glucanase production.

Table 1. Effect of Metal ions on the activity of β-glucanase production

Metal salts	Effective conc. Of metal ions (mM)	Enzyme activity µg/ml/30min	% enzyme activity
Standard	10	43	100
$MnCl_2$	10	76	176.7
$CoCl_2$	10	55	130.23
$CaCl_2$	10	46	127.9
$MgSO_4$	10	39	90.69
NaCl	10	36	83
$FeSO_4$	10	35	81.39
$CuSO_4$	10	34	79.06

From the observation table it indicates that Mn^{+2} exhibits maximal enzyme activity showing positive modulation of enzyme. Co^{+2} and Ca^{+2} also cause activation of the enzyme. $Na^{+,}$ $Mg^{2+}$and Fe^{+2} reduce the activity of the enzyme. The strong inhibitory effect was observed with $Cu^{2+.}$

Discussion:

Intracellular β- glucanase enzyme was successfully isolated from whey yeast. Isolated yeast was identified as *Galactomyces* sp. as per the results from phylogenetic tree and BLAST analysis of the sequence data. The time course for maximum enzyme production by isolated yeast was 36 h fermentation period. β- glucanase from whey yeast has optimum P^H 5.5. The enzyme exhibited optimum temperature of 50° C and is thermostable upto 70° C. The enzyme activity increased exponentially from 600 - 1200 mg substrate concentration. Mn^{+2}, Co^{+2} and Ca^{+2} are activators of the enzyme whereas Cu^{+2} cause maximum inhibition of enzyme activity. Yeast cell culture grown in the presence of nitrogen sources in the form of $NaNO_3$ and yeast extract enhanced the level of enzyme production by isolated yeast sp.

Acknowledgement:

Financial assistance received from Dr. D.Y. Patil Biotechnology & Bioinformatics Institute gratefully acknowledged. We are also thankful to Ms. Sandhya Khatal for her kindly help to generate the phylogenic tree.

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