

Research Article

Isolation, Production, Quantitative assay and Optimization of Chitin deacetylase from Yeast

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Abstract: Chitosan is a novel biological macromolecule with unique properties, whose importance is increasing apparently. Chitosan has application in many areas like in food additives, cosmetics, pharmaceuticals and agriculture etc. Because of its high antimicrobial activity against a wide range of pathogenic and spoilage microorganisms, including fungi, Gram-positive and Gram-negative bacteria, it is used as food preservative. In spite of this, it is used for Removal of dye, Color stabilization in food industry and as Animal feed additive. Recently, in industries, chitosan is manufactured by thermochemical deacetylation of chitin with conc. alkali. But, to avoid degradation of the polymer chain and to circumvent environmental pollution caused by thermochemical deacetylation, an alternative procedure has been investigated. The enzymatic deacetylation of chitin is one such procedure which could potentially be employed, especially when a controlled and well-defined process is required. Chitin deacetylase (Chitin amidohydrolase, EC 3.5.1.41) is the enzyme that catalyses the conversion of chitin to chitosan by the deacetylation of GlcNAc residues. During my research work, numerous microbes like fungi, bacteria and yeast were screened to select the suitable microorganism for the production of chitin deacetylase. Among the various isolates, SN-1 was found to be desirous microbe for the production of chitin deacetylase based on their enzyme activity. The isolate SN-1 was found to be a yeast based on its morphological and gram staining characteristics.

Keywords: Chitin, Chitosan, Thermochemical deacetylation, Chitin deacetylase

INTRODUCTION

Bioconversion of chitin, nature's most abundant polysaccharide after cellulose is accomplished by enzyme chitin deacetylase. The constantly increasing demand for relatively non-expensive microbial chitin deacetylase and their enhanced utilization in various sectors of industries is the main motivating force for research on different microbial chitin deacetylases. Extensive basic and applied research on chitin deacetylase revealed their commercial and industrial significance. In spite of their commercial importance, the high cost of production of these enzymes has hindered the industrial application of chitin bioconversion to chitosan. Chitosan, because of its ever increasing demand in various areas such as biomedicine, food ingredients, cosmetics and pharmaceuticals are produced by a harsh thermochemical process to meet the requirement. But this thermochemical process cause environmental pollution and is non-eco-friendly. In contrast, the use of chitin deacetylase enzyme for the bioconversion of chitin to chitosan offers the possibility of a controlled, non-degradable process which result in homogenous deacetylation of chitin. The chitosan produced by enzymatic method have a more regular pattern of deacetylation [1].

Chitin deacetylase (EC 3.5.1.41) is an enzyme that catalyzes the chemical reaction of hydrolysis of acetamido group of N-acetyl-D-glucosamine in chitin [2]. It belongs to family of hydrolases i.e acting on C-N

bonds other than peptide bonds in linear amide. It is also known as chitin amidohydrolase and participates in aminosugar metabolism [3]. Chitin deacetylase has been reported in several microorganisms like fungi, yeast, bacteria and it is also present in some insect species [4]. It was first identified and partially purified from the fungus *M. rouxii* [5]. Fungal chitin deacetylase have been studied more amply than those from insects and from marine bacteria. On the basis of diverse locations in fungi, chitin deacetylase have been divided in to two subgroups: intracellular chitin deacetylase and extracellular chitin deacetylase. Intracellular chitin deacetylase are secreted in to periplasm and is found in *M.rouxii* and *A.coerulea* [5]. Extracellular chitin deacetylase are secreted in to culture medium and is found in *C.lindemuthianum* and *A.nidulans* [6, 7]. Chitin deacetylase are secreted during an exclusive period corresponding to their special biological roles. Chitin deacetylase from fungal species might be secreted during different periods. For instance, an extracellular chitin deacetylase from *C.lindemuthianum* was exclusively secreted during fungal hyphae penetration in to plants to modify chitin that could be recognized a plant resistance system. In contrast, an intracellular chitin deacetylase from *M.rouxii* was produced during fungal cell wall formation [8]. They were expressed exclusively during sporulation of *S.cerevisiae* [9] and produced during vegetative growth of *C.neoformans* [10]. In insects, chitin deacetylase have been detected in *Anopheles gambiae*, *Apis mellifera* [11], *Drosophila melanogaster* [12],

Helicoverpa armigera [13], *Mamestra configurata* [14], *Tribolium castaneum* [11] and *Trichoplusia ni* [15]. Insect chitin deacetylase are associated with the midgut peritrophic membrane (PM) and evenly distributed throughout the entire length of PM, as found in case of *T.ni* [15], *H.armigera* [13] and *M.configurata* [14]. The presence of these enzymes was detected only during the feeding period, in the midgut tissue of larvae. When the larvae had stopped feeding in their later stage this protein became absent in the midgut tissue [15]. These biological roles of chitin deacetylase make it an imperative enzyme. Thus, the aim of this study was to isolate chitin deacetylase producing microorganisms from various samples and to screen them for their highest chitin deacetylase activity.

MATERIAL AND METHODS

The microorganisms for chitin deacetylase production were isolated from various samples like soil, agricultural waste and sewage etc. from different locations of Kurukshetra, Haryana (India). The samples were diluted and the dilutions from 10^{-2} to 10^{-6} were used to spread on Nutrient agar plates and incubated at 37°C for 2 days. The isolated colonies were picked up and streaked on Nutrient agar plates to get pure culture.

Screening of Microorganisms

Screening of chitin deacetylase producing microbes was carried out in chitin deacetylase screening plates containing (g/l): Chitin-1.0g, Sodium nitrate-2.0g, K_2HPO_4 -1.0g, KH_2PO_4 -1.0g, PN-0.50g, $MgSO_4 \cdot 7H_2O$ -0.50g, Yeast extract-0.50g and Agar-20g (pH-7.0) [16]. After 2-3 days of incubation, the colonies showing color production were confirmed as chitin deacetylase producers and used for further studies.

Production of Chitin deacetylase

A basic liquid medium having the following composition (g/l) Yeast extract-3g, Glucose-10g, Peptone-5g was used for the production of chitin deacetylase [17]. 500ml Erlenmeyer flask containing 100ml of basal medium were inoculated with 1ml of microbial culture and incubated at 37°C for 2-3 days. The medium was centrifuged and supernatant was used as crude enzyme source.

Colorimetric assay for Chitin deacetylase activity

The quantitative assay of chitin deacetylase was done by using glycol chitin as a substrate prepared by the procedure of Araki and Ito (1975) [5] from glycol chitosan (Hi-Media). The reaction mixture containing 500µl of crude enzyme and 500µl of glycol chitin in 50mM sodium tetraborate buffer was incubated at 37°C for 30 minutes. The reaction was terminated by $KHSO_4$. For color development MBTH (3-methyl-2-benzothiazolone hydrazone) was added [18]. The absorbance was read at 650nm. Standard Glucosamine-HCl was used for preparation of standard curve. One unit of enzyme was defined as the amount

of enzyme required to produce 1mg of glucosamine per minute when incubated with glycol chitin as substrate.

Optimization of Chitin deacetylase production

The selected isolates were subjected to different cultural conditions to derive the optimum conditions for chitin deacetylase production. All the experiments were carried out in 500ml Erlenmeyer flask containing 100ml of production medium. Growth and chitin deacetylase production were estimated at regular intervals for the following parameters:-

Effect of pH and Temperature

pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and

Temperature: 25, 30, 35, 40, 45, 50, 55, 60°C were used for optimization.

Effect of Carbon, Nitrogen-sources and Inorganic salts:-

C-source: Glucose, Cellulose, Galactose, Fructose, Sucrose, Maltose, Lactose and Starch.

N-source: Beef extract, Yeast extract, Peptone, Urea, Ammonium sulphate and Sodium nitrate.

Inorganic salts: $MgSO_4$, $FeSO_4$, $ZnCl_2$, $CuSO_4$, K_2HPO_4 , $CoCl_2$, $MnSO_4$ were supplemented to the production medium individually to observe their effect on chitin deacetylase production.

Effect of Incubation time, Inoculum size and Agitation speed:-

Incubation time: 24, 48, 72, 96 and 144h

Inoculum size: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ml

Agitation speed: 150, 200, 250, 300, 350 and 400 rpm were used to check their effect on chitin deacetylase production.

RESULT AND DISCUSSION

Chitin deacetylase is the key enzyme in bioconversion of chitin to chitosan. The enzymes of microbial origin were found to be more advantageous than the harsh thermochemical process for chitosan production. In the present study, isolation and screening of chitin deacetylase producing microorganisms were carried out and production conditions for enhanced chitin deacetylase production were optimized. The serially diluted samples were screened for chitin deacetylase producing microorganisms. The isolates showing yellow color production in screening medium were found as chitin deacetylase producers (fig 1). All the positive strains were then purified using repeated subculturing on Nutrient agar plate. The purified isolate was then maintained in Nutrient agar slants.

All the positive isolates were then screened by MBTH method for quantitative assay of enzyme activity. The best isolate was studied in detail for enzyme chitin deacetylase production by submerged fermentation using chitin as substrate. On the basis of

morphology and gram staining, the isolate SN-1 (best chitin deacetylase producer) was found to be yeast with large budded cells.



Fig-1: Qualitative Analysis of Isolate

In submerged fermentation, the pH 7.0 was found to be optimum for chitin deacetylase production and the effect of various pH is shown in fig 2.

The optimum temperature was found to be 35°C and further increase in temperature decrease the chitin deacetylase activity (fig 3). Supplement of Sucrose as C-source resulted in marginal increase in chitin deacetylase activity. The chitin deacetylase production of isolate SN-1 was suppressed greatly when was grown either on cellulose, galactose and lactose as C-source (fig 4). The nitrogen sources like Yeast extract and inorganic salts like ZnCl₂ were found to stimulate the chitin deacetylase production as shown in fig 5 and fig 6 respectively. The chitin deacetylase production reached max. at 72h of incubation. Further increase in incubation period did not have much impact on chitin deacetylase production rather the enzyme production was found to be decreased. Thus optimum time of chitin deacetylase production was to be 72h after inoculation (fig 7). The Fig 8 and fig 9 shows the effect of inoculum size and agitation speed on the enzyme production respectively. Agitation speed of 300rpm was found to be best for chitin deacetylase production when inoculated with 2-3ml of isolate SN-1 culture.

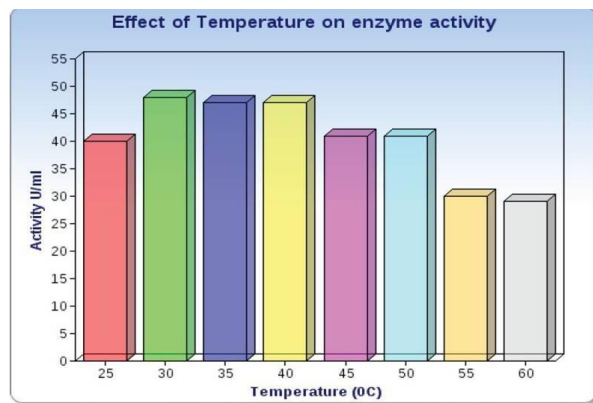


Fig-3: Effect of Temperature on Enzyme Activity

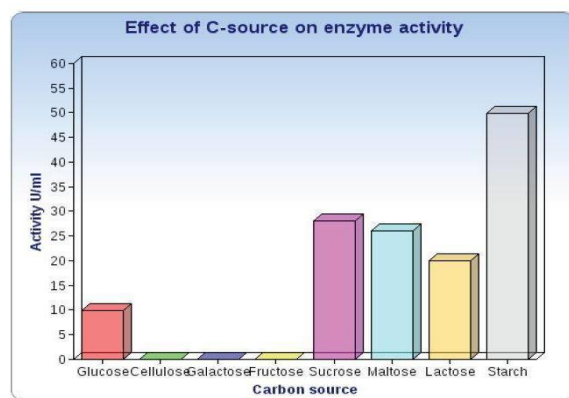


Fig-4: Effect of C-Source on Enzyme Activity

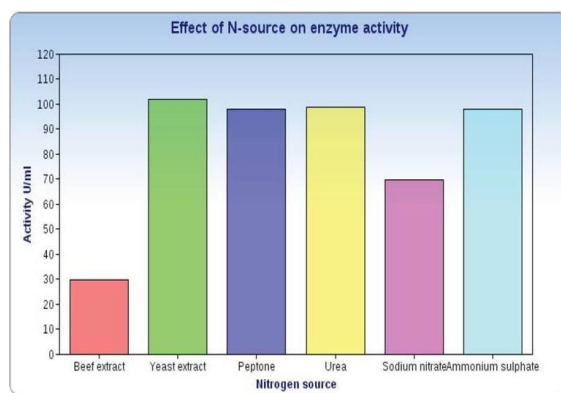


Fig-5: Effect of N-source on Enzyme Activity

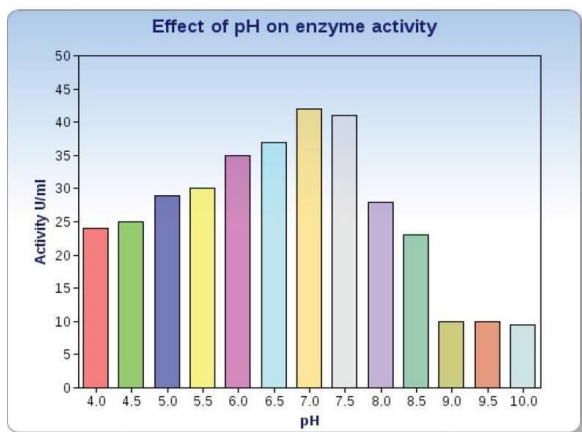


Fig-2: Effect of pH on Enzyme Activity

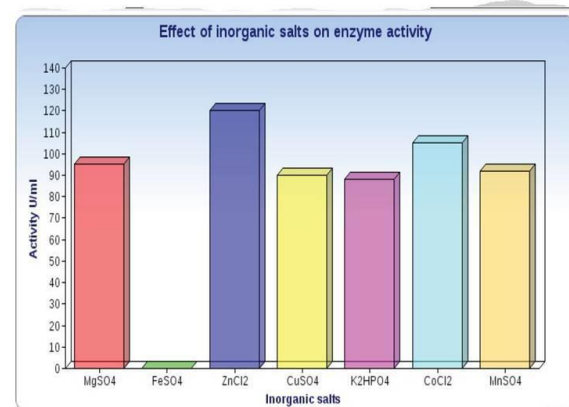


Fig-6: Effect of Inorganic salts on Enzyme Activity

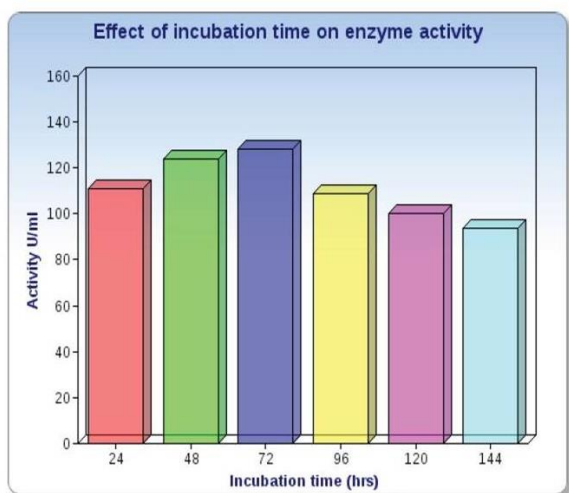


Fig-7: Effect of Incubation Time on Enzyme Activity

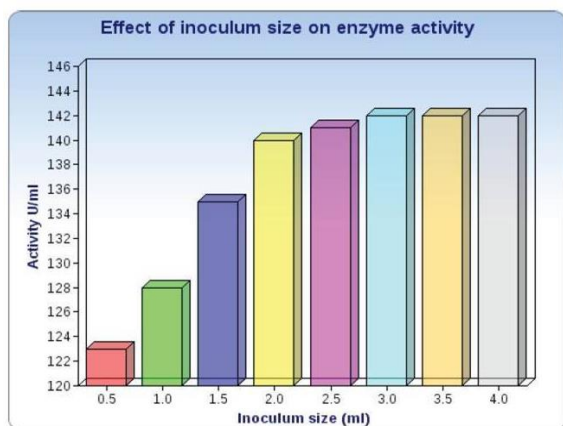


Fig-8: Effect of Inoculum size on enzyme activity

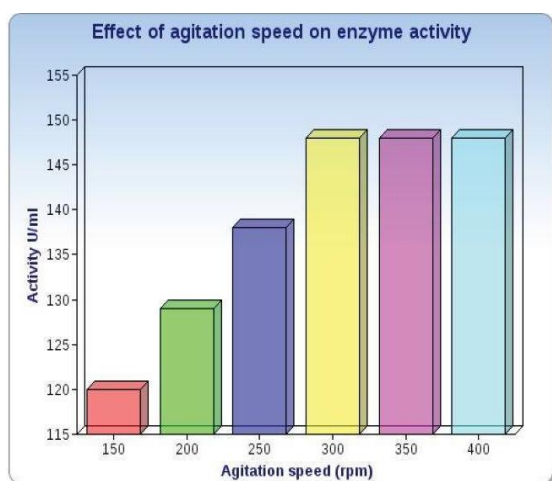


Fig-9: Effect of Agitation speed on Enzyme activity

CONCLUSION

The isolate SN-1 produced significant amount of chitin deacetylase after 72 hrs of incubation in submerged fermentation at 35⁰c and pH-7. The chitin deacetylase show maximum enzyme activity with sucrose as C-source, yeast extract as N-source and ZnCl₂ as inorganic salt when production medium is

inoculated with 2-3ml of overnight grown culture and fermentation is carried out with agitation speed of 300rpm.

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