Isolation, identification and characterization of thermostable 1, 4-α-D-glucanglucohydrolase producing *Aspergillus niger* VGA024

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Abstract
Glucoamylases possess wide range of industrial applications and are considered as one of the economically important enzymes. During the liquefaction and saccharification of starch industrially, glucoamylases which show its efficacy at normal temperature cannot be preferred. This constraint has drawn much attention towards the thermostable glucoamylase enzyme from the non-GMO fungal source. In the current research work, high potent thermostable VGA024 was screened, isolated, purified and preserved in potato dextrose agar slants at 4°C. VGA024 fungal strain was identified by using molecular and morphological techniques and was identified as Aspergillus niger and was deposited in NCBI Genbank with an accession number MT015596.

Harvesting time optimization studies were performed and Aspergillus niger VGA024 showed high enzyme activity i.e. 149 U/ml at 96 h. Similarly, thermostability of the enzyme was assessed and the enzyme showed stability till 90°C with an enzyme activity of 113±4.1. Glucoamylase was partially purified and the molecular mass was assessed by SDS – PAGE which showed 85 kDa.

Keywords: *Asperillus niger*, Glucoamylase, SDS-PAGE, Thermostable.

Introduction

Amylases show a wide variety of industrial applications in the field of brewing, food, textile, paper and medicine. Though various sources of amylase enzymes are available, microbes are mostly preferred due to their ease of operation and high productivity.[12] Amylase enzymes hydrolyse α-glucosidic linkages and based on the catalytic action they are majorly categorized into 3 types *viz.* α-amylase (acting on α-1, 4), β-amylase which acts on second α-1, 4 glycosidic bond from the non-reducing end and glucoamylase which acts on both α-1,4 and α-1,6 bonds.[9]

Glucoamylase is 1, 4-α-D-glucanglucohydrolase (EC 3.2.1.3) exo-enzyme which breaks down α-(1-4) bonds to a higher rate and α-(1-6) bonds present in oligosaccharides, glycogen and starch at a lesser rate. Glucoamylases hydrolyse the glucose units only from the reducing ends of the substrate.[2] Glucoamylase shows a wide variety of industrial applications in the formulations of detergents, production of high fructose syrups, treatment of wool and in the fermentation of starch-rich agricultural residues which can be used as a substrate in the production of biofuels. [9]

Commercially, fungal glucoamylases are produced from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*, *Aspergillus awamori* etc.[1,11] Glucoamylases can be produced in both submerged and solid state fermentation processes.

During the liquefaction and saccharification of starch in industries, high temperatures are maintained during the process. Most of the fungal glucoamylases fail to show stability at high temperatures.[5] Thus, microbes which produce the thermostable glucoamylases have gained much economical importance.[3] Though several attempts *viz.* mutagenesis, cloning and screening of thermophilic microbes are tried, these have several drawbacks i.e. low glucose units' liberation, less glucoamylase productivity etc. Similarly, glucoamylases from thermophilic bacteria are available which can withstand the temperature till 80°C which also have several drawbacks.[6] In this context, need for a fungal theromophilic glucoamylase is more required. Current research work was aimed to isolate a potent high yield glucoamylase producing fungal isolate and characterization.

Material and Methods

Chemicals and Reagents: All the chemicals and reagents required for carrying out the research work were purchased from Merck, Mumbai.

Collection of soil samples: Soil samples were collected from the agricultural lands of Bowrampet village, Telangana. Soil samples were drawn 15 cm below the surface and were collected in sterilized sample containers. The collected soil samples were immediately sent to the laboratory and stored at 4°C.

Isolation and characterization of potent fungal isolate: Soil samples collected were serially diluted and plated on the selective agar medium plates containing (g/L) soluble starch-10, $(NH_4)_2SO_4$-1.4, $MgSO_4$ – 0.2, K_2HPO_4 – 2,

$MnSO_4$ – 0.002, $ZnSO_4$ – 0.0015, $CoCl_2$ – 0.002, Agar – 15 and incubated at 45ºC for 7 days. Colonies showing high zone of clearance were isolated, purified and stored in potato dextrose agar slants at 4ºC.[5]

Fungal strain which is showing high zone of clearance was microscopically examined by using phase contrast microscope (Nikon eclipse E200) and molecular identification till species level was done by using the 18 S rRNA sequence analysis.[4]

Development of inoculum and harvesting time optimization: 100 ml of the selective media broth containing (g/L) soluble starch-10, $(NH_4)_2SO_4$-1.4, $MgSO_4$ – 0.2, K_2HPO_4 – 2, $MnSO_4$ – 0.002, $ZnSO_4$ – 0.0015, $CoCl_2$ – 0.002 was prepared and taken in 250 ml Erlenmeyer flak and sterilized. Potent fungal isolate was inoculated into the flask and kept on orbital shaker (REMI CIS-24 plus) at 200 rpm for 7 days at 45ºC.

To assess the harvesting time, liquid medium samples were drawn at regular intervals of time and glucoamylase assay was performed by using the standard protocol.

Extraction and partial purification of glucoamylase: Liquid medium was centrifuged at 10000 rpm for 15-20 min at 4ºC. Supernatant was collected and further subjected to the partial purification process.[10] The supernatant was saturated to 20% by using ammonium sulphate and centrifuged at 5000 rpm for 15 min. Pellet obtained was again saturated to 100% by ammonium sulphate by centrifuging at 5000 rpm for 15 min. Enzyme activity was calculated for the collected supernatant.[8]

Glucoamylase assay: 25 µl of crude enzyme extract was added to 975 µl of sodium acetate buffer (pH - 5) which contains 1% of soluble starch, 10 mM sodium chloride and 1 mM calcium chloride. The reaction mixture was incubated at 40ºC for 15 min followed by termination of reaction by adding 1ml of dinitrosalicylic acid solution and further boiling for 3 minutes.

The reaction mixture was cooled to room temperature and absorbance was taken at 540 nm by using UV visible spectrophotometer (Elico SL244). Glucoamylase activity can be defined under the standard assay conditions as the amount of enzyme required to release 1µmol of glucose from soluble starch per one minute.[2]

Assessing thermostability of glucoamylase: Thermostability of fungal glucoamylase was assessed by investigating at various temperatures ranging from 30-110ºC. Samples were collected after 1 h and cooled immediately after which the remaining activity was measured by addition of 25µl of enzyme with a concentration of 40 µg/ml to 975 µl of substrate solution containing 50 mM sodium acetate with pH 5.0, 1 mM NaCl, 1 mM $CaCl_2$ and 1% starch solution. Reaction was carried out at 40ºC for 15 min by taking 60 sec cooled sample as control.[2]

SDS – PAGE: The molecular mass of the extracted and partially purified enzyme was detected by using SDS-PAGE. The sample was run in 10% separating gel with constant voltage of 150 V by taking 2-β-galactosidase (116 kDa), phosphorylase b (97 kDa), ovotransferrin (78 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), actinidin (29 kDa) and β-lactoglubolin,(18 kDa) as standard molecular markers.[2,4]

Results and Discussion

With an aim to isolate a potent thermostable glucoamylase producing fungi, soil samples were collected from the agricultural fields of Bowrampet village, Telangana (17.5723ºN, 78.3997ºE). Total 41 fungal isolates were isolated from the collected soil samples, out of which 9 isolates showed good zone of clearance on selective agar plates. Among all the glucoamylase producing fungal isolates, VGA024 showed highest zone of clearance which resembles the hydrolysis of starch. VGA024 was morphologically and microscopically observed which showed similar characters with the *Aspergillus spp*.

VGA024 fungal strain was sent to Bioserve Biotechnologies Private Limited, Hyderabad for sequencing studies to be carried out. The fungal strain was identified till species level by using the Sanger's method. Sequencing results showed 499 bp, DNA sequence was compared with gene sequences in NCBI Genbank and BLAST results showed and confirmed the fungi as *Aspergillus niger*. Similar sequences were collected from NCBI Genbank and maximum likelihood phylogenetic tree was constructed by using the MEGA 7.0 software as in fig. 1. *Aspergillus niger* VGA024 was submitted in the NCBI Genbank and the accession number was assigned as MT015596.

The potent *A.niger* culture was inoculated in selective medium to assess the harvesting time and to characterize the glucoamylase enzyme further. Samples were drawn at regular intervals of time (24, 48, 72, 96, 120 and 144 h). Enzyme was extracted, partially purified and glucoamylase assay was carried out. Samples drawn at 96 h showed highest enzyme activity i.e. 149 U/ml followed by 132 U/ml at 120 h and 126 U/ml at 72 h as in fig. 2.

Glucomaylases from the fungal isolates were used in the saccharification of starch in the industries for conversion of starch into glucose. But limitation of the enzyme optima to 4.0-4.5 and below 65ºC made the researchers to search for a new thermostable enzyme which can withstand at elevated temperatures.[6] The glucoamylase enzyme was tested for its thermostability and the temperature ranges were maintained from 30ºC to 110ºC. The glucoamylase samples were kept at different desired temperatures and assay was carried after 1 h of time as per the standard protocol.

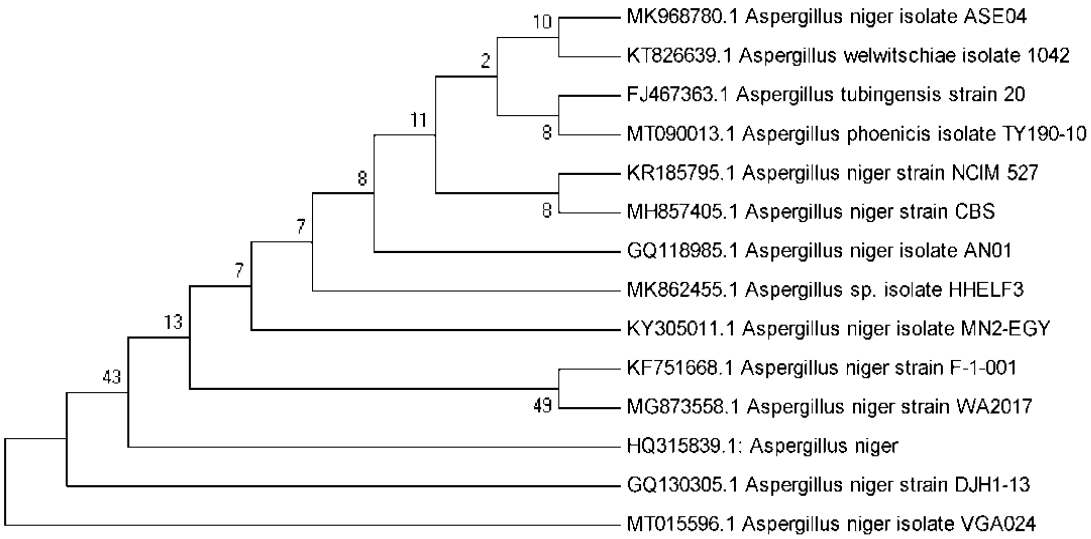

Fig. 1: Maximum likelihood tree showing closely related species based on the similar 18S rRNA sequence.

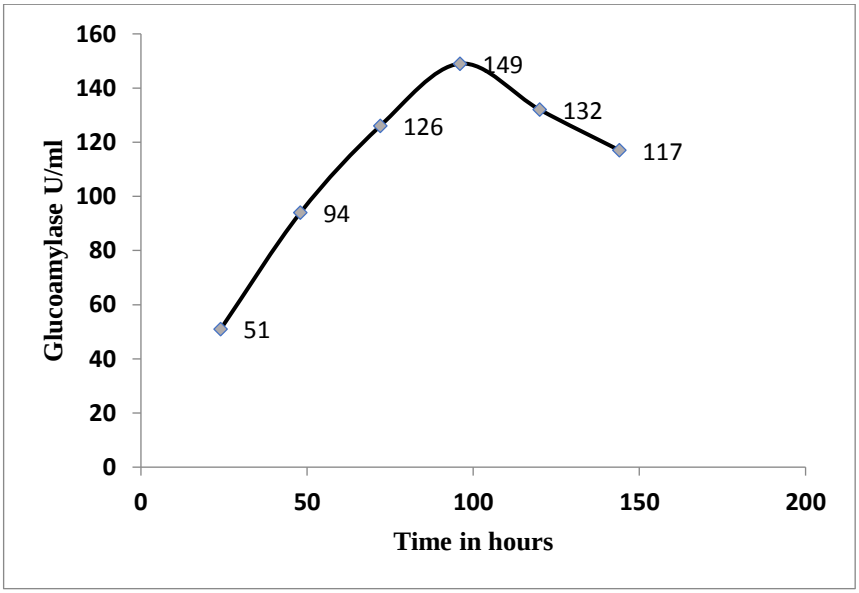

Fig. 2: Harvesting time optimization studies

Results showed that the glucoamylase isolated from *Aspergillus niger* VGA024 has remained unaltered to withstand till 90ºC with an enzyme activity of 113±4.1 (75% of enzyme activity) and later drastic reduction of enzyme activity was observed at 100ºC (57±6.2) and 110ºC (34±5.4) as in fig. 3 Research reports show thermostability of the glucoamylase enzyme isolated from *Sulfolobus solfataricus* at 90ºC with an enzyme activity of 90% with a sudden reduction of activity at 100ºC[7] whereas the glucoamylases isolated from *Aspergillus flavus* and *Thermomyces lanuginosus* showed stability of the enzyme till 65% and 70% of its enzyme activity.[5]

The partially purified glucoamylase was assessed by SDS-PAGE by using the standard molecular markers and the molecular weight was identified as 85 kDa as in fig. 4. Similarly, glucomaylase enzyme isolated from *Clostridium thermosaccharolyticum* and *Aspergillus niger* showed 75 kDa and 62 kDa.[2,13]

Conclusion

Glucoamylase possesses high industrial applications with great economical importance. The high potent fungal strain *Aspergillus niger* VGA024 with an ability to produce thermostable glucoamylase was isolated and identified. Harvesting time optimization studies showed that at 96 h high yield of glucoamylase enzyme was noticed with an activity of 149 U/ml.

Thermostability studies showed that the stability of the enzyme was noticed till 90ºC with an activity of 113±4.1. Molecular mass of the glucoamylase enzyme was assessed to be 85 kDa by SDS-PAGE.

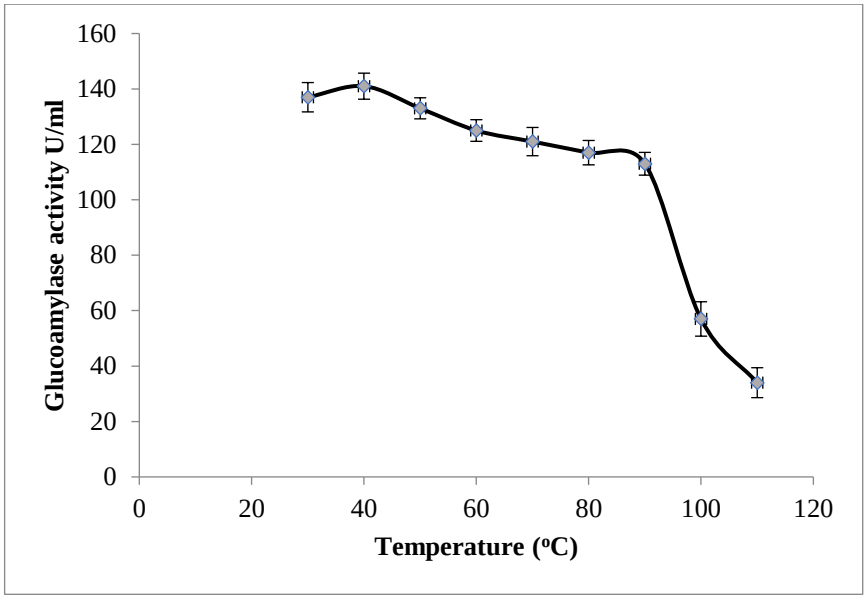

Fig. 3: Assessing the thermostability of glucoamylase enzyme

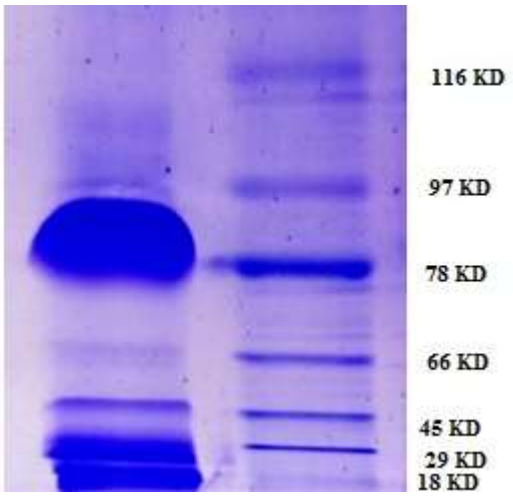

Fig. 4: 10% SDS-PAGE with 2-βgalactosidase (116 kDa), Phosphorylase b (97 kDa), ovotransferrin (78 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), actinidin (29 kDa) and β-lactoglubolin,(18 kDa) as standard molecular markers in Lane 2 and partially purified glucoamylase in Lane 1

Based on these research results, the thermostable glucoamylase enzyme isolated from *Aspergillus niger* VGA024 can be effectively used in the liquefication and saccharification of starch processing.

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