Isolation of a hyperamylolytic mutant strain of Rhizopus oryzae PR7 by Classical Mutagenesis

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Abstract

Purpose: In order to increase the ability to produce isoamylase, the isoamylolytic wild strain of *Rhizopus oryzae* PR 7, MTCC 9642 was subjected to chemical mutagenesis by N-methyl-N'-nitro-nitroso guanidine (MNNG) .

Methods: Among the surviving strains, those strains showing improved amylolytic activity both by *in situ* assay on iodine stained starch agar plate and *in vitro* assay by reducing sugar measurement were selected and sub-cultured subsequently for three generations.

Results: Strain named K1 was found to be the best amylase synthesizing mutant strain. Glycogen (0.5%) was found to be the best inducer followed by pure and indigenous starch types. The selected mutant strain showed maximum production on 96th hour of cultivation and a persistent enzyme production till 120th hour. At this hour glycogen induced production by K1 strain showed a 5.59 times increase in enzyme production than that of the wild strain. Preliminary characterization showed an achievement of increased pH and thermo stability by the enzyme of the mutant strain.

Conclusion: Hence the mutant strain K1 could be used for commercial production of isoamylase using cheap or waste sources of starchy materials.

Keywords : *Rhizopus oryzae*, Isoamylase, MNNG, classical mutagenesis

Introduction

Isoamylase (E.C.3.2.1.68, glycogen-6-glucanohydrolse) hydrolyses 1, 6- α-D-glycosidic linkages of glycogen, amylopectin and α and β limit dextrins, producing linear malto oligosaccharides [1]. It is the only amylase that completely splits the branches forked by α-1, 6 glucosidic linkages in glycogen in an exofashion [2] and has immense commercial importance in saccharide manufacturing and allied industries [3].

Although isoamylase may be produced intra-cellularly or extra-cellularly by various micro organisms [4], except few yeast strains *Rhizopus oryzae* was the only fungus reported to produce extracellular isoamylase [5], [6]. Therefore extensive research is warranted to obtain a hyper producing strain of isoamylase.

In order to increase the production efficacy, the mutant strain could be generated following classical or site directed mutagenesis. Although the former gives more desirable effect, and the strain improvement by the latter is a lengthy and laborious job [7] still classical mutagenesis is a cost effective procedure for reliable short term strain improvement [8] and does not require any profound understanding of the molecular biology and physiology of the microorganisms to be manipulated [9].

Some reports are available on strain improvement by classical mutagenesis for enhanced amylase production [10], [11], [12], but till date no report is available on strain improvement for enhanced isoamylase synthesis by any micro organism.

Hence, in the present study attempts have been made to increase the production of isoamylase by classical mutagenesis of the wild strain of *Rhizopus oryzae* PR7. Some of the parameters for enzyme production and preliminary characterization of the enzyme from mutant strain were also done.

Materials and methods

Chemicals

All chemicals used were purchased from Merck, Germany; Himedia, India and were of analytical grade.

Microorganism

Rhizopus oryzae PR7 MTCC 9642, a saccharifying isoamylase producing strain [13] isolated from the decaying vegetation enriched soil of India was used for production and characterization of the enzyme synthesized.

Cultivation of the strain

The spore suspension was cultivated in petriplates containing basal medium (BM) composed of (g L^{-1}): peptone 0.09% ; $(NH_4)_2HPO_4$ 0.04% ; KCl 0.01% ; $MgSO_4.7H_2O$ 0.01% and 0.5% starch along with 2% agar

for 48 hours at 28-30°C. Triton - X 100 (0.01-0.1%) and Oxgall 0.2% was also used in order to restrict the fungal colonies [14].

Mutagenic treatment

Spore suspension from 5 hyphal discs (0.5 cm) were incubated with aqueous solution of N-methyl-N'-nitro-nitroso guanidine (MNNG) (1mg in 10 ml water) from 30 minutes at room temperature followed by thorough mixing and centrifugation. The pellet was washed thrice aseptically with sterile distilled water. The clean spores were collected from the pellet and then cultivated on starch-agar plates for 48-72 hours at 28°C.

Selection of mutant strain

The surviving colonies were transferred to fresh starch-agar plates using Triton-X 100 as a colony restrictor. The plates were incubated at 28^0C for 48 hours. The *in situ* amylolytic activity of the colonies of the *Rhizopus oryzae* on the plates were measured by using alcoholic iodine solution containing iodine (0.2 gm), potassium iodide (2gm) dissolved in 25% (v/v) methanol [15]. The plates showed a transparent starch free halo around the colony [16].The ratio of the diameter of halo to the diameter of colony of each strain was measured. Strains with increased enzyme production were selected, sub-cultured and assayed subsequently for second and third generations. The strain showing highest enzyme production was selected after cultivating for upto third generation.

Cultivation of selected strain

The selected mutant strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl-1): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.H_2O$ 0.1 and starch 0.25 (pH 8.0) at 28°C for 72 hrs in static condition.

Enzyme extraction and enzyme assay

The culture broth was centrifuged at 10,000 rpm for 5 min and the supernatant was used as the crude enzyme. To measure the activity of isoamylase, the assay mixture (1ml) containing an equal volume of properly diluted enzyme and 1% (w/v) glycogen in 50mM phosphate buffer (pH-5.0) was incubated at 55°C for 5 min. The reducing sugar released was measured by DNS method [17]. One unit of isoamylase activity was expressed as the amount of enzyme releasing 1 µmol of reducing sugar per ml per minute using glucose as standard.

Optimization of production parameters

Both the wild and selected mutant strain K1 was cultivated in presence of various substrates namely pure starch, glycogen and cheap indigenous starches like millet powder, arum and water chestnut dust (0.5%) at various temperature and pH for 24-120h. After an interval of 24h, aliquots were withdrawn and enzyme activities of both wild and mutant strains were measured.

Preliminary characterization of enzyme from mutant strain

To detect the optimum temperature and pH , the enzyme synthesized by the mutant strain K1 was incubated with 1% (w/v) glycogen in 50mM phosphate buffer at various temperature (25-85°C) and at various pH (4.0 to 9.0) maintained by various buffers namely 50mM citrate, phosphate and Tris glycine at 55°C respectively. The thermostability was checked by exposing the enzyme protein at various temperatures (25-85°C) for 10 minutes followed by the measurement of the residual activity. Similarly the pH stability was determined by leaving the enzyme protein with buffers of various pH (4.0 to 9.0) for 12 hrs and measuring the residual activities.

Results and discussion

Out of about 150 isolated strains, 32 strains were preliminary selected and out of them strain K1 was finally selected for highest enzyme production for three successive generations (Table 1a,b,c). The strain K1 showed maximum isoamylase production and a positive correlation between *in situ* halo formation and *in vitro* assay (Figure 1).

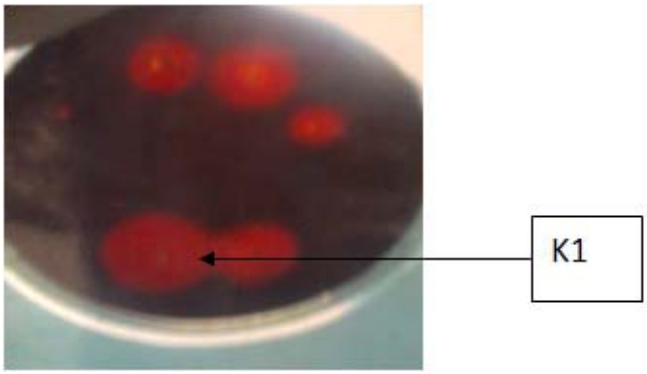

Figure 1. Halo around the colonies of mutant strain K1 on iodinated starch agar plate.

Table 1a. Assay of First Generation Strains

Name of Strain	**Relative activity (%)**
Wild	100
A 2	55.6
B 2	39
B 8	61
C 2	116
C 3	106
C 5	50
C 6	111
D 1	67
D 3	28
D 4	22
D 5	56
D 6	111
D 7	17
E 1	33
E 4	117
E 5	133
F 2	106
F 5	56
F 8	116
G 1	50
G 4	61
G 5	78
G 8	56
H 8	67
I 5	89
I 7	111
J1	105
J 4	94
J 6	72
K 1	**139**
K 2	111
K 8	122

Table 1b. Assay of Second Generation Strains

Name of Strain	Relative activity (%)
Wild	100
C 3	110
C 6	120
D 6	85
E 4	95
E 5	130
F 2	85
F 8	50
I 7	70
J 1	115
K 1	140
K 2	135
K 8	105

Table 1c. Assay of Third Generation Strains

Name of Strain	Relative activity (%)
Wild	100
C 3	106
C 6	117
E 5	78
J 1	89
K 1	**138**
K 2	78
K 8	111

Effect of cultivation time

Although a remarkable increase in enzyme activity (about 5.59 times) could be seen after mutagenesis, the selected mutant strain K1 showed highest enzyme production after 96 hours of growth while for the wild strain it was after 72 hours (Fig 2). But no sudden drop of activity could be seen in mutant strain as in the wild type, thus substantiating the use of the enzyme for a long period. A cultivation time of 96 hr was also required by isoamylase production by *Hendersonula* sp. [18].

Effect of carbon source

Glycogen proved to be a better substrate than starch for isoamylase production in case of both the wild and mutant K1 strain (Fig 3). Similar results were reported by Harada *et al* [19]. Of the cheap sources of indigenous starches used as substrate, millet was found to be the best source followed by arum for the production of enzyme by K1. Thus these cheap sources could be very economically utilized for enzyme production.

Effect of temperature and pH

The mutant strain like the wild type strain showed a preference towards pH 8.0 and 28°C for highest growth and isoamylase production (data not presented). Unlike the wild counterpart, the mutant strain did not show any tendency of pellet formation above and beyond this temperature, which might be due to some modification at their genetic level [16].

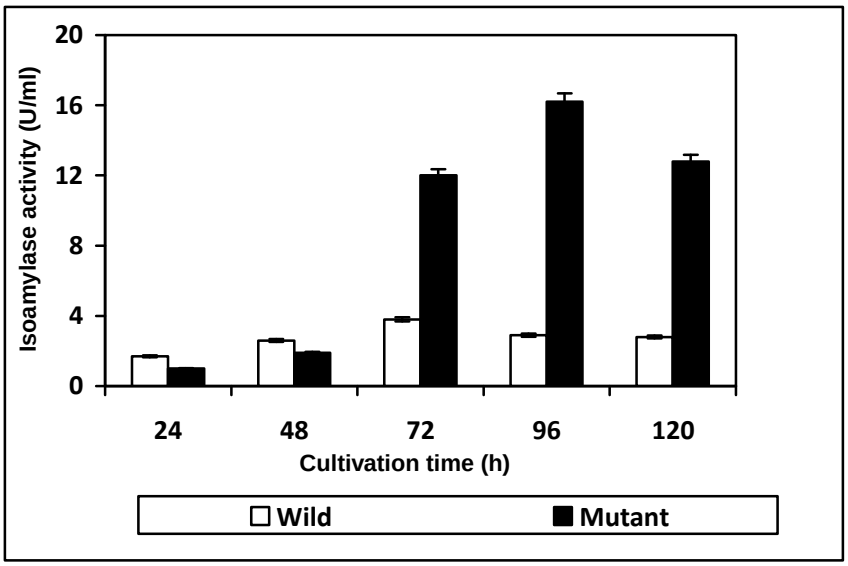

Fig 2. Isoamylase production by wild (PR7) and mutant (K1) strains of *Rhizopus oryzae*.from starch induced culture.

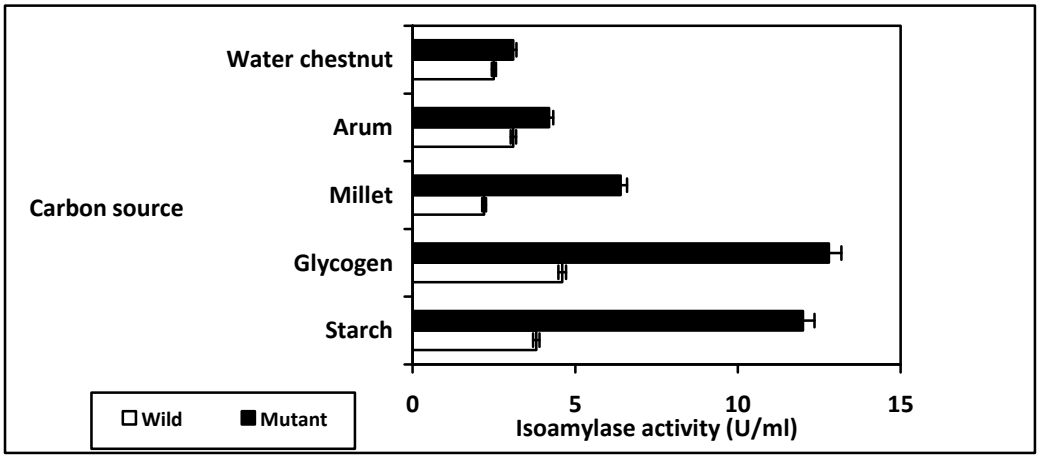

Fig 3. Effect of carbon sources on isoamylase production by wild (PR7) and mutant (K1) strains of *Rhizopus oryzae* after 72h of cultivation.

Characterization of the enzyme

The enzyme was found to have its temperature optima at 55°C (Fig 4), which was identical to that of its wild type variety [6]. But the thermostability of the enzyme was found to increase, as 65% of the enzyme activity could be retained after exposure to 70°C for 10 minutes. On the other hand, the optimum pH was found at 5.0 but the enzyme showed stability at a broader range than that of its wild counterpart. Probably this conformational stability of enzymes was achieved through change at secondary structure of the enzyme protein imparted by classical mutagenesis.

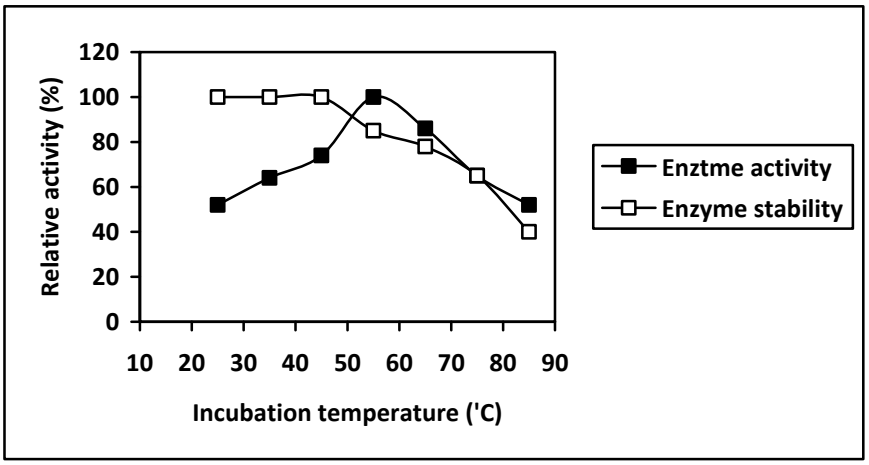

Fig 4. Effect of incubation temperature on activity and stability of the enzyme from mutant strain K1.

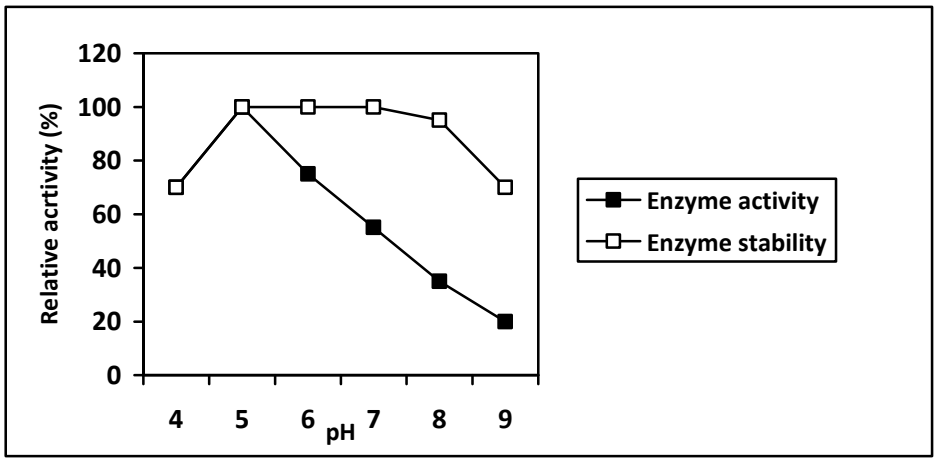

Fig 5. Effect of pH on activity and stability of the enzyme from mutant strain K1.

Conclusion

As industrial production and application of any enzyme requires hyper producing strain and increased stability of the enzyme, *Rhizopus oryzae* K1, a hyperamylolytic mutant producing thermostable and pH stable isoamylase could be employed for commercial production of microbial isoamylase. Moreover, use of cheap indigenous carbon source would further valorize waste utilization and add economy to this enzyme production.

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