British Biotechnology Journal
2(2): 60-72, 2012

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Increase in Endoglucanase Productivity and Mycelial Stability of *Rhizopus oryzae* by Classical Mutagenesis

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Research Article

Received 21st December 2011
Accepted 21st March 2012
Online Ready 3rd April 2012

ABSTRACT

Aim: To develop a mutant strain with high endoglucanase productivity and optimization of some cultivation parameters.
Place and Duration of Study: Microbiology Research Laboratory, Department of Zoology, Molecular Biology & Genetics, Presidency University, College Street, Kolkata: 700 073, India, between Aug, 2010 and March 2011.
Methodology: The wild strain of *Rhizopus oryzae* PR7 MTCC 9642 was subjected to classical mutagenesis by suspending 5 hyphal discs (0.5 cm) in 10ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) solutions of various concentrations (125-1000µg). The *in situ* cellulolytic activity of the colonies of the mutant strains on the plates were measured by using alcoholic iodine solution and the highest enzyme producing mutant was selected. The mutant strain was later cultivated in presence of various domestic wastes at various pH, temperature, time. The morphological alteration was also checked by staining with fluorescent dye.
Results: Out of 50 mutants, strain A7 was selected that showed about 33% increase in endoglucanase synthesis utilizing orange bagasse as sole carbon source in a shake flask screen. The strain was found to have the same pH and temperature optima, but could achieve highest level of enzyme production earlier than that by its wild counterpart. Being a dimorphic fungus, the wild type strain of *Rhizopus oryzae*, showed a transformation to yeast like pelleted form, whereas the mutant strain A 7 showed persistent filamentous structure indicating the achievement of a structural stability in presence of environmental stress.
Conclusion: The present mutant strain could ferment orange bagasse and showed an increased production of endoglucanase with minimized time consumption with greater

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mycelial stability against various environmental stresses. These achievements will definitely add economy in industrial production of endoglucanse at a nominal cost.

Keywords: Dimorphism; endoglucanase; N-methyl-N'-nitro-N-nitrosoguanidine; mutagenesis; Rhizopus oryzae.

1. INTRODUCTION

The bioconversion of cellulosic materials has been receiving attention in recent years and it is now a subject of intensive research to the development of a large-scale conversion process beneficial to mankind (Kumakura, 1997). Enzymatic degradation of cellulose to glucose requires the co-operative action of three synergistically acting enzymes: namely endoglucanase (CM cellulase, EC 3.2.1.4), which cleaves internal glucosidic bonds, exoglucanase (Avicelase, EC 3.2.1.91) that cleaves cellobiosyl units from the ends of cellulose chains; and β-glucosidase (EC 3.2.1.21), which cleaves glucose units from cello oligosaccharides (Wood, 1989). Among these enzymes, endoglucanase is the most important enzymes for its wide applications in food, feed, textile, detergent industries (Bhat and Bhat, 1997). Although a number of well-studied fungal strains were reported to have cellulolytic activities (Acharya et al., 2008 , Singhania et al., 2010), extensive search is still going on to isolate a hyper producing microbial strain that could be used for bulk production of cellulases at a nominal cost (Karmakar and Ray, 2011).

A number of biotechnological approaches have been taken to enhance the enzyme production efficacy of the strain and random mutagenesis has been used for decades as a tool for increasing genetic variability and improving the properties of microbial strains (Clarkson et al., 2001). Although strain improvement is a lengthy and laborious job (Anjum Jia et al., 2010) and chemical mutagens may induce mutations within a sequence originating mutagen-specific patterns of mutations, still classical mutagenesis and selection are cost effective procedure for reliable short term strain improvement (Iftikhar et al., 2010). The greatest advantage of this method is the simplicity that does not require any profound understanding of the molecular biology and physiology of the microorganisms being manipulated (Gromada & Fiedurek, 1997). Many strains have been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulases (Kuhad et al., 1994, Szengyel et al., 2000, Chand et al., 2005, Vu et al., 2009, Javed et al., 2011) that could be utilised for production of commercially efficient enzyme (Chand et al., 2005).

In order to make the enzyme production process economically viable, many cellulosic agro wastes were tested as sole carbon source. Although much work has been done on the production of cellulase from lignocellulosics (Acharya et al., 2008, Singh et al., 2009; Karmakar and Ray, 2010), emphasis has been placed much on bagasse (Chand et al., 2005, Omosajola et al., 2008).

Hence attempts have been made to utilise common domestic wastes including orange bagasse to produce endoglucanase. Moreover, so far the literature survey is concerned, no report is available on hyper productive cellulolytic strain of *Rhizopus,* the present study aims in increasing the production of endoglucanase by the mutant strain of *Rhizopus oryzae* PR 7

through classical mutagenesis followed by characterization and optimization of cultivation parameters of the mutant strain in presence of cellulosic wastes.

2. MATERIALS AND METHODS

2.1 Organism

Rhizopus oryzae PR7 MTCC 9642 (Karmakar and Ray, 2010) was used in these studies. The fungus was grown in 1% peptone dextrose agar (PDA) plates for 48 h at 28-30°C. The inocula were prepared by making hyphal discs (0.5 cm diameter). Each disc was used to inoculate 10 ml of medium (Ray and Chakraverty, 1998)

2.2 Chemicals

All reagents used in the study were of analytical grade of which N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and carboxymethyl cellulose were purchased from Sigma (USA). The agro wastes used were collected from temple effluents and were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure carboxy methyl cellulose.

2.3 Mutagenesis and Selection of Mutant Strain

The mutated fungal culture was prepared by suspending 5 hyphal discs (0.5 cm) in 10ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) solutions of various concentrations (125-1000µg). After thorough cyclomixing, the spore suspension was allowed to stand for half an hour at room temperature. Then the spore suspension was cultivated in Petri plates containing cellulose –agar medium 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 (Khattab & Bazaraa, 2005).

The *in situ* cellulolytic 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 (2 gm) dissolved in 25% (v/v) methanol (Kasana et al., 2008). The plates showed a transparent cellulose free halo around the central translucent zone of the colony. Comparing the activity of the wild organism the primary screening of the hyper producing mutant strains was done. The colonies showing increased cellulose digesting ability as evident from the increased ratio of diameter of cellulose free halo around the fungal colony to the diameter of the fungal colony were selected.

Being a eukaryotic system, *Rhizopus oryzae* has a natural tendency of reverting back to its wild form, the selected mutant strains thus raised were again subjected to mutagenesis following same methodology and the selected mutant strains of second generation were cultivated in liquid culture medium supplemented with carboxymethyl cellulose containing basal medium at 37°C for 24-96 hours.

2.4 Cultivation of Strain

The strain (wild and mutant) was cultivated in liquid medium in 100 mL Erlenmeyer flasks each containing 10 ml Basal Medium (BM) composed of (g l^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.7H_2O$ 0.1 and carboxy methyl cellulose (CMC) 0.5 (pH 6)

and to study the effect of the agro wastes pure CMC was replaced by dried flower and orange bagasse.

2.5 Enzyme Assay

To measure the activity of endoglucanase from both wild and mutant strains, the grown culture was filtered through filter paper (Whatman No. 1), the filtrate was centrifuged at 10,000 rpm for 5 min at 4℃ and the supernatant was used as the crude enzyme. The assay mixture (1 ml) containing 0.5ml of enzyme and 1% (w/v) carboxy methyl cellulose dissolved in 0.5 ml of 10 mM phosphate buffer (pH 6) was incubated at 33±5℃ for 10 min. The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with heat inactivated enzymes. One unit of endoglucanase was defined as that amount of enzyme that liberated 1 micro mole of glucose per millilitre per minute of reaction.

2.6 Optimization of Cultivation Parameters

The effect of different temperature was studied by incubating the culture containing flasks at various temperatures (10 - 50℃). The strain was grown in different flasks containing media with various pH (4-9) to check the optimum pH for enzyme production. Similarly, the effect of agrowastes was studied by supplementing various agrowastes in the cultivation medium. The most suitable concentration of orange bagasse was determined by varying its concentration (0.25-35% w/v) in the growth medium. The effect of cultivation time was determined by picking up the culture containing flasks with optimized media at various time intervals (12- 96 hours), followed by an assay of the enzyme activity.

2.7 Photo Micrographic Study

Both the wild and mutant strains were stained with acridine orange solution (1mg) dissolved in 10ml of 0.1(M) phosphate buffer (pH 6.0) for 20 minutes followed by washing with 0.1(M) phosphate buffer (pH 6.0) following the method of Ruchel and Schaffrinski, 1999 and were visualized under Axioscop-40 (Carl Zeiss) microscope at 40X (Karmakar et al., 2011) under a fluorescent field 500 nm. The photomicrographs were taken in Axio Cam MRC (Rev.3.mi). Each experiment was performed thrice and their values were averaged.

3. RESULTS AND DISCUSSION

MNNG was previously reported as an effective mutagen for strain improvement of *Rhizopus sp.* for enhanced enzyme activity (Iftikhar et al., 2010). The Petri plates inoculated with spore solution treated with different concentration of MNNG showed a gradual decrease in survival rate and colony count with increasing concentration of the mutagenic agent. The plate having 250µg concentration showed maximum number of viable colonies (Figure 1) and successive experiments were performed with this dose of mutagen.

The killer curve (Figure 2) showed the sub lethal exposure time was 30 minutes.

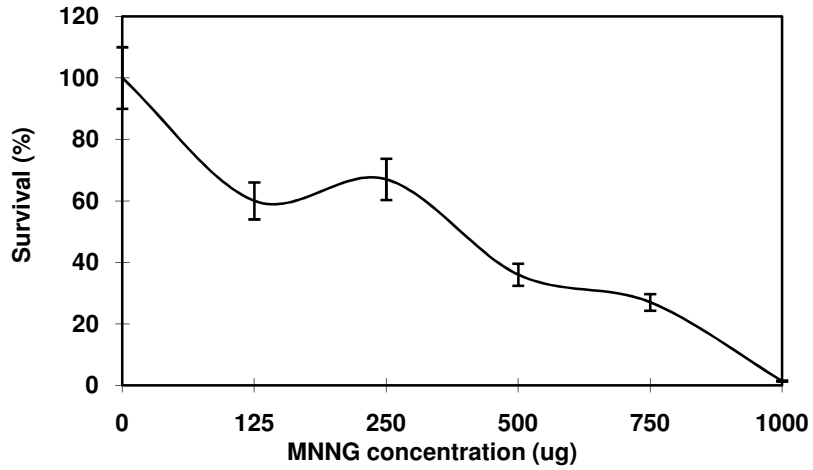


Fig. 1. Effect of mutagen (MNNG) concentration on the survival of *Rhizopus oryzae*
Mean±SEM= Mean values ± Standard error of means of three experiments

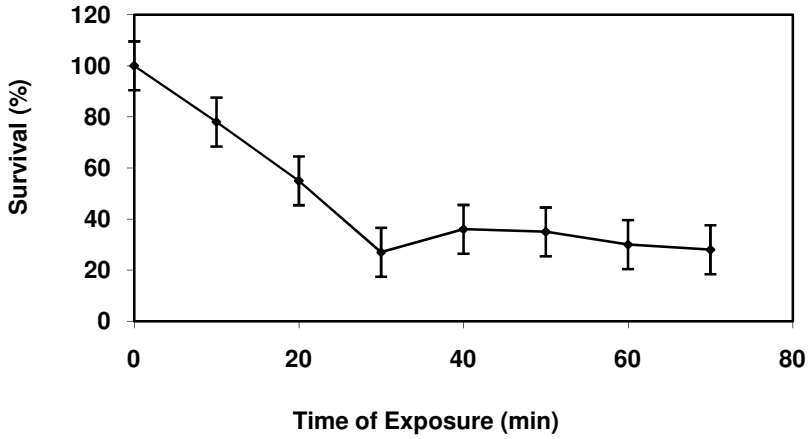


Fig. 2. Effect of mutagen (MNNG) on *R. oryzae* to formulate the kill curve
Mean±SEM= Mean values ± Standard error of means of three experiments

3.1 Effect of Mutation on Endoglucanse Production

Increased diameter of the halo around each fungal colony on the iodine stained cellulose-agar plate indicates increased production of cellulose by the strain. Out of such 50 mutant colonies, strain A 7 was selected since it showed the highest halo diameter (Kasana, 2008). The production enhancement from the wild type strain was confirmed after determining the endoglucanase activity from liquid state culture also. Similar cellulase production enhancement in fungal strain were found in (about 30% increase) *Fusarium oxysporum* (Kuhad et al., 1994), 2.03 fold in *Aspergillus* s*p.* (Vu et al., 2009) and 41.17% in *Humicola insolens* (Javed et al., 2011).

3.2 Effect of Various Carbon Sources

Among the inducers tested, orange bagasse was proved the best (Fig. 3) and about 33% increase in endoglucanase production could be obtained after mutagenesis. Orange bagasse containing a large amount of cellulose (Giese et al., 2008) could be utilized as a substrate for production of fungal endoglucanase. Utilization of this agro waste could add economy in enzyme production.

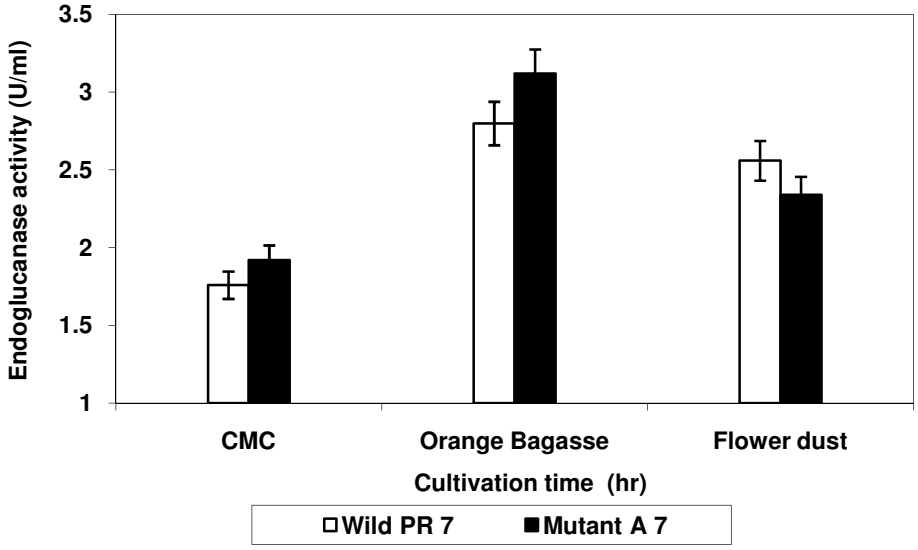


Fig. 3. Effect of various carbon sources on endoglucanase production by wild and mutant strains

Mean±SEM= Mean values ± Standard error of means of three experiments

3.3 Effect of Various Concentrations of Orange Bagasse as Carbon Sources

Highest production was found to be at the concentration of 2% (w/v) of orange bagasse (Figure 4), above which the production was reduced in case of the wild strain probably due to the adverse effect of higher load of nutrient supplements present in these substrates (Omojasola et al., 2008), or as a result of hindrance of mass transfer of oxygen by higher amount of solid substrate (Ghosh and Ray, 2011). But enzyme production was found to remain unchanged for mutant strain even in higher substrate concentration which revealed the ability of the mutant strain to withstand higher load of nutrient supplements.

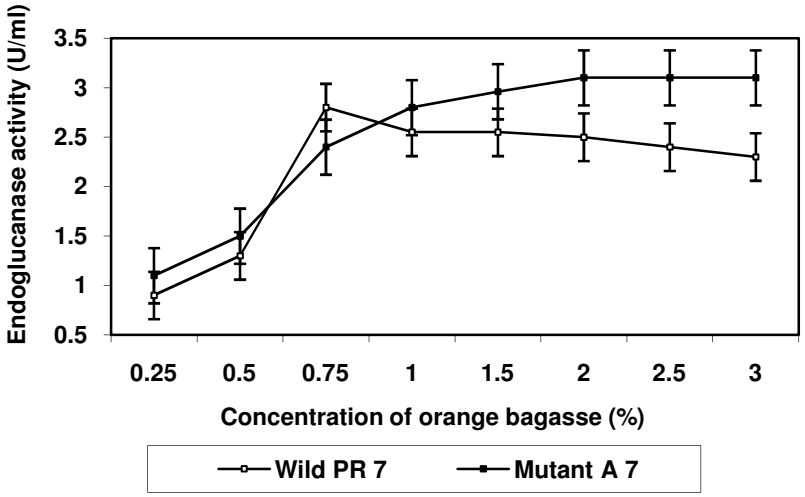


Fig. 4. Effect of concentration of carbon source on endoglucanase production by wild and mutant strains

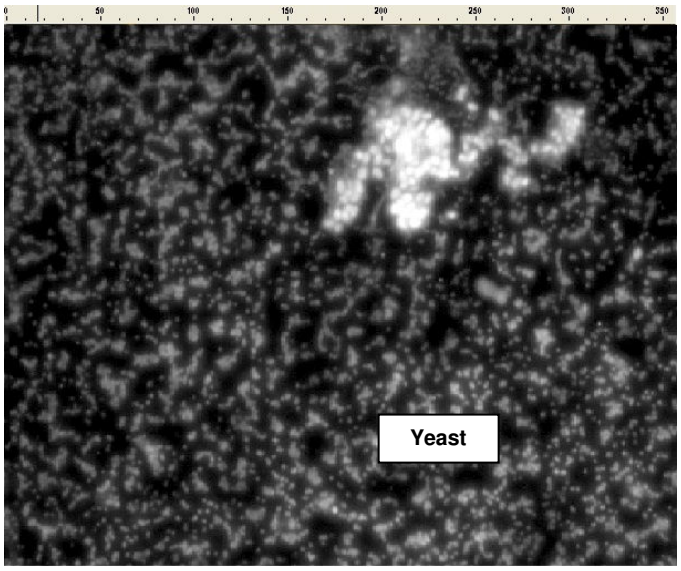
Mean±SEM= *Mean values ± Standard error of means of three experiments*

3.4 Effect of pH on Mycelial Morphology of the Fungus and Enzyme Production

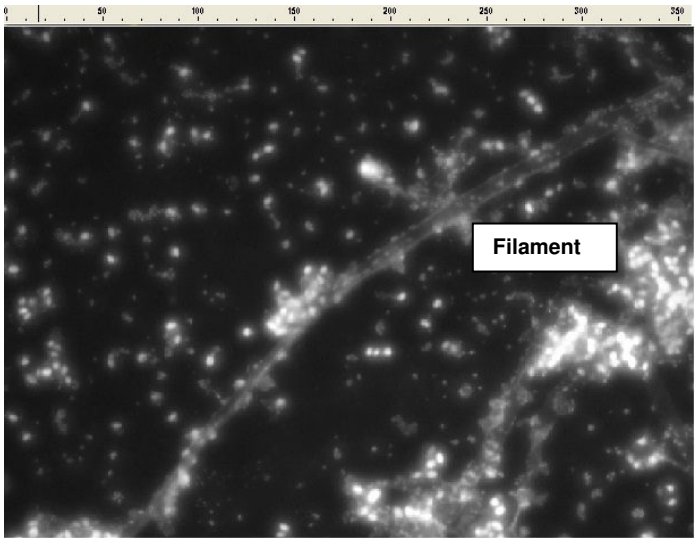
Although both wild and mutant strain showed highest enzyme production at pH 6.0, it was found to decrease gradually above and below this pH (Table 1). Being a dimorphic fungus, the wild strain showed a tendency to develop yeast like pelleted structure with restricted spore formation at pH below and above the optimum pH. But the effect of increased and decreased pH was not so prominent on mutant strain A-7 since the mutant strain showed greater pH stability and showed increased resistance to changed pH as evident from its filamentous structure (Figure 5).

Table 1. Effect of pH in orange bagasse supplemented cultivation media of wild and mutant strains of *Rhizopus oryzae* on endoglucanase activity and morphology of the fungus

pH	Wild Type (PR 7)		Mutant (A 7)	
	Endoglucanase activity (U/ml)	Morphology	Endoglucanase activity (U/ml)	Morphology
4	0.93±0.01	Yeast	1.30±0.05	Intermediate
5	2.09±0.09	Intermediate	2.73±0.12	Filament
6	2.80±0.07	Filament	3.10±0.13	Filament
7	2.48±0.10	Yeast	2.47±0.09	Filament
8	2.25±0.08	Yeast	2.08±0.07	Filament
9	1.7±0.07	Yeast	1.60±0.06	Intermediate



A



B

Fig. 5. Effect of mutation on morphological change in *Rhizopus oryzae* under pH stress

A: Restricted spore formation and total yeast like appearance at pH 8 in Wild PR7 strain

B: More spore formation and total filamentous appearance at pH 8 in Mutant A7 strain (Scale- 0-350 µm)

In the present strain the switch from filamentous form to yeast-like growth formation could be in response to different environmental cues (Karmakar and Ray, 2011) which is probably controlled by complex genetic pathways (Elías-Villalobos et al., 2011). It might be presumed that mutation in the present strain had probably stopped the normal dimorphic change towards yeast like state, even in presence of high pH, might have been accomplished by the normal functioning of Tup1 like gene, as present in other dimorphic fungus causing a drastic reduction in the filamentation capacity of the strain (Elías-Villalobos et al., 2011).

3.5 Effect of Temperature on Enzyme Production

The optimum temperature for enzyme production by both wild and mutant strains was found to be at 37°C, above which the enzyme production reduced (Fig 6). This reduction was abrupt in case of mutant strain. Although transformation towards yeast like growth in presence of higher temperature was obvious for wild type strain, appearance of an intermediate form was found in mutant A 7 strain. This confirmed the fact that somehow the mutational change imparted the mycelial stability of the fungus even in the presence of environmental stress.

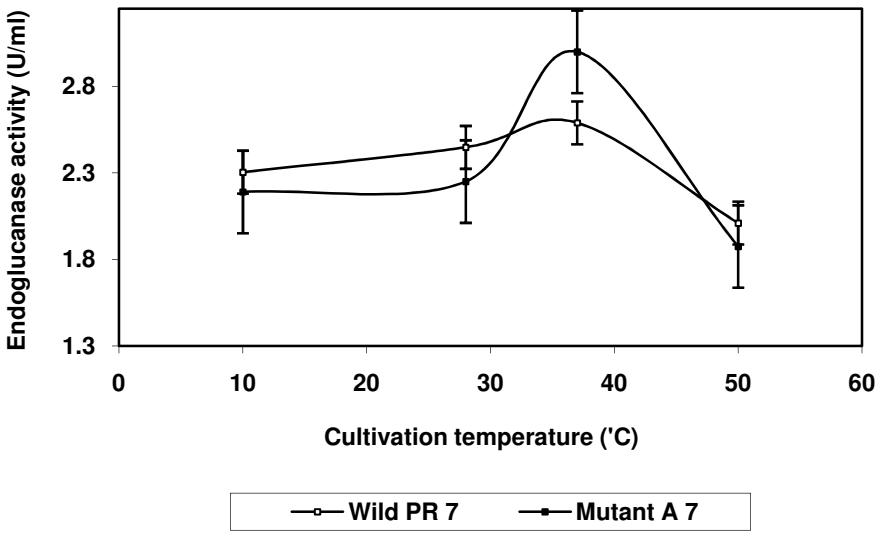


Fig. 6. Effect of temperature on endoglucanase production in orange bagasse supplemented cultivation media of wild and mutant strains of *Rhizopus oryzae*
Mean±SEM= Mean values ± Standard error of means of three experiments

3.6 Effect of Cultivation Time on Enzyme Production

Although the highest production was achieved from wild strain at 48th hour of growth, surprisingly highest enzyme production was achieved from mutant A7 within 24 hour of growth (Fig 7). Similar tendency of reduction in cultivation time for enzyme synthesis by the mutant strain was found by Rasul et al. 2011; who opined that the production of the enzyme at shorter duration is a considerable quality of the mutant strain to minimize time consumption in production process at industrial scale.

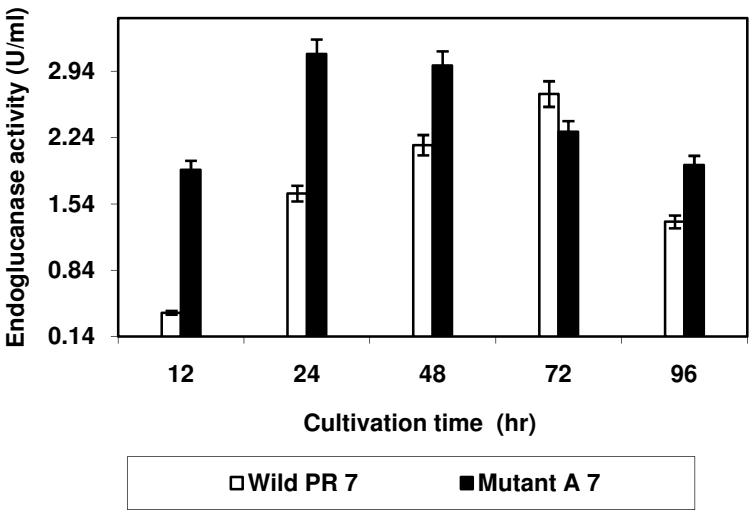


Fig 7. Enzyme production kinetics of wild and mutant strains of *Rhizopus oryzae*
Mean±SEM= *Mean values ± Standard error of means of three experiments*
Orange bagasse - 2 % (w/v), pH 6.Temperature- 37℃.

3.7 Effect of Additives on Enzyme Production

Although there was a marked increase and decrease in enzyme production in presence of Mn^{2+} and heavy metals (Hg^{2+} and Cu^{2+})respectively, the effects were not so prominent in case of the mutant strains (Table 2), which indicated that the mutation somehow made the strain non responsive to the additives.

Table 2. Effect of various additives on endoglucanase production by wild and mutant strains of *Rhizopus oryzae*

Additive	Endoglucanase activity (U/ml)	
	Wild (PR 7)	Mutant (A 7)
None	2.79±0.12	2.88±0.11
Na^{+}	2.48±0.10	2.28±0.10
K^{+}	2.01±0.09	1.92±0.08
Mg^{2+}	2.48±0.08	2.40±0.09
Mn^{2+}	3.87±0.15	3.12±0.16
Fe^{3+}	3.41±0.11	3.00±0.15
Ca^{2+}	2.63±0.08	2.40±0.10
Sr^{2+}	1.86±0.04	1.92±0.07
Pb^{2+}	2.94±0.09	2.16±0.12
Hg^{2+}	1.08±0.05	0.96±0.04
Cu^{2+}	0.93±0.02	0.96±0.03

4. CONCLUSION

Despite a worldwide increasing demand for cellulases, the high cost of production of these enzymes has hindered the industrial application of cellulose bioconversion (Narasimha et al., 2006). The production cost might be reduced by using a hyper productive strain that would be able to utilize cellulosic agro wastes for the production of enzyme. The present hyper productive mutant strain could ferment orange bagasse and showed an increased production of endoglucanase with minimized time consumption. It also acquired a mycelial stability against various environmental stresses. These achievements will definitely add economy in industrial production of endoglucanase at a nominal cost.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Science and Technology, West Bengal for the financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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