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Production and Improvement of Alkaline α - Amylase by Bacterial Isolate from Chilika Lake

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A B S T R A C T

The present study suggest that the Chilika Lake isolate i.e., *Bacillus* sp. may act as a potent strain for alkaline α- amylase production. In case of parent strain the enzyme production was higher with banana peels as the substrate; in case of mutant, potato peels used as substrate. Optimization of the fermentation parameters and the use of suitable carbon, nitrogen and metal chloride supplements resulted in increase in the enzyme yield. The enzyme production was significantly high at optimum temperature which was found to be 37 °C and the optimum inoculums size 2%. Maximum production was found when the medium is supplemented with starch as carbon source, yeast extract as nitrogen source and NaCl as mineral chloride. The enzyme was found to be active over a wide range of pH and showed the optimum activity at pH 11. This isolate both wild and mutant could thus be industrially exploited for the synthesis of alkaline α- amylase which have immense industrial application.

Introduction

α-Amylases (1-4, α D- glucanohydrolase EC 3.2.1.1) are extracellular endo-enzymes that hydrolyze internal α–1-4- glycosidic linkage in starch and related substrate in an endo-fashion producing oligosaccharides, glucose and α–limit dextrin (Fogarty *et al.*, 1999). Alpha-amylases find potential applications in pharmaceuticals, baking, brewing, textile, paper, syrup industries and detergent manufacturing processes (Bajpai and Bajpai, 1989; Hewitt and Solomons, 1996; Sivaramakrishnan *et al.*, 2006). In recent years, the market of detergent additive enzymes is one of the largest markets for industrial enzymes (Niehaus *et al.*, 1999; Schallmey *et al.*, 2004). Several enzymes such as alkaline protease, cellulase, lipase and amylase are usually put in detergents as additives (Niehaus *et al.*, 1999; Horikoshi, 2006). Amylases are the second type of enzymes used in the formulation of enzymatic detergent and 90% of all liquid detergents contain these enzymes (Gupta *et al.*, 2003). These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custards and chocolates to dextrin and other smaller oligosaccharides

(Mukherjee *et al.*, 2009). Amylases having activity at alkaline pH maintain the necessary stability under detergent conditions (Kirk *et al.*, 2002).

Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. Termamyl® (Novozymes) derived from *Bacillus licheniformis* and its derivative Duramyl® (Novozymes) have been used for that purpose (Olsen and Falholt, 1998). Duramyl® (Novozymes) was developed by site-direct mutagenesis. UV- light has also been reported to be mutagenic in variety of organisms and in recent years, attempts have been made for the overproduction of microbial enzyme by induced mutagenesis (Singh *et al.*, 2011). The present investigation deals with the enhancement of the production of α-amylase by subjecting the isolate to mutagenesis by UV radiation.

Materials and Methods

Sample collection and analysis

Sediment sample was collected from Chilika Lake i.e. Balugaon in sterile plastic polythene and brought to the Department of Microbiology, O.U.A.T., Bhubaneswar (Odisha) for further study. The pH (Akpor *et al.*, 2006) and moisture content (Jackson, 1967) were calculated accordingly.

Bacterium and growth conditions

The bacterial culture was isolated on nutrient agar medium, pH 9.0 and one colony was selected and re-streaked on nutrient agar plate to check the purity of the culture and then maintained in nutrient agar slants for further experiments. The isolate was checked for amylolytic activity based on zone of clearance on starch agar plate and named as CHB7.

Mutagenesis

Bacterial suspension (0.5 ml) was transferred to sterile nutrient agar plates and exposed to UV irradiation (260-270nm) for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min. The distance between lamp and petri-plates was adjusted to 55 cm. UV exposed plates were placed in the incubator at 37 °C for 24 h. Colonies of CHB7 isolate after UV treatment were recorded to determine survival of the target strain. Sub culturing of resistant colonies was done and then re-exposed to UV irradiation to obtain mutant colonies. The screened mutant derivatives were assayed quantitatively for alkaline α –amylase activity by plate assay method (Singh *et al.*, 2013).

Qualitative analysis of enzyme production

Qualitative analysis of enzyme production by 3 mutants was performed at pH 6.0 - 12.0 and based on the highest zone of clearance only one potent isolate was selected for further study.

Partial characterization of bacterial isolates

After screening, the amylase producing isolates (parent and mutant) were subjected for partial characterization by cultural (color, texture, margin, elevation and size), morphological (Gram variability), standard biochemical tests (Lysine, TSI, Arginine, Ornithine, Mannitol motility, Indole, Methyl Red, Voges-Proskauer, Citrate, Nitrate, Esculin, Urease). The results were analyzed in PIBWin Software (Bryant, 2003) for probabilistic identification.

Preparation of inoculum

A volume of 100ml of nutrient broth taken in a 250ml Erlenmeyer flask was inoculated with a loop full of cells from a 24 h old slant

and kept at 37 °C in a bacterial incubator. After 18 h of incubation, 1ml of this nutrient broth culture was used as the inoculum.

Solid state fermentation

Agro residues: Rice Husk (RH), Rice Bran (RB), Potato Peels (PP), Banana Peels (BP), Sugarcane Bagasse (SCB), Corn Cob (CC) and Cauliflower Stalk (CS) were collected from local market, Siripur. They were properly washed and chopped into small pieces. The residues were separately spread on trays and oven dried at 70 °C for 24 h (Unakal *et al.,* 2012). After grinding into fine powdered form, they were preserved at room temperature. Agro residues (2.5 g) were kept separately in a 250 mL Erlenmeyer flask and then moistened with 10mL mineral salt medium to 75% moisture content and sterilized at 121°C for 15-20 min. Media supplemented with peptone 0.6%, KCl 0.05% (w/v), $MgSO_4.7H_2O$ 0.05% (w/v) and starch (1.0%), pH 9.0 was used for amylase production for target bacterial strain along with screened mutant. The fermentation process was started by adding 1 mL of overnight bacterial culture. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and incubated at 37°C for 24 to 48 h with occasional gentle shaking (Sajjad and Choudhry, 2012). Both parent (CHB7) and mutant (MCH/40) strains were analyzed for enzyme production under different physical and cultural conditions.

Enzyme extraction

After 24-48 h incubation, 20mL 0.05M sodium phosphate buffer (pH-8.0) was added to 1g fermented carrier, the mixture was shaken for 1h at 37 °C and 150 rpm on a rotary shaker, filtered with muslin cloth and centrifuged at 10,000 rpm at 4°C for 15min. The supernatant was used as the crude enzyme (Sajjad and Choudhry, 2012).

Assay of enzyme activity

DNSA method (quantitative assay)

Quantitative assay for amylase activity was carried out by measuring the amount of reducing sugar according to the DNSA method (Miller, 1959). Amylase activity was determined by incubating a mixture of 1 ml of aliquot of each enzyme source and 1% soluble starch dissolved in 0.05 M sodium phosphate buffer, at pH 8.0, at 37 °C for 20 min. The reaction was stopped by adding 1.5 ml of 3, 5-Di-nitro salicylic acid, and then followed by boiling for 5 min at 99.8 °C at water bath. The final volume was made up to 4.5 ml with distilled water and the reducing sugar released was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releasing 1□ mol maltose equivalent per minute under the assay conditions. Reducing sugar (maltose) concentration was determined from a standard curve under same condition using maltose.

$$\text{Amylase activity ((IU/mL)/min)} = \frac{\text{Amount of sugar released} \times 1000}{\text{mol.wt of maltose} \times \text{time of incubation}}$$

Estimation of protein concentration

The protein concentration was estimated by using Bovine serum albumin as standard (Lowry *et al.,* 1951).

Optimization at different period of fermentation

As production of enzyme is well correlated with the period of fermentation, the effect of fermentation period on amylase production was carried out. Varied fermentation period of 24, 48, 72, 96, 120 h of the bacterial isolate were carried 37 °C in the assay medium for amylase production and amylase activity was

found out for both parent (CHB7) and mutant (MCH/40) strains.

Optimization of pH for amylase production

The effect of pH on amylase production of both parent (CHB7) and mutant (MCH/40) strains was determined by growing the isolates in the assay medium with different pH values ranging from 6.0-12.0. The amylase assay was carried out by the method described earlier.

Optimization at different temperature condition

The effect of different temperatures an amylase production by both parent (CHB7) and mutant (MCH/40) were determined by growing in assay media at varied temperatures (17, 37 and 47 °C). In this experiment the pH and time of incubation remains constant at 9.0 and 96h for the bacterial isolate. The amylase activity was carried out to determine the concentration of the enzyme.

Optimization at different inoculum size of fermentation

As production of enzyme is well correlated with the inoculum size, the effect of inoculum size on amylase production was carried out. Varied inoculum size (1- 5ml) was used. In the assay medium for amylase production and amylase activity was found out for parent (CHB7) and mutant (MCH/40) strains.

Optimization at different Carbon Sources

To study the effect of different carbon sources (glucose, fructose, lactose, mannitol, starch, sucrose, dextrose, xylose) both parent (CHB7) and mutant (MCH/40) strains were inoculated into the assay medium with different carbon sources at pH-9.0 at 37 °C for 96 h incubation to find out amylase activity under different cultural conditions.

Optimization at different Nitrogen sources

To ascertain the effect of culture conditions on amylase production, the study was carried out at different nitrogen sources like peptone, yeast extract, casein, urea, $NaNO_3$, $(NH_4)_2SO_4$. In this experiment, both parent (CHB7) and mutant (MCH/40) were inoculated in respective assay medium containing different nitrogen sources with pH 9.0 at 37 °C in a incubator for 96 h to obtain a uniform growth.

Optimization at different metal chlorides ions

Amylase activity was assayed in the presence of 10mM metal chlorides (NaCl, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $CaCl_2$, and $FeCl_3$).

Thin layer chromatography (TLC)

Soluble starch (amylose) (1%) was digested with amylase in sodium phosphate buffer pH 8.0 at 37 ºC for 45 minutes. Previously chilled ethanol was added to the enzyme and substrate mixture to stop the reaction. The end products were then analyzed on silica gel 60 (GF254) (Merck) thin-layer chromatography. After developing the products with a solvent system of butanol-acetic acid-water (3:1:1) by volume, the spots were visualized by spraying it with 20% Sulphuric acid in ethanol and baking it in an oven at 120 ºC for 30 min. The procedure of Aygan *et al.*, (2008) with slight modification was followed.

Results and Discussion

The pH and moisture content of the Chilika sediment was found to be 9.0 and 80% respectively which shows that the sample is alkaline. The parent isolate showed big,

white, oval shape colony with flat elevation, irregular margin and rough texture. It was gram negative and endospore positive. It was observed that parent strain isolate CHB7 showed a zone of clearance of 6mm on starch agar plate at pH 9.0 and 10.0 by hydrolyzing the starch, indicating the production of alkaline amylase by the isolate. The parent strain CHB7 was exposed to UV-radiation for various time lengths. Further the resistant were sub cultured followed by starch agar plate assay at pH 9.0. From table 1 it was evident that the parent strain was mutated after being exposed to UV radiation and showed good starch hydrolysis which was proved by the different zone of clearance shown by mutant strain. Among all mutants; MCH/20, MCH/40 and MCH/50 showed highest zone of clearance, which were 9, 9 and 8mm respectively.

From the qualitative estimation results obtained for amylase by all the bacterial isolates, it was obtained that mutant (MCH/40) showed highest enzymatic activity as shown in table 2. Therefore biochemical test and enzymatic assay were carried out taking parent (CHB7) and the mutant (MCH/40) strains (Table 3). From table 4, it was observed that sugar utilization results for both parent and mutant strains were the same. From the biochemical and sugar utilization tests the organism (Parent and mutant) was identified as *Bacillus* sp.

The standard curve of maltose and the protein concentrations are presented below in figures 1 and 2 respectively.

Table.1 Zone of clearance shown by mutant strains after treatment with U.V radiation

Sl.no.	Name of the isolate based on time of exposing U.V radiation (min)	Net zone of hydrolysis*
1.	MCH/5	5.0
2.	MCH/10	6.0
3.	MCH/15	6.0
4.	MCH/20	9.0
5.	MCH/25	5.0
6.	MCH/30	6.0
7.	MCH/35	7.0
8.	MCH/40	9.0
9.	MCH/45	5.0
10.	MCH/50	8.0
11.	MCH/55	5.0
12.	MCH/60	3.0

*Each value is the average of three replicates.

Table.2 Starch plate assay by parent and mutant strains at various pH

Isolates	Zone of clearance in (mm)*						
	pH6.0	pH7.0	pH 8.0	pH 9.0	pH 10.0	pH 11.0	pH 12.0
Parent	2.0	4.0	5.0	6.0	6.0	9.0	10.0
MCH/20	4.0	4.0	6.0	9.0	9.0	10.0	11.0
MCH/40	2.0	2.0	5.0	10.0	8.0	9.0	13.0
MCH/50	2.0	6.0	6.0	10.0	9.0	10.0	12.0

*****Each value is the average of three replicates.

Table.3 Biochemical tests for parent and mutant

Strains	In	MR	VP	Ci	NR	Es	Orn	Lys	Ur	Mani	Mo	TSI	Arg
Parent CHB7	–	+	–	–	+	+	–	–	+	+	–	+	+
MutantMCH/40	–	+	–	+	+	+	–	+	+	+	+	+	+

In- Indole test; MR- Methyl red test; VP- Voges Proskauer test; Ci- Citrate utilisation; NR- Nitrate reductase; Ur- urease test; Es- Esculin hydrolysis; Mo- Motility test; Man-manitol; TSI- triple sugar iron test; Arg- arginine; Orn- Ornithine; Lys- Lysine; (+): Positive; (-): Negative

Table.4 Sugar utilization tests

Strains	Glucose	Sucrose	Xylose	Mannose	Cellobiose	Salicin	Galactose	Inositol
Parent CHB7	+	+	–	–	–	–	–	–
Mutant MCH/40	+	+	–	–	–	–	–	–

Fig.1

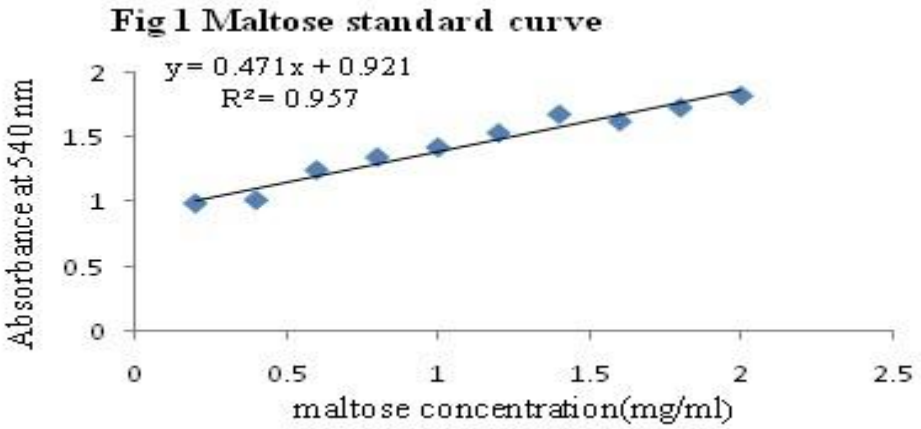


Fig.2

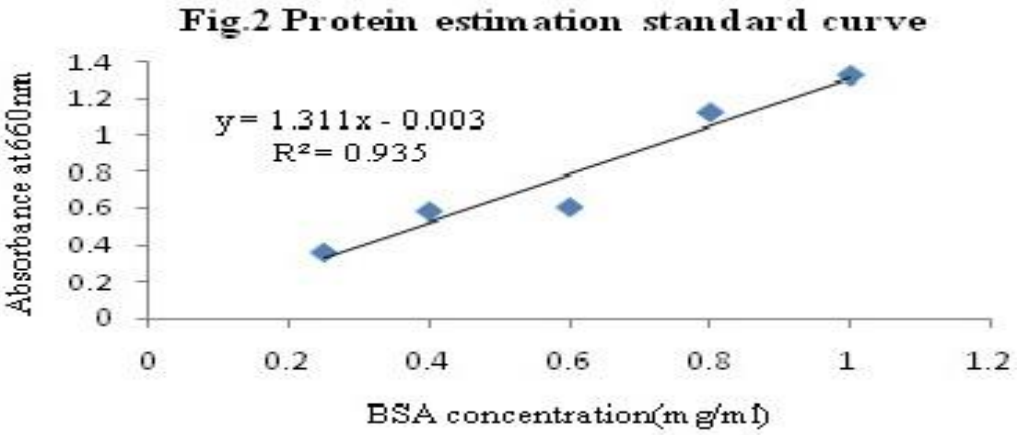
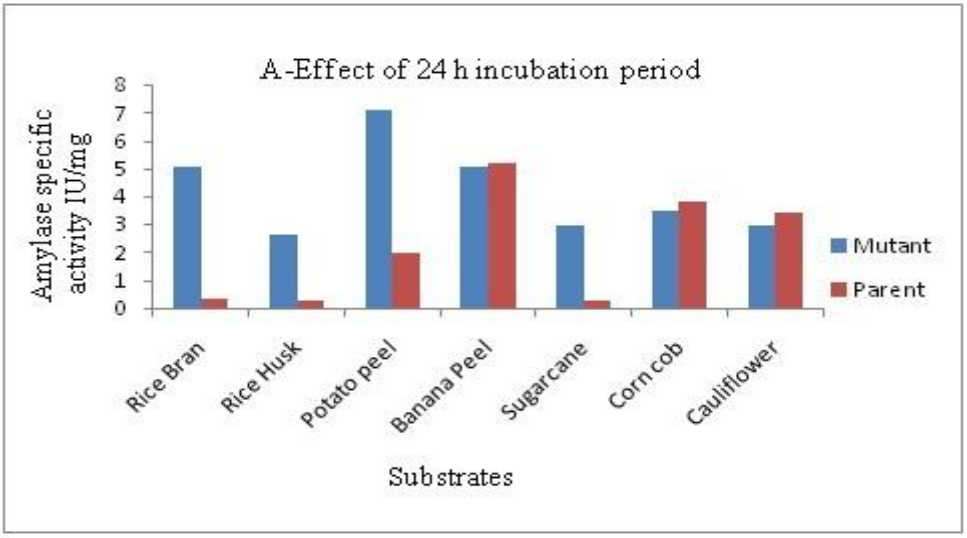
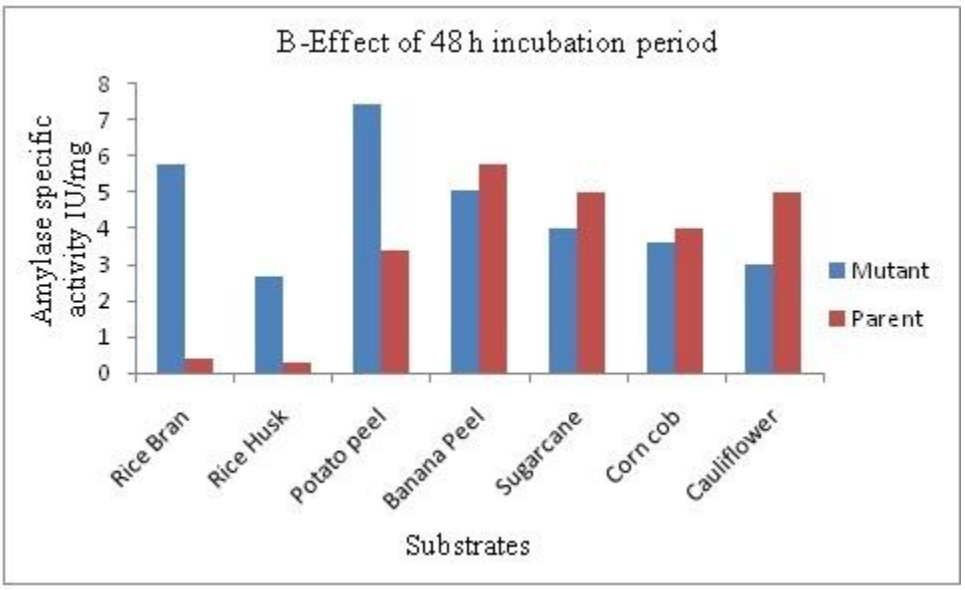
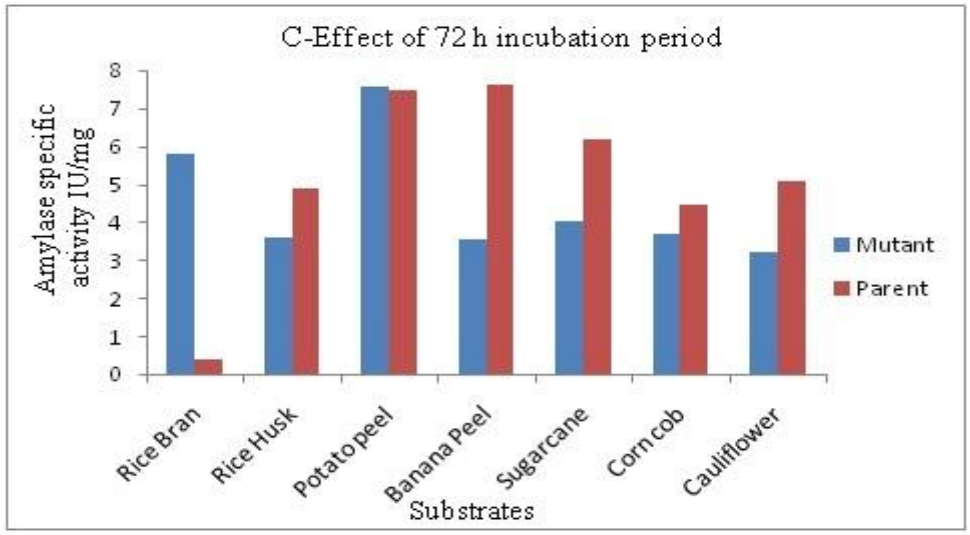
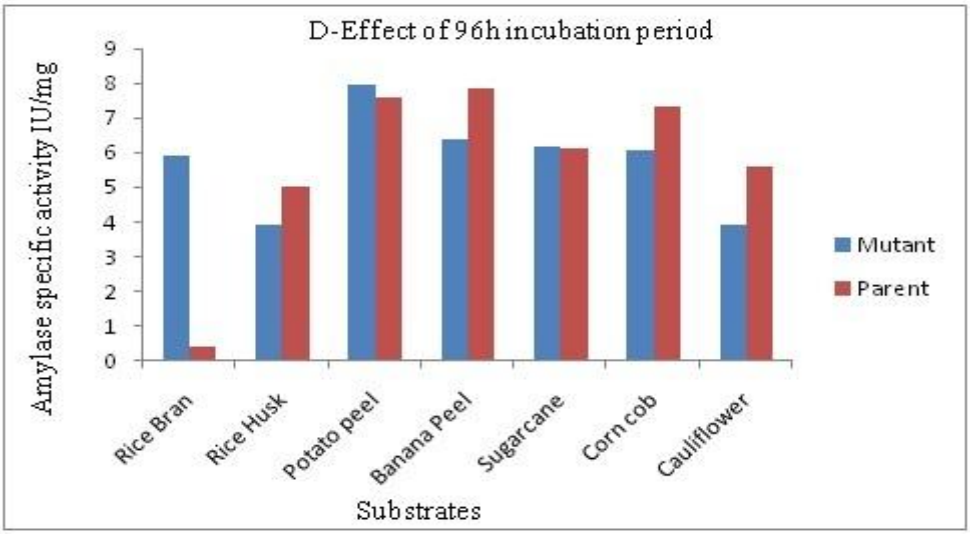


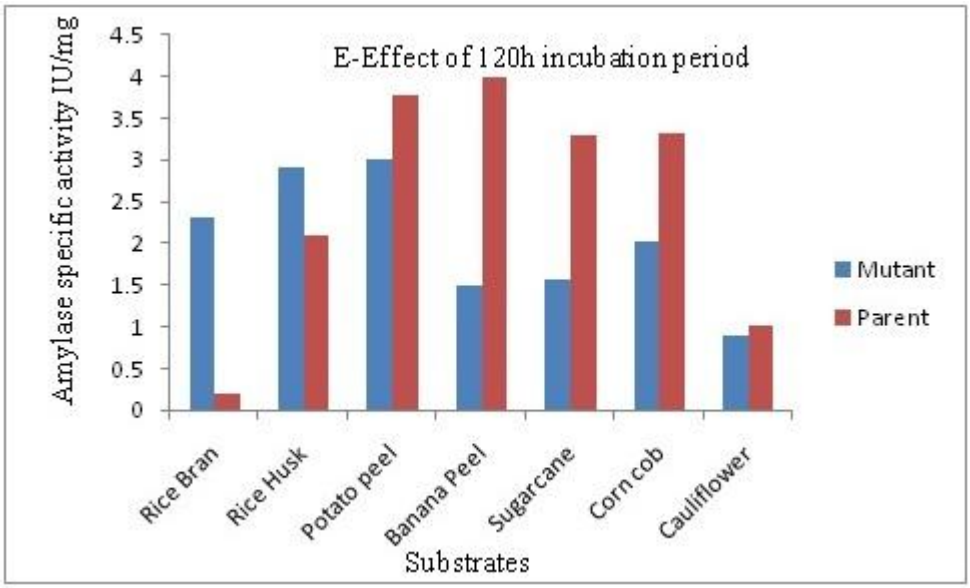
Fig.3 Effect of fermentation period on parent and mutant strains











Effect of incubation period on α -amylase production in solid state fermentation: A-24 h, B-48h, C-72h, D- 96 h and E- 120 h.

Fig.4 Effect of pH on parent and mutant strains

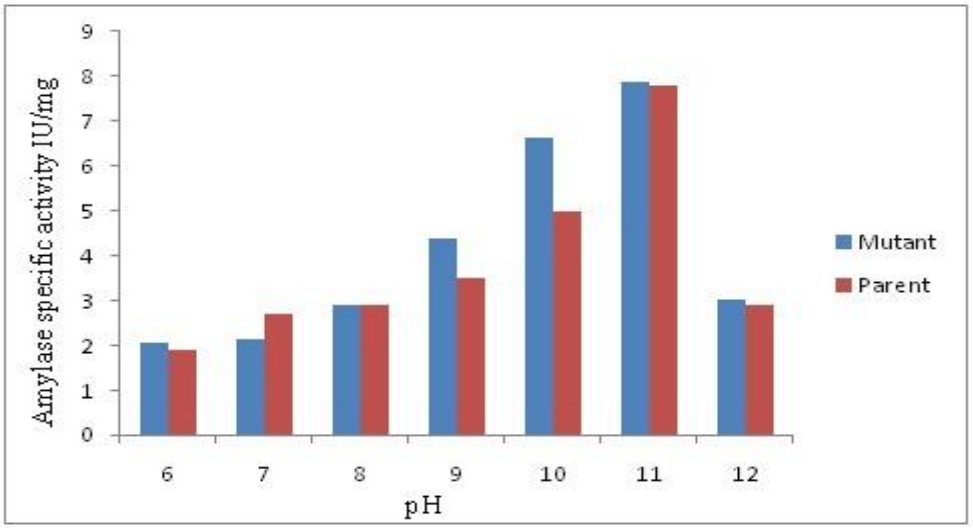


Fig.5 Effect of temperature on parent and mutant strains

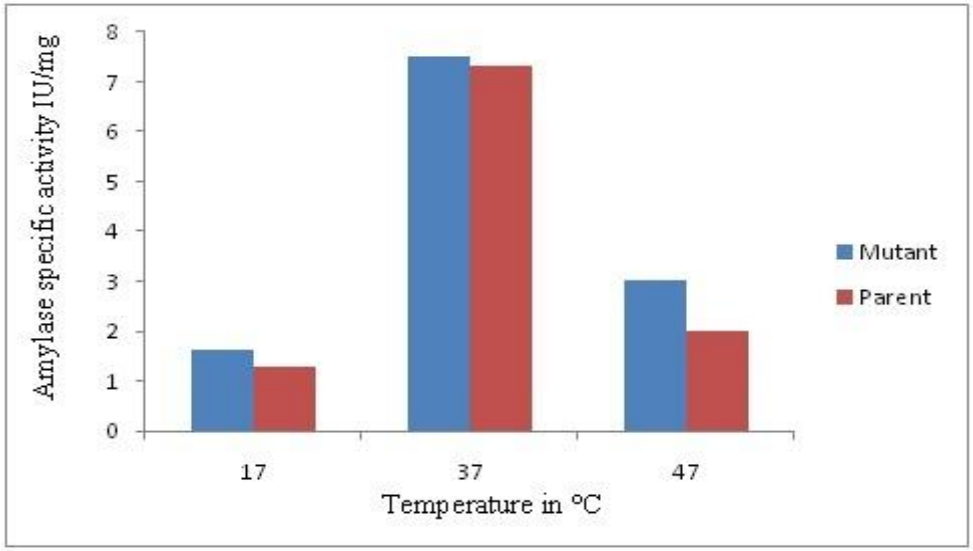


Fig.6 Effect of inoculum size on parent and mutant strains

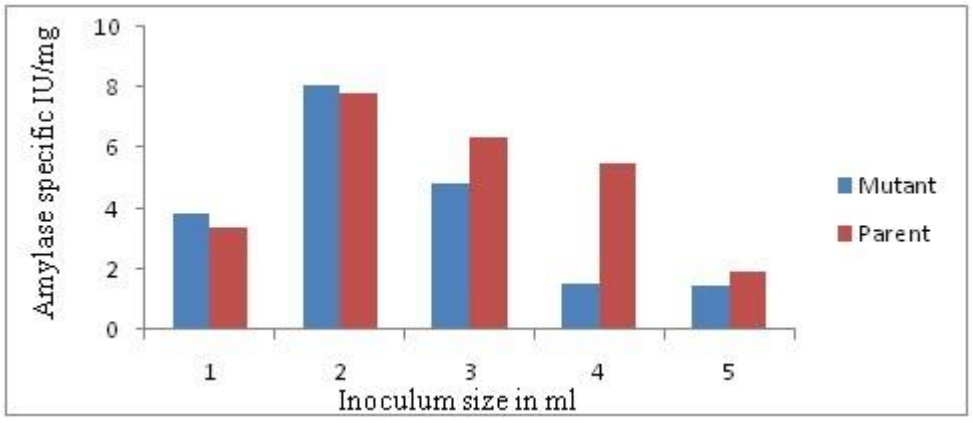


Fig.7 Effect of carbon sources (0.2%) on parent and mutant strains

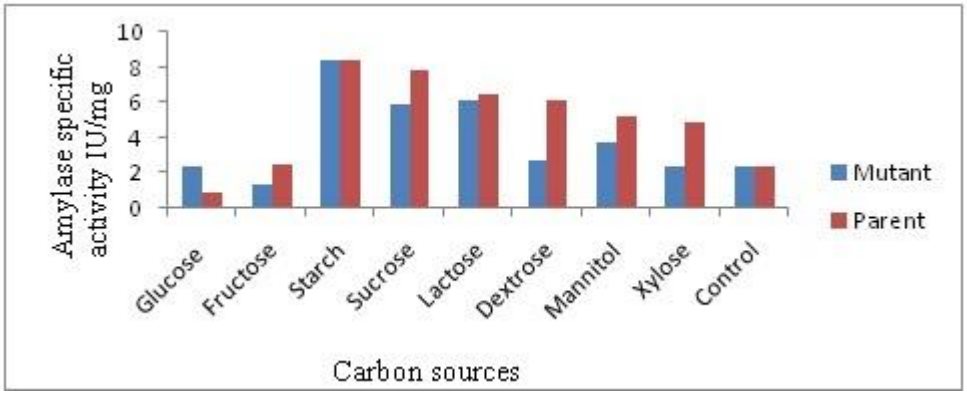


Fig.8 Effect of nitrogen sources (0.2%) on parent and mutant strains

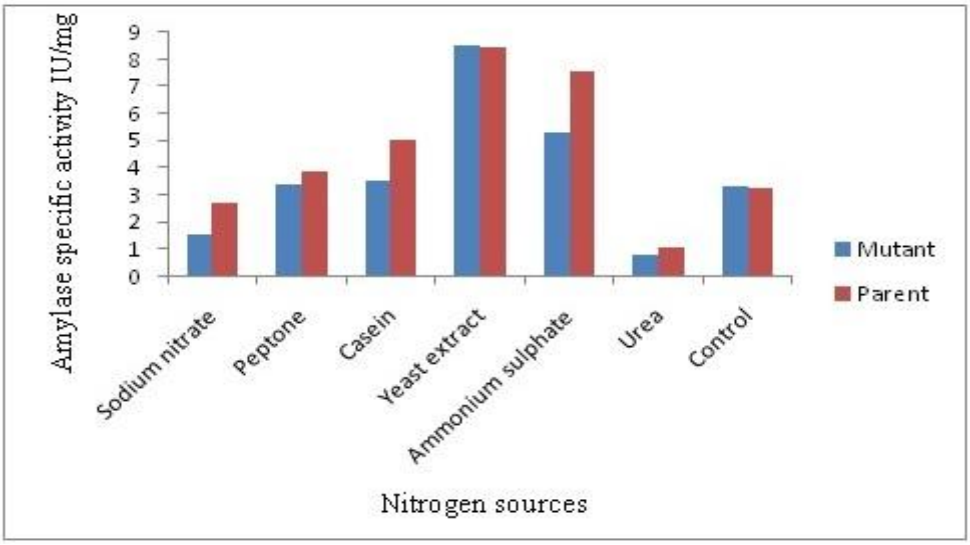


Fig.9 Effect of mineral chlorides (0.2%) on parent and mutant strains

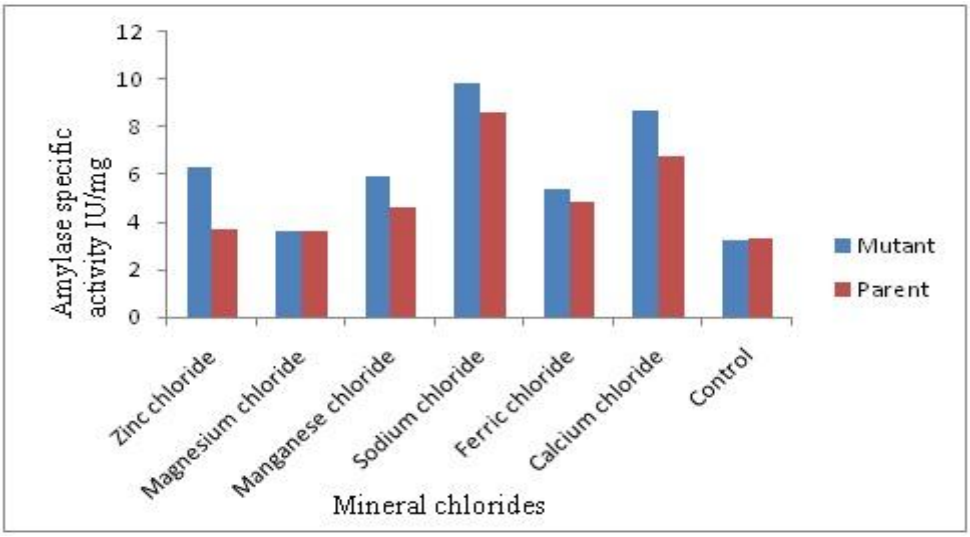
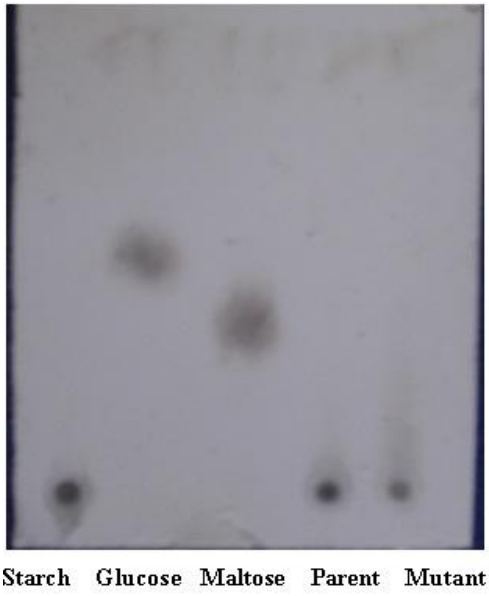


Fig.10 TLC analysis



Effect of various process parameters on amylase production

Enzyme production depends on many factors that are directly proportional to the growth of the microorganisms. The bacterial isolates: parent (CHB7) and mutant (MCH/40) strains were investigated for the higher production of alkaline amylase in varied incubation period which revealed that maximum production was shown at 96 h of incubation by CHB7 (7.87 IU/mg) and MCH/40 (7.93IU/mg) using banana peel and potato peel respectively at 37 °C (Fig. 3). After that there was a slight decrease of amylase production at 120 h which may be due to lack of nutrients and changes in medium pH. Maximum production of alkaline amylase was seen at pH 11.0 by CHB7 (7.78 IU/mg) and mutant MCH/40 (7.89 IU/mg) (Fig. 4).

The amylase production was maximum at 37 °C for CHB7 (7.33 IU/mg) and mutant MCH/40 (7.51) (Fig. 5). Maximum production of amylase at 37 °C has also been reported (Haq *et al.*, 2010). A concentration of 2ml showed highest production of alkaline amylase for CHB7 (7.79 IU/mg) and mutant MCH/40 (8.0 IU/mg) respectively (Fig. 6). A similar finding where 1.5 ml inoculum of bacterial culture produced maximum amount of α amylase has been reported (Haq *et al.*, 2012).

From the quantitative study (Fig. 7) it was observed that amylase production was more on starch than any other carbon sources which corroborates with the findings of Tsegaye and Gessesse (2014). CHB7 and MCH/40 showed higher production of amylase of 8.399 IU/mg and 8.372 IU/mg on starch respectively. From figure 8 it was observed that yeast extract showed the highest amylase production for CHB7 (8.44 IU/mg) and mutant MCH/40 (8.55 IU/mg) respectively as compared to the other nitrogen sources. Similar finding was also reported previously by Bhattacharya *et al.*, (2011).

In presence of NaCl, CHB7 (8.619 IU/mg) and MCH/40 (9.876 IU/mg) showed higher production of amylase (Fig. 9). Using thin-layer chromatographic analysis, it was ascertained that the amylase produced by our

bacterial cultures was α amylase owing to the presence of maltose and very low amounts of other oligosaccharides as the main end-products of starch hydrolysis (Fig. 10). The mutant produced higher alkaline amylase than the wild strain showing 1.245 fold increase as compared to wild strain showing an increase of 1.095 fold.

This research work contributes towards the development of an economical alkaline α amylase production process (solid state fermentation) using agro-industrial residue.

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