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PRODUCTION OF AMYLASE FROM INDIGENOUSLY ISOLATED STRAIN OF *Aureobasidium pullulans* AND ITS HYPER PRODUCING MUTANT

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ABSTRACT

New isolate obtained from leaf surfaces of *Spinacia olevacea, Brassica oleracea* and *Coriandrum sativum* was identified as *Aureobasidium pullulans* on the basis of cultural, morphological, biochemical and molecular characterization. The factors such as inoculums size, incubation time, sources of starch, and concentration of starch, sources of carbon and nitrogen, effect of initial pH and effect of temperature affecting production of amylase were optimized in present study. The optimum pH, temperature and incubation period for enzyme production were 5, 25^{O}C and 5 days respectively. Among different sources of starch tested potato starch was shown to be the best. Potato starch at 1% was recorded to be the best concentration of starch for enzyme production. Sodium nitrate was ideal nitrogen source. At optimum conditions *Aureobasidium pullulans* Cau 19 has shown amylase activity 800U/L, which is twofold higher than before optimization. For the improvement of *Aureobasidium pullulans* Cau 19, the parental strain, after optimization of the cultural conditions, was subjected to UV irradiation for 8 min. Total 286 mutants were checked, out of which 9 mutant strains showing comparatively greater hydrolysis zone were selected for further study. *Aureobasidium pullulans* UVm 276 mutant has shown amylase activity which is 2.78 fold that of the wild type strain. Thus these findings have more impact on enzyme economy for biotechnological applications of microbial amylases.

Keywords: *Aureobasidium pullulans*, Amylase, Optimization, UV irradiation

INTRODUCTION

Amylases are among the most important enzymes used in several industries such as paper industry, detergent industry, food and pharmaceutical industries in various processes such as starch liquefaction and saccharification, textile desizing, manufacture of high fructose containing syrups, treatment of starch processing waste water (**Gupta *et al.*, 2003**). The emergence of newer technology of immobilizing enzymes on metal nanoparticles would further enhance the applicability of enzymes like amylase. **(Li *et al.*, 2017; Ahmad and Sardar, 2015**). We have recently reported the preparation of bioconjugate using biologically synthesized gold nanoparticles and purified amylase from *Aureobasidium pullulans.* (**Mulay and Deopurkar, 2017)**.The amylases are produced by plants, animals, and microorganisms (**Pandey *et al.*, 2000**), largely microbial amylases are commercially available, due to ease of cultivation of microorganisms and processing to isolate and purify enzyme. Interest in the amylolytic yeasts has increased in recent years as their potential value for conversion of starchy biomass to single cell protein or ethanol (**Gupta *et al.*, 2003)**.. Also most of the yeasts from environment are safe and are conferred GRAS (generally regarded as safe) status. *Aureobasidium pullulans* is yeast like fungus that has been used for industrial production of wide variety of enzymes used in biotechnology particularly in process involving starch hydrolysis. The search for newer yeast is biotechnologically important for the development of efficient, economical and environmentally safe amylolytic hydrolysis of starch.
We report the isolation and characterization of amylase producing yeast from phyllosphere of different plants. These isolates were mutagenized to enhance the production of amylase.

MATERIALS AND METHODS

Collection of samples

Fifty leaves samples, each were collected from locally (Baramati a city in Pune district in the state of Maharashtra, India.) available plants viz. Spinach (*Spinacia olevacea*), Cauliflower (*Brassica oleracea*) and Coriander (*Coriandrum sativum*). Care was taken to protect the phyllosphere from external factors by collecting the leaf samples in presterilized polythene bags.

Isolation of *Aureobasidium pullulans*

Aureobasidium pullulans strains were isolated from leaves of Spinach, Cauliflower and Coriander using procedure described by Pollock *et al.* **(Pollock *et al.*, 1992)**. Sample leaves were washed gently with water, cut into small pieces. A gram of cut leaves were suspended in 10 mL of sterile distilled water and kept on shaking for 3 days at 28^{O}C on a rotary shaker at 120 rpm. one mL was inoculated in 100mL of enrichment medium, which contained (g/L) : 2.0g of yeast extract, 0.5g of $(NH_4)_2HPO_4$, 1.0g of NaCl, 0.2g of $MgSO_4.7H_2O$, 3.0g of K_2HPO_4, 0.01g each of $FeSO_4$, $MnSO_4$ and $ZnSO_4$ pH7, 20.0g Sucrose and 10 mg mL^{-1} Chloramphenicol. It was incubated for two days on rotary shaker at 25 ^{O}C. About 100 µL of liquid culture was spread on the agar medium of same composition. Plates were incubated at 25^{O}C for 3 days. Isolated colonies were subjected to cultural, morphological, biochemical and molecular characterization. Cultural characteristics of all *A. pullulans* strains were studied on Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) after incubation for 4 days.

Screening of *Aureobasidium pullulans* for amylase production

The amylolytic activity of isolated strains of *A. pullulans* was determined by spot inoculating 100 µL culture suspension (10 ^{7} cells/mL) on Czapek –Dox agar medium containing 1% of starch, and incubated for 4 days at 25^{O}C. After incubation, plates were flooded with iodine solution. The diameter of halo, formed after addition of iodine was measured indicating the amylolytic activity of the *A. pullulans* strains **(Kathiresan and Manivannan, 2006)**. Out of several isolates, *A. pullulans* Cau19 showing the highest amylolytic halo was selected for further studies.

Characterization of natural isolates of *Aureobasidium pullulans*

After careful microscopic examination of *A. pullulans* Cau19, culture was extensively characterized with respect to its ability to utilize spectrum of carbon sources, nitrogen sources and its ability to produce various enzyme such as cellulase, xylanase, gelatinase, protease and urease. Yeast nitrogen base agar and Yeast carbon base agar were used for incorporating carbon (1%) and Nitrogen (1%) compound respectively. The ability of *A. pullulans* Cau19 to produce various enzymes was checked using Yeast nitrogen base agar (YNBA) containing appropriate substrates (1% w/v) followed by specific detection procedure as described below.

Amylase: The culture plates were flooded with iodine solution to detect the zone of clearance due to amylase production.

Cellulase: Cellulose (1.5%w/v) was added to agar medium and cellulose was detected in terms of zone of clearance.

Xylanase: Congo red solution (0.5%) was added to reveal the zone of clearance due to xylan hydrolysis.

Gelatinase: Gelatinase production was indicated by clear zone around the growth by addition of 15% $HgCl_2$ in 20% HCl.

Protease: The culture plates were flooded with 10%HCl to reveal the zone of clearance due to protease production.

Urease: Ten mL of urea broth containing phenol red was dispensed into tubes and inoculated with one mL of inoculum, incubated at 28^{O}C. The tubes were examined every half an hour for a change of color to red.

Isolation and amplification of genomic DNA

Isolation and amplification of Genomic DNA was carried out using Prepman Ultra* sample preparation reagent (Applied Biosystems, Applera, USA). In brief, a single colony was added into 200µL of Prepman ultra* reagent. It was treated at 99^{O}C in thermal cycler for 20 min. Tube was vortexed for 10 seconds and centrifuged at 10,000 rpm for 10 min. Three µL of the supernatant was used as a template for PCR reaction. Genomic DNA was amplified by PCR (ABI 9700 geneamp PCR). Primers targeting the 18S rRNA gene were used for amplification. Amplification mixtures were submitted to 30 cycles of thermal cycling. PCR products were analyzed on 0 .8% agarose gel. Reaction was set with 2µL of purified PCR product using forward and reverse primer. The tubes were submitted to the thermal cycler (ABI 3170 PCR) and followed 25 cycles of reaction conditions, product was purified. To the reaction mixture, 2.5µL of 125 mM EDTA and 60µL of absolute ethanol were added to the reaction mixture. This preparation was incubated at room temperature for 15 min. and centrifuged at 10,000 rpm at 4^{O}C for 20 min. To the pellet, 60 µL of 70% ethanol was added and centrifuged at 10,000 rpm for 10 min. Ethanol was decanted and sample was air dried. Ten (10µL) of Hi-Di formamide loading buffer was added to each reaction tube containing single stranded PCR product of cycle sequencing reaction. After heat shock denaturation for 3 min at 95^{O}C and quick cooling on ice, 10µL of each reaction mixture was added in to an individual well and subjected to electrophoresis in automated DNA sequencer. The sequence thus generated through automated sequencing was used to search for homologous sequences in the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov), with the help of BLASTN (Basic Local Alignment Search Tool) database search tool. The results were expressed in percentage of homology between submitted sequence and the most relevant sequences from the data base.

Submerged fermentation for amylase production

A. pullulans Cau19 was inoculated in Sabouraud Dextrose broth and grown for 48 hour at 25^{O}C on rotary shaker (120 rpm). This culture was used as inoculum (1% V/V) after adjusting cell count to 10^7/mL in all production media containing 1% starch. Production cultures were incubated for 7 days at 25^{O}C on rotary shaker (120 rpm). The factors such as inoculums size, incubation time, sources of starch, and concentration of starch, sources of carbon and nitrogen, initial pH and temperature of incubation were optimized, in shake flask studies using 100 ml of medium in 500ml of flask.

Determination of amylase activity

Amylase was assayed by adding 0.1 mL of enzyme (crude extract/ fermented broth supernatant) in 0.9 mL of a 1% soluble starch in phosphate buffer (pH7, 0.1M) and incubated for 20 minutes at 37^{O}C. This reaction was stopped by adding 1mL of 3,5dinitorsalicylic acid followed by boiling for 10 min to develop red color. The final volume was made to 10ml with distilled water and absorbance measured at 540nm **(Miller, 1959).** One unit of amylase activity was defined as the amount of enzyme which liberated 1µmole of a reducing sugar per minute under the assay conditions. All the experiments were performed in triplicates.

Protein estimation

Proteins were estimated using the method of **Lowry *et al.* (1951)**. Bovine serum albumin was used as a standard (BSA).

Growth measurement

Growth was measured in terms of cell count and dry weight **(Marlida *et al.*, 2000)**.

Mutagenesis using UV

A. pullulans Cau19 was exposed to UV light for 8 min. Irradiated culture was inoculated into Sabouraud's Dextrose broth and incubated at 28^{O}C for 48 hours at 120 rpm. The culture was plated on starch agar to evaluate amylase production.

Product analysis of amylase of *Aureobasidium pullulans* Cau 19

The partially purified amylase was incubated at 37^{O}C with 1% soluble starch in 0.1M phosphate buffer (pH 7). Samples were removed after 30 min incubation and hydrolyzed products were analyzed by thin layer chromatography. 50 µL sample was loaded on pre coated silica gel plate. 1 % solution of Glucose and Maltose were used as standards. Substrate blank i.e. 1 % starch solution was also loaded on the same plate. The solvent system was isopropanol-acetic acid- water (6:3:1). The chromatogram was developed with aniline diphenylamine reagent prepared by mixing 20 mL of 85% phosphoric acid with a solution of 4 mL of aniline & 4 g of diphenylamine in 200 mL of acetone.

RESULTS AND DISCUSSION

Isolation, Screening and Identification of Amylolytic *Aureobasidium pullulans*

From 150 leaves samples, 15 different isolates of *A. pullulans* were isolated and screened for amylase production. The result for eight isolates of *A. pullulans* strains were as shown in Fig.1. The *A. pullulans* strain Cau14 (isolated from Cauliflower) and *A. pullulans* strain Cor1 (isolated from Coriander) revealed diameter of halos 2.5 mm and 4 mm respectively. Remaining six cultures showed zone of clearance more than 5 mm. Among these strains Cau19 displayed zone diameter of 10 mm while strain Spi10 showed zone diameter of 9 mm. These results clearly indicated that Cau14 and Cor1 were poor producers of amylase whereas Cau19, Cau11and Spi10 were better producer *Aureobasidium pullulans* Cau19.

Characterization with respect to Carbon, Nitrogen assimilation and production of different enzymes

Table 1 Carbon & Nitrogen assimilation by *Aureobasidium pullulans* Cau19

Carbon Assimilation	
Glucose	+
Fructose	+
Sucrose	+
Maltose	+
Lactose	+
Mannitol	+
Starch	+
Methanol	-
Nitrogen Assimilation	
Ammonium sulphate	+
Ammonium nitrate	+
Sodium nitrate	+
L –Asparagine	+
Peptone	+
Yeast extract	+
Enzyme Activity	
Gelatinase	-
Protease	+
Cellulase	-
Amylase	+
Urease	+
Xylanase	+

A. pullulans Cau19 strains was biochemically characterized with respect to its ability to assimilate different carbon and nitrogen sources and its ability to produce different enzymes. The results are given in table-1. It was found that this strain could not use methanol as a carbon source. The results are in agreement with the result of **Takahashi *et al.* (1981)**, who reported that methanol did not support rapid growth of *A. pullulans* strain14. Culture used monosaccharide like glucose, fructose, and disaccharide such as sucrose, maltose, lactose and alcoholic sugar mannitol for its growth. As previously illustrated the culture was selected on the basis of zone of clearance on starch agar plates, after the addition of iodine reagent. This is reflected in ability of this culture to use starch for its growth. Growth on carbon sources revealed the production of invertase, β galactosidase and glucoamylase by *A. pullulans* Cau19. Earlier report by **Chi *et al*. (2009)** and **Deshpande *et al.* (1992)** states that *A. pullulans* produced various enzymes including protease, amylase, lipase, cellulase and xylanase etc.

As far as nitrogen sources are concerned *A. pullulans* Cau19 could use ammonium nitrates, asparagine and peptone as nitrogen source for its growth, when each of this nitrogen source was included as sole nitrogen source in the medium.

The organism was further identified using 18S rDNA sequence methodology. Part of the 18S rDNA sequence was amplified and sequenced. The obtained sequence was compared with the sequence available in NCBI. Phylogenetic relationship of *A. pullulans* isolates (♦) from cauliflower and spinach with representative members is shown in Fig.2 .Isolates are submitted to Gene bank data base with accession number of *A. pullulans* strain Cau19 (JN807328) and *A. pullulans* strain Spi10 (JN807329). The bootstrap consensus tree was constructed by the Neighbour-Joining method using MEGA 5.05 software from the distance data generated by multiple alignments of the nucleotide sequences.

Effect of different cultural conditions on production of amylase:

Fig. 3 shows the time course of production of amylase and growth of *Aureobasidium pullulans* Cau19 in production medium at 25^{O}C, pH 5.5 under shaking condition (120 rpm) for 5 days. Amylase production by *A. pullulans* Cau19 was detected after 2 days of growth (0.136 U/mL) and showed steady rise till it reached a peak (0.456 U/mL) in 5 days of growth. After 5 days, there was a sharp decrease in enzyme production (0.106 and 0.033 U/mL on 6th and 7th days of incubation respectively). Fig.3. reveals that maximum amylase activity produced by *A. pullulans* Cau19 was at the end of its logarithmic growth. **Suganthi *et al*. (2011)**, reported that maximum amylase activity was produced after six days of incubation by *Aspergillus niger* BAN 3E. **Nahas *et al*. (2002)** reported that the mycelial growth on starch reached a maximum after five days and maximum amylase activity was produced after two days of cultivation. Very high activity in fermentation medium at the end of exponential phase of producing organism is not very rare phenomenon. There are such observations of maximum production after exponential growth e.g. **Clementi *et al*. (1980)** and **Sandhu *et al*. (1987)** reported that the yield of exo and endo amylases increases appreciably, in *Saccharomyces fibuligera* during growth and attains its maximum in stationary phase. It is very likely that the enzymes are growth associated but are in the form of wall associated proteins which may subsequently be released in the surroundings perhaps as a result of senescence related autolysis. The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on the growth rate and enzyme production **(Kunamneni, 2005)**.

Inoculum level reported to play critical role in submerged fermentation. In this investigation maximum amylase production was obtained with 1% of inoculum (10^{7} cells /mL). Further increased in inoculum level resulted in gradual decrease in enzyme production. This may be due to the limiting nutrients at higher inoculum size. At low level of inoculum the production of enzyme was insignificant. Thus 1% inoculum was selected for further study. (Data not shown).

The starch refers to very heterogeneous polysaccharide composition; the content of amylose, amylopectin, degree of branching, occurrence of nonsaccharide components all lead to natural heterogeneity in starches derived from different biological sources **(Aberle *et al*., 1994).** On this background it was of interest to see the growth and production of amylase by *A. pullulans* Cau19 grown on different starches. As seen from the Fig. 4. *A. pullulans* Cau19 produced amylase as high as 0.462 U/mL with specific activity of 5.5 U/mg and the growth of the culture was also maximum with potato starch. Contrary to this, tapioca starch resulted in poor growth and poor yield of amylase from *A. pullulans* Cau19. Interestingly the specific activities of amylase produced on potato starch as well as on tapioca starch were almost the same (5.5 and 5.6 U/mg). Both corn and wheat starch resulted in less yield of amylase (0.317 and 0.264 U/mL respectively) but specific activities were very high (about 7.0 U/mg). One wonders if high specific activity with corn and wheat starch reflects the more selective amylase synthesis; it is too preliminary to conclude at this stage. As with the heterogeneity of starches, there appears to be heterogeneity of amylase from different strains of *Aureobasidium* e.g. **Li *et al.* (2007)** reported best production of amylase by *Aureobasidium pullulans* N13d with potato starch while **Taniguich *et al.* (1982), Okolo *et al*. (1995)** report that potato starch is not easily hydrolyzed.

As can be seen from the Fig.5. the maximum growth (0.297 g/100mL) was obtained with 1% starch in the medium; it is quite understandable that at 0.5% starch the biomass was less. However as the starch concentration was further increased from 1% to 1.5, 2 and 2.5 % the biomass yield decreased to 0.123, 0.092 and 0.076 g/100mL respectively. Initial increase in the biomass yield (with starch concentration from 0.5 to 1) is obviously due to nutritional increment. However when starch concentration increased viscosity of the medium also increases hampering the O_2 transfer rate. Perhaps this could explain the decreased biomass as starch concentration reached up to 2.5%. Contrary to this, report by **Mishra and Behera (2008)** states that amylolytic activity and growth kinetics of *Bacillus* strain isolated from kitchen waste increases by increase in the starch concentration up to 2%.

Maximum yield of amylase was obtained when cells were grown in the medium containing sodium nitrate as a sole nitrogen source (0.846 U/mL), whereas amylase production was less in medium containing ammonium sulphate as a sole nitrogen source (0.185 U/mL). The amylolytic activity with sodium nitrate was 4.6 times higher as compared to that of the ammonium sulphate. Sodium nitrate proved to be good inorganic nitrogen source for the production of amylase by the *A. pullulans* Cau19 followed by asparagine (0.502 U/mL) and peptone (0.462 U/mL) (Fig.6.). **Avdiiuk and Varbanets (2008)** reported that the best nitrogen source for α amylase production by *Bacillus subtilis* 147 was sodium nitrate. Fig.6. reveals that the amylase yield by *A. pullulans* Cau19 was significantly low in the medium supplemented with ammonium sulphate, ammonium nitrate and urea (0.185, 0.264, 0.291 U/mL respectively). According to **Haq *et al*. (2002)** urea releases ammonium ions slowly, thus was not good source of nitrogen. This observation was attributed to the low urease activity of the organism. The inhibitory effects of some of the salts may be related to the pH changes associated with their use in the medium. **Swain *et al.* (2006)** reported to find suppressed α –amylase production by newly isolated *Bacillus subtilis w*hen 1% ammonium sulphate was used in the fermentation medium which is analogous with our findings. The biomass yield was high with sodium nitrate as sole nitrogen source (0.357 g/100mL) where as biomass yield was low with urea as a sole nitrogen source (0.044g/100 mL). Biomass yield with sodium nitrate was 10 times higher as compared to that of the urea. Specific activity of amylase produced in media with asparagine as sole nitrogen source was higher (35.93 U/mg) as compared to the specific activities obtained in media with sodium nitrate, urea, peptone, ammonium nitrate and ammonium sulphate.

Different carbon sources at 1% (w/v) concentrations were used to study their effect on growth and amylase production. Results are shown in Fig.7. Production media containing starch as a sole carbon source was the best for amylase production, maximum activity was 0.842 U/mL. Similar results were also been reported for the best amylase production in the presence of starch as carbon source at 37^{O}C for *Bacillus sp.***(Kar and Ghose, 2008)**. The amylolytic activity by *A. pullulans* Cau19 with starch as a carbon source was 9.15 times higher as compared to that of the maltose. Amylase production was minimum in media containing maltose as a carbon source with amylase activity, 0.092 U/mL. Decrease in amylase production might be due to inhibitory effect of maltose on amylase production. With glucose as a sole carbon source enzyme production was approximately 0.80 U/mL The ability to synthesize amylase even in presence of easily metabolisable monosaccharide (glucose and fructose) indicates the absence of catabolite repression in *A. pullulans* Cau19. This is parallel to the report for production of amylase from *Aspergillus oryzaein,* where catabolite repression was not observed in presence of glucose **(Yabuki *et al.*, 1977)**. Contrary to this, glucose caused only minimal level of amylase production by *Aspergillus oryzae* **(Ars and Baile, 1977).** From the Fig.7. it is cleared that, of the carbon sources tested, starch, glucose, fructose, mannitol, xylose and sucrose enhanced in the enzyme yield as compared to maltose and lactose. Lactose was the only carbon source that inhibited the growth as well as amylase production. The biomass yield was higher with fructose and xylose as sole carbon source (0.914 and 0.801 g/mL) respectively, where as biomass yield was minimum with lactose as a sole carbon source (0.223g/100mL). Biomass yield with fructose was 4.1 times higher as compared to that with the lactose. Specific activity of amylase produced in media with starch as a sole carbon source was higher (13.4 U/mg) as compared to the specific activity obtained in media with maltose (0.79 U/mg).

When the fermentation was carried out at different temperatures (20^{O}C to 45^{O}C), enzyme production was maximum at 25^{O}C (0.841 U/mL). Further increase in temperature resulted in decrease in production of amylase. Decrease in amylase production may be due to denaturation of enzyme above 45^{O}C. *A. pullulans* Cau19 produced low level of amylase at 20^{O}C (0.343 U/mL). The influence of temperature on amylase production is related to the growth of the organism. It was observed that, biomass dry weight was increased from 20^{O}C to 25^{O}C ie 0.256 to 0.489 g/100mL, but further increase in temperature showed decrease in biomass dry weight. The optimal temperature for amylase production was reported at 30^{O}C for *Streptomyces albbidoflavus* and *Streptomyces tendae* TK-VL-333 **(Narayana and Vijayalakshmi , 2008).** Amylase activity increased progressively with increasing temperature from 20^{O}C, reaching maximum at 60^{O}C, in *Aspergillus niger*. **(Omemu *et al.*, 2005)**, whereas the optimum was found to be 35^{O}C for *Aspergillus falvus var. columnaris* **(Ellaiah *et al***., **2002)**.

Since enzymes are very sensitive to pH, determination of the optimal pH is essential to the production of amylase. In the present study, the effect of pH on the production of enzyme was thus studied by carrying out fermentation over range of pH (2-11). It is evident from the Fig.9. that amylase production by *A. pullulans* Cau19 increased with increase in pH from 3.5 to 5, followed by gradual decrease thereafter. The amylase activity was highest at pH 5 (0.814U/mL).This result is parallel to the findings of **El-Safey and Ammar (2002),** who also reported that fungal cultures give optimum enzyme production at pH 5 using various substrate. The biomass yield was maximum at pH 5.0 (0.557g/100mL) which is disparate to the findings of **Olama and Sabry (1989),** where the amylase activity and the biomass yield was maximum at pH 7.0 in *Aspergillus flavus* and *Penicillum purpurescence*. The culture grown in medium of initial pH 6.5, 8, 9.5 showed enzyme yields of 0.456, 0.353, 0.263 U/mL and biomass dry weight 0.460, 0.314, 0.057 g/100mL respectively. Thus high alkalinity of medium declined the amylase production. This might be due to inhibitory effect of alkaline pH on the growth of *A. pullulans* Cau19 as well as on enzyme production. However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 **(Yabuki *et al*., 1977)**. *A. pullulans* Cau19 did not produce significant amylase at initial pH 2, and very less amylase was produced at pH 11 (0.053 U/mL). It must however be pointed out that *A. pullulans* Cau19 produced amylase over a wide pH range (3.5 to 9.5). Specific activities of amylase produced in media of initial pH 5.0, 6.5, 8, and 9.5 were higher (11.72, 7.4, 10.26, and 5.3U/mg respectively) as compared to the specific activities obtained in media with initial pH 2 (0.07 U/mg protein) and pH 3 (3.03 U/mg protein) pH 11 (1.6 U/mg).

Analysis of amylase produced by *Aureobasidium pullulans* Cau 19:

Fig.10. shows the result of thin layer chromatographic separation of hydrolytic products using crude amylase of *A. pullulans* Cau19. As can be seen glucose was the only end product detected while maltose was completely absent in the starch hydrolytic product here. It is therefore suggestive that *A. pullulans* Cau19 amylase is predominantly glucoamylase. This result suggest that amylase produced by *A. pullulans* Cau19 can act on both α -1,4 and α -1,6 glycosidic linkages in the starch molecule. Our results are concordant with results of **Li *et al*. (2007b)** for glucoamylase by marine derived *Aureobasidium pullulans* N13d. Absence of maltose is the major advantage in the industrial production of glucose syrup.

Mutagenesis using UV:

Mutagenesis has been the classical approach for strain improvement. *A. pullulans* Cau19 was exposed to UV for 8 min. (corresponding to 90% lethal dose as previously determined) and then plated on starch agar plate to screen for amylase activities using iodine as detection agent. More precisely mutants selected on the basis of wider zone of starch clearance on starch agar plate were selected. The production of amylase by these mutant cultures was investigated after growing culture in liquid medium as described earlier. Fig.11. shows that UV 275 and UV276 mutants produced almost 3 folds more amylase as compared to that produced by wild type. Six mutant strains of *A. pullulans* cau19 (UVm259, UVm270, UVm274, UVm275, UVm276 and UVm281) produced amylase greater than two fold of amylase by wild type strain. This enhancement may be due to possible changes in the promoter zone of the gene coding for amylase due to ultraviolet exposure. The radiation might have deregulated the transcription of the mRNA corresponding to an increased production. Since ultraviolet radiation affects mainly the hydrogen bonds of pyrimidine bases (cytosine + thymine) the most vulnerable regulatory sequence must have been those containing the highest concentration of C+T **(Nicolas Santiago *et al*., 2006)**. It can be assumed that amylase production might be under the control of such regulon. It should be pointed out that Yoneda and Maruo reported generation of hyper producing strains with double or triple α amylase synthesis by *Bacillus subtilis*. **(Yoneda and Maruo, 1975).** On this background *A. pullulans* Cau19-276 with triple production of amylase appears to be significant strain improvement, more so when this mutant strain is stable.

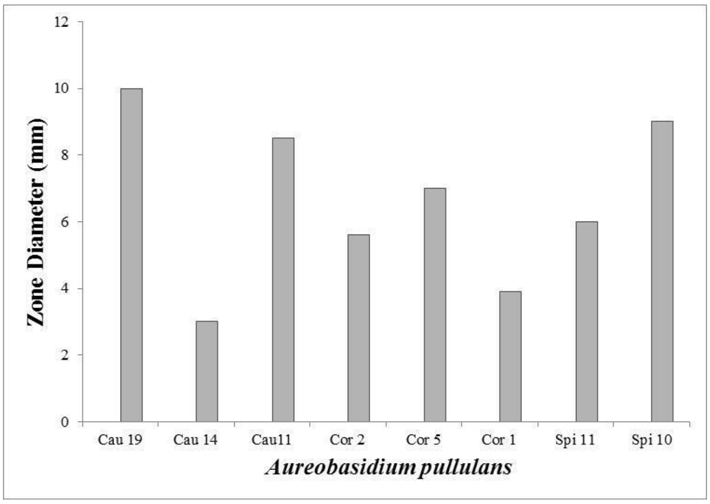

Fig. 1 The amylolytic activity by different strains of *Aureobasidium pullulans*. The amylolytic activity of isolated strains of *A. pullulans* was determined by inoculating (100 µl) an equal number of cells of each isolates (10^{7} cells/ml) on Czepex –dox agar medium containing 1% of starch. The agar plates were incubated for 4 days at 25^{O}C· The diameter of halo formed after addition of Iodine was measured and represented the amylolytic activity of the *A. pullulans* strains.

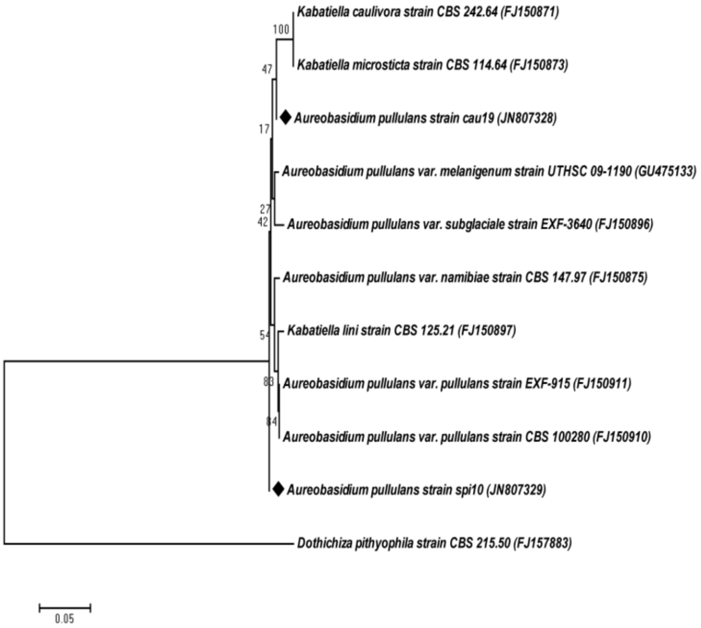

Fig. 2 Phylogenetic tree showing the placement of *Aureobasidium pullulans* and some related species based on analysis of the 18s rRNA gene. Sequences not generated during this study were obtained from GenBank; accession numbers are shown in parentheses. The tree was constructed by neighbor –joining analysis of aligned sequences. *Dothichiza pithyophila* was used as an out group. Numbers at nodes indicate percentages of bootstrap sampling from 1000 replication.

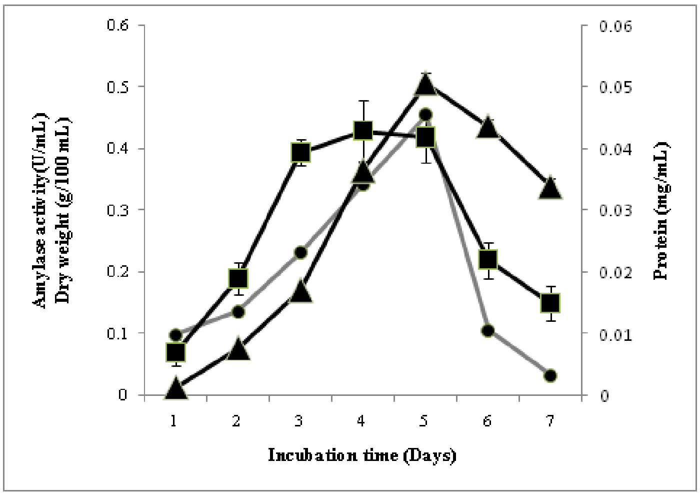

Fig. 3 Time course of amylase production by *Aureobasidium pullulans* Cau 19 cultivated in production medium (0.1% yeast extract, 0.5% peptone, 1% starch, 0.05%$MgSO_4$, 0.5%KH_2PO_4, 0.15%NaCl, 0.00125% $CaCl_2$) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days.(■) amylase activity,(●)biomass dry weight and (▲) protein . Each data point represents mean of three replications and bars extending from means represent standard errors of that mean.

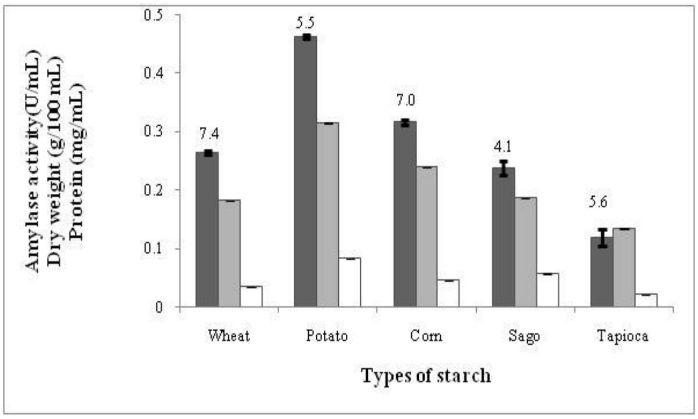

Fig. 4 Effect of different types of starch on amylase production by *A. pullulans* Cau19. Cells were grown in production medium (0.1% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$ in addition to 1% starch source) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days. (■) amylase activity, (■) biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Numbers above the columns represent the specific activity of amylase.

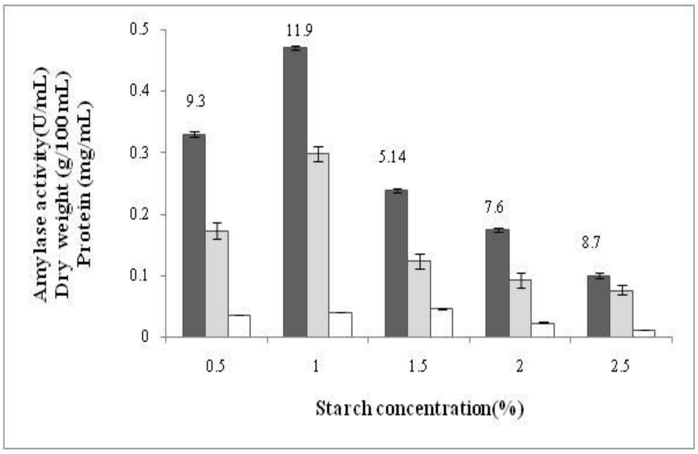

Fig. 5 Effect of starch concentration of on amylase production by *A. pullulans* Cau19. Cells were grown in medium (0.1% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$ and indicated starch concentration) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days. (■) amylase activity, (■) biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Numbers above the columns represent the specific activity of amylase.

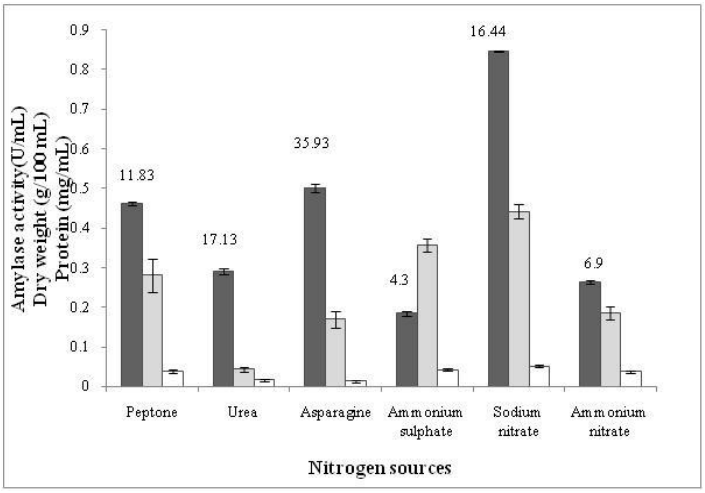

Fig. 6 Effect of nitrogen sources on amylase production by *A. pullulans* Cau19.
Cells were grown in production medium (0.1% yeast extract, 1% starch, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$ in addition to 0.05% weight of nitrogen from different nitrogen source) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days.(■) amylase activity, (□) biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Numbers above the columns represent the specific activity of amylase.

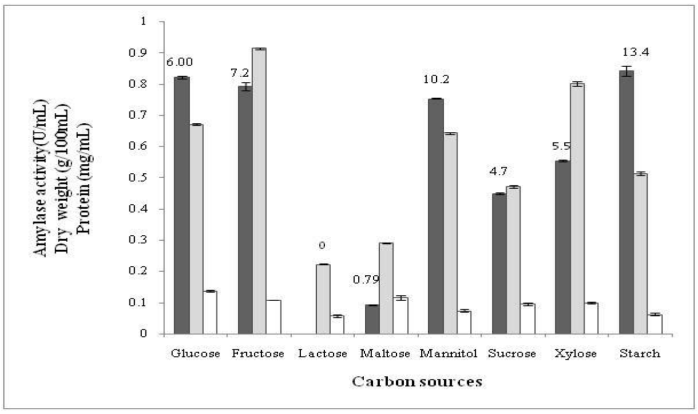

Fig. 7 Effect of carbon sources on production of amylase by *A. pullulans* Cau19.
Cells were grown in medium (0.1% yeast extract, 0.5% peptone, 0.05 % $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$ and 1% indicated carbon sources) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days. (■) amylase activity, (□) biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Number above the columns represent the specific activity of amylase.

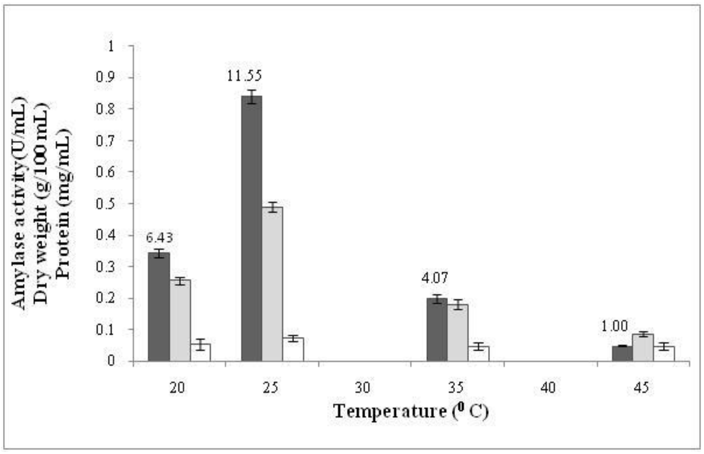

Fig. 8 Effect of temperature on amylase production by *A. pullulans* Cau19.
Cells were grown in production medium (0.1% yeast extract, 0.5% peptone, 1% starch, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days (■) amylase activity, (□)biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Numbers above the columns represent the specific activity of amylase.

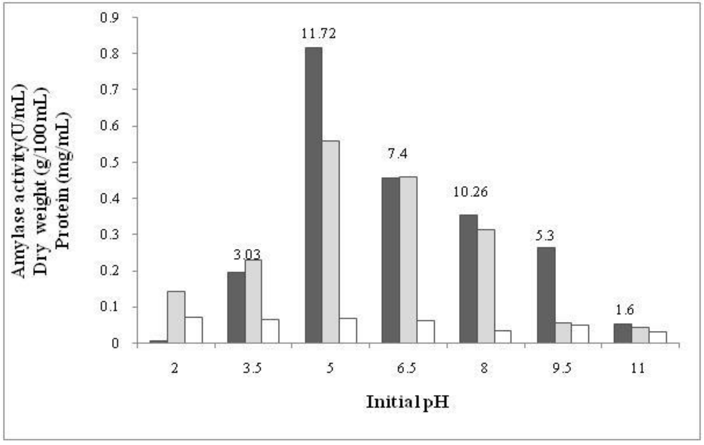

Fig. 9 Effect of pH on amylase production by *A. pullulans* Cau19.
Cells were grown in medium (0.1% yeast extract, 0.5% peptone, 1% starch, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$) at 25^{O}C, under shaking condition (120 rpm) for 5 days. (■) amylase activity, (□) biomass dry weight and (□) protein. Each data point represents mean of three replications and bars extending from means represent standard errors of that mean. Numbers above the columns represent the specific activity of amylase.

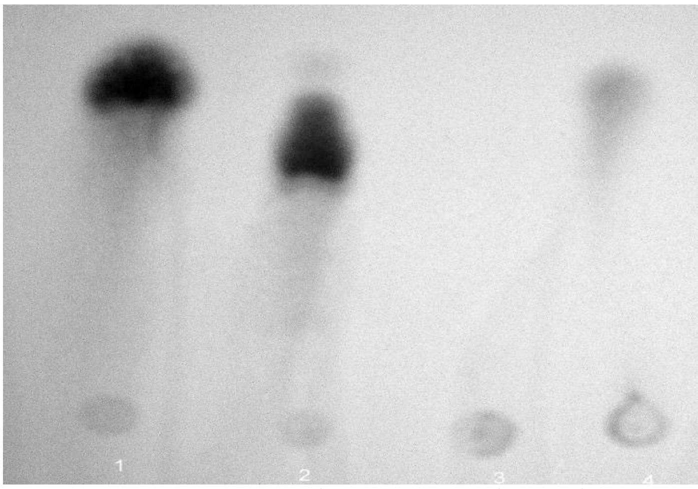

Fig. 10 Thin layer chromatogram of the end products of starch after hydrolysis with the crude amylase from *A. pullulans* Cau19.

Amylase was incubated with 1% soluble starch in 0.1 M acetate buffer (pH 5.6) for 30 minutes at 37^{O}C. Lane 1: hydrolyzed sample; Lane 2: maltose; lane 3: substrate control (1% potato starch solution); Lane 4: glucose. The end product of starch hydrolysis was analyzed by using TLC plate (Silica gel 60 , MERCK Germany) with the solvent system isopropanol –acetic acid –water (6:3:1) and detection reagent containing 20 g/L diphenylamine in acetone, 850 g/L phosphoric acid (5:5:1by volume).

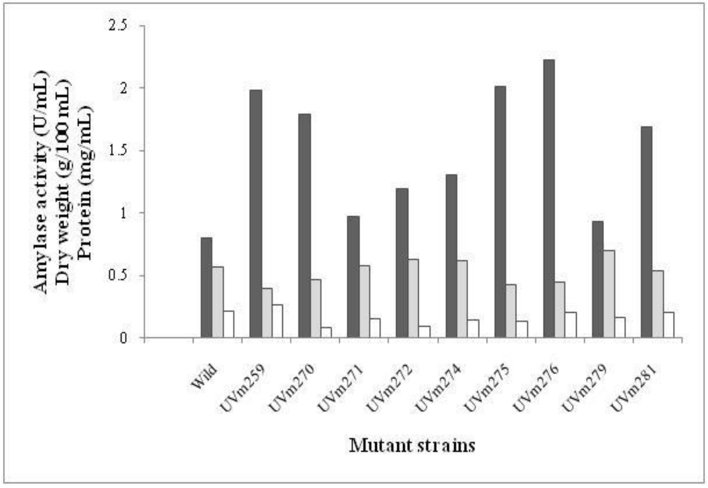

Fig. 11 Amylase production by selected mutant of *A. pullulans* Cau19.

Cells were cultivated in production medium (0.1% yeast extract, 0.5% peptone, 1% starch, 0.05% $MgSO_4$, 0.5% KH_2PO_4, 0.15% NaCl, 0.00125% $CaCl_2$) at 25^{O}C, pH-5.5, under shaking condition (120 rpm) for 5 days. (■) amylase activity, (▨) biomass dry weight and (□) protein. Each data point represents mean of three replications .

CONCLUSION

Among the ten tested *A. pullulans* strains, *A. pullulans* Cau19 had the highest amylase activity. Isolate *A. pullulans* Cau19 was identified by morphological, biochemical, and molecular analysis. The culture conditions were optimized for amylase production. At optimum conditions *A. pullulans* Cau19 has shown amylase activity 800 U/mL, which is twofold higher than before optimization. For further improvement of parental strain mutagenesis was carried with U.V. irradiation. *A. pullulans* Cau19-UVm276 mutants has shown amylase activity which is approximately 3 fold greater than that of wild strain.

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