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 oleracea, 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°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 biocatalysis 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 oleracea), 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°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 (NH₄)₂HPO₄, 1.0g of NaCl, 0.2g of MgSO₄.7H₂O, 3.0g of KH₂PO₄, 0.01g each of FeSO₄, MnSO₄ and ZnSO₄ pH7. 20.0g Sucrose and 10 mg mL⁻¹ Chloramphenicol. It was incubated for two days on rotary shaker at 25°C. About 100 µL of liquid culture was spread on the agar medium of same composition. Plates were incubated at 25°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°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 (% 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; 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°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 Prepmen Ultra™ sample preparation reagent (Applied Biosystems, Applera, USA). In brief, a single colony was added into 200µL of Prepmen Ultra™ reagent. It was treated at 99°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°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°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°C on rotary shaker (120 rpm). This culture was used as inoculum (1% V/V) after adjusting cell count to 10/mL in all production media containing 1% starch. Production cultures were incubated for 7 days at 25°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°C. This reaction was stopped by adding 1mL of 5,5dinitrosalic 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 (Marilida 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°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°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, Cau1 and 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

<table>
<thead>
<tr>
<th>Carbon Assimilation</th>
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<tbody>
<tr>
<td>Glucose +</td>
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<tr>
<td>Fructose +</td>
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<tr>
<td>Sucrose +</td>
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<td>Maltose +</td>
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<tr>
<td>Lactose +</td>
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<tr>
<td>Mannitol +</td>
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<td>Starch +</td>
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<td>Methanol -</td>
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<tr>
<th>Nitrogen Assimilation</th>
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<tbody>
<tr>
<td>Ammonium sulphate +</td>
</tr>
<tr>
<td>Ammonium nitrate +</td>
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<tr>
<td>Sodium nitrate +</td>
</tr>
<tr>
<td>L–Asparagine +</td>
</tr>
<tr>
<td>Peptone +</td>
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<td>Yeast extract +</td>
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<tr>
<th>Enzyme Activity</th>
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<tr>
<td>Gelatinase -</td>
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<tr>
<td>Protease +</td>
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<tr>
<td>Cellulase -</td>
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<td>Xylanase +</td>
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**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 reports by Chi et al. (2007), Le Lannou et al. (1992) and Deshpande et al. (1992) revealed that amylase production by higher as compared to that of the ammonium sulphate. Sodium nitrate eased due to nutritional increment. Whereas the optimum was (1980) O

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°C, pH 5.5 under shaking condition (120 rpm) for 5 days. Amylase production by A. pullulans Cau19 was observed after two days of cultivation. The yield of amylase activity produced by A. pullulans Cau19 was at the end of its logarithmic growth. (1992) reported that maximum amylase activity produced by A. pullulans Cau19 was after six days of incubation by Aspergillus niger BAN 3E. (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. (1980) and Sandhu et al. (1987) that the yield of exo and endo amylases increases appreciably, in Saccaromyces fibuligera during growth and attains its maximum in stationary phase. It is very likely that the enzymes are grown associated but are in 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 (10² cells/mL). Further increase 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, amylpectin, 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 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 amylosylase activity; 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 than Taniguchi 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 of 1%) is attributed to growth of mycelia. However when starch concentration increased viscosity of the medium also increased hampering the O₂ 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%.

When each of this nitrogen source was included as sole nitrogen source in stationary phase. In the present investigation, maximum production after exponential growth e.g. (2006) reported that the mycelial growth of A. pullulans Cau19 followed by asparagine (0.502 U/mL) and peptone (0.462 U/mL) (Fig.6.). Abdul and Varbanets (2008) reported that the best nitrogen source for a amylase production by Bacillus subtilis 147 was sodium nitrate. Fig.6. reveals that the amylase yield by A. pullulans Cau19 was significantly low when ammonium sulphate was used as sole nitrogen source (0.185 U/mL). As seen from the Fig. 4. amylose production by A. pullulans Cau19 in production medium at 25°C, pH 5.5 under shaking condition (120 rpm) for 5 days. Amylase production by A. pullulans Cau19 was observed 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) that the yield of exo and endo amylases increases appreciably, in Saccaromyces fibuligera during growth and attains its maximum in stationary phase. It is very likely that the enzymes are grown associated but are in 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)

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The biomass yield was maximum at pH 7.0 in Aspergillus flavus and Penicillium purpureascence. 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 5.3 U/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 Aspergillus pullulans. 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 N134. 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 a 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.

![Image](image1.png)

**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 (10^5 cells/ml) on Czapek –dox agar medium containing 1% of starch. The agar plates were incubated for 4 days at 25°C. The diameter of halo formed after addition of iodine was measured and represented the amylolytic activity of the A. pullulans strains.

![Image](image2.png)

**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.

![Image](image3.png)

**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%MgSO4, 0.5%KH2PO4, 0.15%NaCl, 0.00125% CaCl2 ) at 25°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% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂ in addition to 1% starch source) at 25°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 on amylase production by *A. pullulans* Cau19. Cells were grown in medium (0.1% yeast extract, 0.5% peptone, 0.05% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂ and indicated starch concentration) at 25°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% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂ and indicated nitrogen source) at 25°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% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂ and 1% indicated carbon sources) at 25°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. 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% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂) at 25°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% MgSO₄, 0.5% KH₂PO₄, 0.15% NaCl, 0.00125% CaCl₂) at 25°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°C. Lane 1: hydrolyzed sample; Lane 2: maltose; lane 3: 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 acetic, 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% MgSO4, 0.5% KH2PO4, 0.15% NaCl, 0.00125% CaCl2) at 25°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 out with UV irradiation. A. pullulans Cau19-UVM0276 mutants has shown amylase activity which is approximately 3 fold greater than that of wild strain.

REFERENCES


