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ORIGINAL ARTICLE

Partial purification and characterization of amylase enzyme under solid state fermentation from *Monascus sanguineus*

Padmavathi Tallapragada *, **Rashmi Dikshit, Anusha Jadhav, Umme Sarah**

Department of Microbiology, Centre of PG Studies, Jain University, 18/3, 9th Main, Jayanagar 3rd Block, Bangalore, India

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Abstract Amylase is an important enzyme having a varied range of industrial applications from food to cosmetics, from pharmaceutical to detergent industry, etc. The present study was carried out considering these important applications of amylase enzyme. *Monascus sanguineus* also has not been explored for its efficiency to produce amylase enzymes under solid state fermentation.

In the present study, various substrates were screened and among them beetroot as a solid substrate has given maximum yield (0.029 U/mL). Enzyme activity was further optimized by response surface methodology (RSM) and maximum experimental yield of 0.014 U/mL was obtained at optimized conditions of pH 5, incubation temperature of 50 °C and 10 min incubation time. A MATLAB software package was used for the graphical and regression analysis of the experimented data. Enzyme kinetics was calculated with different concentrations of starch and observed K_m value was 0.055 mM from linear regression analysis. The enzyme was moderately inhibited (44.7%) by NaCl and KCl (0.105 U/mL) with minimum inhibition (14.8%) observed with SDS. Molecular weight calculation and amylase confirmation in protein sample was done by SDS–PAGE and Zymography. Calculated molecular weight was 56 kDa. Alkaline amylase produced by *M. sanguineus* has exhibited high efficiency towards removal of stains on cloths in combination with commercial detergent (Surf excel) at 20 °C.

It can be concluded that the fungus *M. sanguineus* is a good source of amylase production under solid state fermentation. Application of amylase produced by *M. sanguineus* in detergent industry was also carried out and it was proven very effective in stain removal from the fabrics.

1. Introduction

α-amylases (E.C.3.2.1.1) are enzymes known to catalyse the hydrolysis of internal α-1,4-glycosidic linkages in starch into smaller moiety, such as glucose, maltose, etc. Amylases play a significance role in biotechnology which constitutes a varied

* Corresponding author.
E-mail addresses: vam2010tpraviju@gmail.com, t.padmavathi@jainuniversity.ac.in (P. Tallapragada).

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range of industrial applications such as pharmaceutical, food, paper industry, cosmetics, detergent, etc. sharing approximately 25% of the total world enzyme market. Plants, animals and microorganism are major sources to obtain α-amylases enzyme [4].

Monascus is a homothallic fungus classified into class *Ascomycetes* and family Monascaceae. *Monascus* spp. are well known producers of extracellular enzymes such as amylase, glucoamylase, β-glicosidase and various potent enzyme inhibitors. These bioactive compounds have major importance in food and pharmaceutical industries. In the last few decades, there had been an escalating trend for the utilization of agro-wastes as carbon source under solid state fermentation (SSF) to produce varied range of enzymes and other beneficiary secondary metabolites from micro-organism [15]. Filamentous fungi show hyphal mode of growth pattern and is capable of surviving under water deficient and higher osmotic pressure conditions. These factors make it an efficient bio converser of solid substrates in natural micro flora. Various industries such as food, beverage and other agro industries generate huge amount of waste residues which are difficult to dispose off. These wastes instead can be utilized as nutrient source for the growth and production of various metabolites from microbial sources [14]. The biosynthesis of amylase enzyme has been carried out under submerged fermentation, but recent studies had proven that solid state fermentation technique could be an efficient approach to produce and optimize the yield from microbial sources. For developing a fermentation process for amylase production, certain factors such as pH stability, thermo stability, Ca^{++} ion independency need to be taken care as these are key factors for the biosynthesis as well as the yield [4].

Response surface methodology (RSM) is a rapid and reliable technique which is a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables [9].

The aim of the present work was to study the production of amylase enzyme under solid state fermentation from *Monascus sanguineus* and statistically optimize the enzymatic activity. Characterization, kinetics and its application was also studied.

2. Material and methods

2.1. Isolated culture

Monascus strain was isolated from pomegranate (*Punica granatum*). The strain was identified as *M. sanguineus* and maintained on Potato Dextrose Agar (PDA) medium [6].

2.2. Inoculum preparation

Properly grown culture on PDA media was scrapped off and diluted in distilled water in order to make spore suspension. Spore suspension was used as inoculum.

2.3. Cultural conditions of amylase production under solid state fermentation (SSF)

For solid-state fermentation, five substrates were chosen viz. orange peel, beet root peel, onion peel, groundnut oil cake, and coconut oil cake. These were obtained from a local market of Chamarajpet, Bangalore, India, and were used as the basic solid substrate for enzyme production under solid-state fermentation. These substrates were dried and crushed into fine powder. Five gram of substrate was weighed and placed in conical flask and 10 ml of basal media was added to it and kept for autoclaving at 121 °C for 20 min. The basal media composition was as follows: Soluble Starch 5 g; Yeast extract 2 g; KH_2PO_4 1 g; $MgSO_4$ $7H_2O$ 0.5 g in 1000 mL of distilled water. The pH was maintained at 6. After autoclaving, these flasks were inoculated with 10% (v/v) spore suspension. Fermentation was carried out at 30 °C for 15 days [19].

2.4. Enzyme extraction and estimation

Fermented substrates were dried at 50 °C for 24 h. Phosphate (PO_4) buffer (pH 7) was prepared. Twenty-five mL of PO_4 buffer was added into each flask containing the dried substrates. The enzyme was extracted in shaking conditions at 150 rpm overnight followed by centrifugation at 10,000 rpm for 15 min and obtained clear supernatant was used as crude enzyme. Enzyme assay was carried out using DNS method. 0.1 mL of crude enzyme extract was taken to which 0.9 mL of phosphate buffer was added followed by incubation for 30 min at room temperature. One mL of freshly prepared DNS solution was added and incubated in boiling water bath for 10 min. The absorbance was recorded at 540 nm after diluting it with 2.5 mL water [1].

2.5. Optimization of enzyme activity by response surface methodology

The experimental design was formulated according to central composite design (CCD) method of RSM using MATLAB software version 7.5.0 (R2007b) from the Math works, Inc., USA for the selected 3 factors namely pH, temperature and incubation time. A set of 20 experiments was necessitated with each variable at 5 levels (Table 1).

2.6. Ammonium sulphate precipitation

The enzyme present in crude cell free extract (60 mL) was purified by Ammonium sulphate precipitation to 60% saturation at 4 °C and left for 4 h. The precipitate was recovered by centrifugation at 10,000 rpm for 10 min at 4 °C and dissolved in the minimum volume of 100 mM phosphate buffer (pH 7.0). It was then transferred in a pre-activated dialysis bag and immersed in phosphate buffer (10 mM) at 4 °C overnight. Buffer was changed at every 1 h interval in order to achieve proper purification [2]. The dialysate was transferred into small screw-capped tubes and stored at −18 °C.

Table 1 Level of the independent variables for design of experiment.

	Level				
Variables	−1	−2	0	+1	+2
pH	5	6	7	8	9
Temperature (°C)	10	20	30	40	50
Incubation time (min)	10	20	30	40	50