



ORIGINAL ARTICLE

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



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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.

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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

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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 -glucosidase 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; $MgSO_4 \cdot 7H_2O$ 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

2.7. Protein estimation by Lowry's method

Extracellular amylase from partially purified samples of beetroot peel was estimated by Lowry's [12] method. Optical density of the reaction mixture was observed at 660 nm against a blank prepared with 0.1 mL buffer.

2.8. Determination of molecular mass by SDS-PAGE

Poly-Acrylamide Gel Electrophoresis (PAGE) of the partial purified amylase enzyme was performed according to Laemmli [11]. After electrophoresis, the gel was immersed in fixing solution. Staining of the band was done with coomassie brilliant blue, R-250 (CBB) for 2 h and later de-stained. The molecular weight of the amylase was estimated using standard protein molecular weight marker consisting of Bovine Serum Albumin (66 kDa).

2.9. Zymography/activity staining of enzyme

To obtain Zymograph, non-denaturing PAGE was performed. The gel obtained from electrophoresis was dipped in 1% starch solution for 20 min followed by the addition of few drops of Lugol solution to it (0.67% Potassium Iodide and 0.33% Iodine). The gel was observed for a yellow band [7].

2.10. Enzyme kinetics

Different concentrations of starch (0, 10, 15, 20, 25 mM) were used to estimate the kinetic parameters, K_m and V_{max} using double reciprocal Lineweaver–Burk plot. Michaelis–Menten equation was used to fit the data for the kinetic constant in non-linear manner and curve fitting was done using MATLAB software. The enzyme assay was carried out using DNS method at 540 nm as described above [7].

2.11. Effect of inhibitors/activators on amylase activity

Different inhibitors and activators in the concentration of 5 mM were used to investigate the amylase activity. NaCl, KCl, $CaCl_2$, Sodium dodecyl Sulphate (SDS), Ethylene Diamine Tetraacetic acid (EDTA) were used for enzyme assay. Enzyme was incubated with above set compounds for 30 min. After incubation, the enzyme activity was observed by DNS assay as described earlier [7].

2.12. Wash performance analysis of enzyme for cold washing

Use of purified enzyme extract in laundry detergent formulations was studied on small square white cotton cloth pieces (8×8 cm) stained with Spinach gravy and chocolate. The stained cloth pieces were allowed to sit overnight and taken in separate flasks [20]. The following sets were prepared and studied:

- i) Flask with distilled water (100 mL) + stained cloth (cloth stained with Spinach gravy and chocolate, independently).
- ii) Flask with distilled water (98 mL) + stained cloth + 2 mL Surf excel detergent (1% w/v).

- iii) Flask with distilled water (96 mL) + stained cloth + 2 mL Surf excel detergent (1% w/v) + 2 mL enzyme extract.

The above flasks were incubated at 28 °C for 30 min in a rotatory shaker. After incubation, cloth pieces were taken out, rinsed with cold tap water and dried. Untreated cloth pieces stained with gravy and chocolate were taken as control.

2.13. Statistical analysis

A MATLAB software package was used for the graphical and regression analysis of the experimented data and for examining the response surface and contour plots. Statistical parameters were estimated using analysis of variance (ANOVA).

3. Results and discussion

3.1. Screening of the substrate for alpha amylase production

The maximum enzyme yield was observed with beetroot peel powder (0.0287 U/mL) followed by orange peel (0.0284 U/mL) and the minimum activity was observed with onion peel (0.0171 U/mL). Beetroot is known to be a rich source of carbohydrates, unique crimson red colour β -betalain pigments and various micronutrients such as folic acid, potassium, manganese, etc. There has been an increased exploitation of organic residues from various sectors of agriculture and industry over past few decades. Organic agro waste residues such as vegetable and fruit peel, bran, bagasse, fruit seeds, etc. have taken a significant place in bioprocess technique as potential raw materials. These agro wastes show an excellent solid layer and in addition provide essential nutrients to support microbial growth and production of secondary metabolites [13].

3.2. Optimization of enzyme activity by response surface methodology

To get optimal enzymatic activity, three important factors i.e. incubation temperature (°C), incubation time (min.) and pH were varied and the obtained data were statistically analysed. The equation of the model explaining three variables for amylase activity (U/mL) is given below and obtained coefficients are provided in Table 2.

Table 2 Model co-efficient estimated by linear regressions of amylase activity on Beetroot peel.

	Co-efficient (10^{-3})	Standard error	t-value	p-value
A	16.3597	0.0156	1.0455	0.32039
B	-0.9472	0.0032	-0.2931	0.77544
C	0.0599	0.0003	0.1994	0.84592
D	-0.5888	0.0003	-1.9590	0.07857
E	-0.0013	3.6×10^{-5}	-0.0348	0.97294
F	0.0262	3.6×10^{-5}	0.7305	0.48187
G	-0.0029	3.6×10^{-6}	-0.8000	0.44228
H	0.0261	0.0002	0.1289	0.89996
I	0.0006	2×10^{-6}	0.3139	0.76002
J	0.0054	2×10^{-6}	2.6572	0.02401

$$\begin{aligned}
 &\text{Amylase activity (U/ml)} \\
 &= 16.3597 - 0.9472 \times \text{pH} + 0.0599 \\
 &\quad \times \text{Incubation temperature} - 0.5888 \times \text{Incubation time} \\
 &\quad - 0.0013 \times \text{pH} \times \text{Incubation temperature} + 0.0262 \times \text{pH} \\
 &\quad \times \text{Incubation time} - 0.0029 \times \text{Incubation temperature} \\
 &\quad \times \text{Incubation time} + 0.0261 \times \text{pH}^2 + 0.0006 \\
 &\quad \times \text{Incubation temperature}^2 + 0.0054 \times \text{Incubation time}^2
 \end{aligned} \tag{1}$$

Interactive effect of incubation temperature and incubation time is given in (Fig. 1a). The amylase activity reduced gradually with the increase in incubation time with the decrease being sharper at higher incubation temperature. Maximum amylase activity was observed with highest incubation temperature and minimum incubation time. The interactive effect of incubation time and pH concentration also showed significant variation. The activity decreased sharply with incubation time at low pH, though the dip observed at higher pH was slightly low. The maximum activity was observed with lowest pH and minimum incubation time (Fig. 1b). Interactive effect of pH and incubation temperature on the amylase activity for beetroot peel is given in Fig. 1c. The activity doesn't show much variation with incubation temperature but increases slightly

with an increase in the pH value. Thus, from the above obtained data it can be concluded that incubation temperature doesn't have much significant effect on the amylase activity but the incubation time has a negative effect on the activity.

From the analysis of variance (Table 3), the model for amylase was highly significant ($p < 0.01$) and the R^2 (determination coefficient) value for the model, being the measure of the goodness of fit of the model, was 0.8405 which showed that 84.05% of the total variation in the observed response value could be explained by the model, or by experimental parameters and their interactions.

The validation of data for amylase activity (U/mL) from *M. sanguineus* was carried out with the help of a polynomial model. The optimum values for the test variables in coded factors were found to be pH of 5 at 50 °C of incubation temperature and 10 min of incubation time predicting maximum amylase activity of 0.011 U/mL. Experiment was carried out with the above predicted optimum conditions and an amylase activity of 0.014 U/mL was observed. This demonstrated a good match between the experimental value and predicted value thus substantiating the proposed model.

The tested ranges of inoculum and the initial medium pH did not give any significant variation in α amylase activity. There are few reports available regarding amylases and glu-

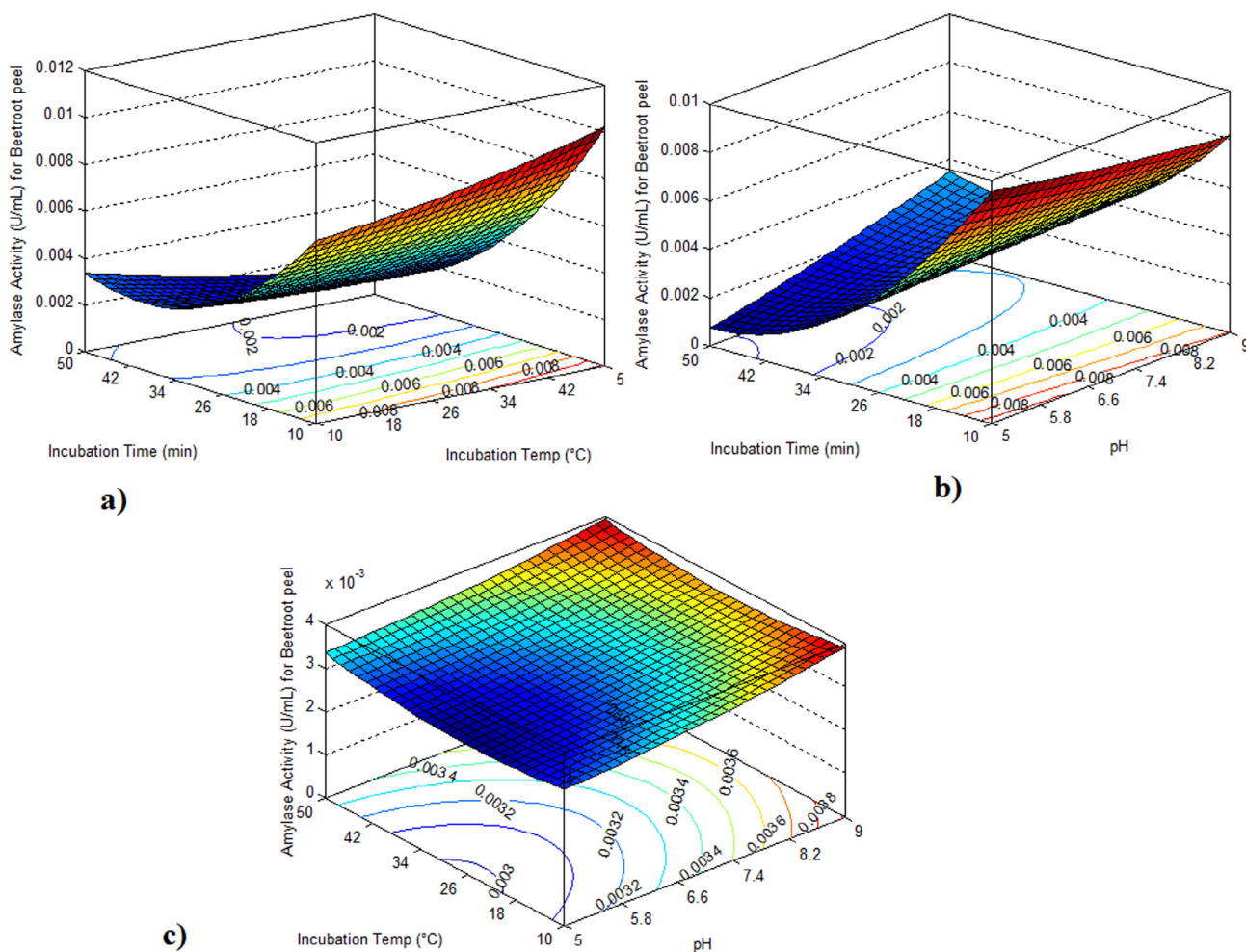


Figure 1 3D response surface plot showing interactive effect of pH, temperature, incubation time on amylase activity; (a) incubation time and temperature, (b) incubation time and pH, (c) temperature and pH.

Table 3 Analysis of variance results for RSM quadratic model for the three entities under study on beetroot peel.

Sum of squares	Degree of freedom	<i>f</i> -value	<i>p</i> -value	Mean square error	<i>R</i> ²	Adj <i>R</i> ²
0.00001	9	5.8561	0.00537	0.000001	0.8405	0.6970

coamylase from some of the *Monascus* sp., but not much work has been carried out on *M. sanguineus*.

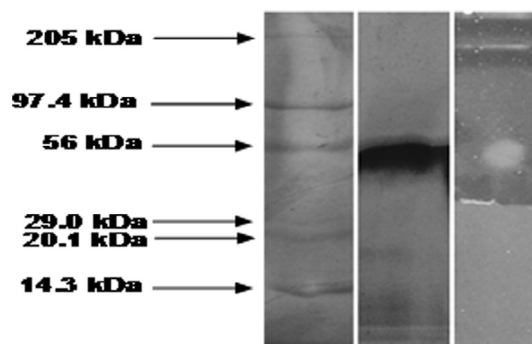
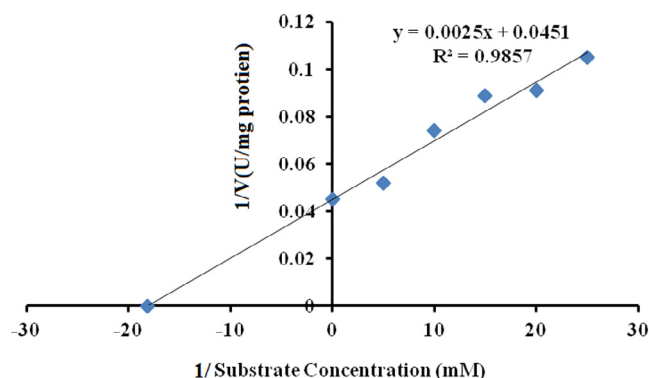
3.3. Partial purification, molecular weight and Zymography of the enzymes

Partial purification of amylase from crude enzyme extracts obtained from solid state fermentation of beetroot peel was achieved using (NH₄)₂SO₄ precipitation 60% saturation. The α-amylase present in the crude extract was purified by ammonium sulphate precipitation which resulted in 6.46 fold purification (Table 4). Partially purified enzyme amylase was associated with other molecules depicting two major bands as revealed from SDS-PAGE. The molecular weight of amylase from *M. sanguineus* was calculated by plotting a graph between linear logarithms of relative molecular mass vs Rf value. The molecular weight of amylase obtained from the band of SDS-PAGE was 56 kDa. Zymogram of partial purified enzyme confirmed amylase presence in fermented beetroot from *M. sanguineus* (Fig. 2).

Several authors have reported different molecular mass for amylase from different organisms approximately in the range of 40–70 kDa. Similarly, molecular weight of fungal amylase isolated from *Aspergillus niger* JGI 24 was 43 kDa as reported by Varalakshmi et al. [18]. Demirkan et al. [5] has reported α-amylase produced by *B. amyloliquefaciens* and the purified enzyme having a molecular weight of 52 kDa. Faber [8] reported the molecular weight of α-amylase from *B. licheniformis* as 55 kDa.

3.4. Kinetic constants of α-amylase

K_m and *V_{max}* (kinetic constants) of amylase were estimated by linear regression technique utilizing Lineweaver–Burk plot. For this, the concentration [S] of the substrate (starch) was plotted against the enzyme activity (V). The linear line followed the Lineweaver–Burk plot which in turn was used to calculate the kinetic constants. *K_m* and *V_{max}* (kinetic constants) of amylase were estimated by linear regression technique utilizing Lineweaver–Burk method, the maximum value of enzymatic activity (*V_{max}*) was approximately 22.075 U/mL proteins (Fig. 3) as extrapolated by the Lineweaver–Burk plot. As per the theory, the value of *k_m* is the substrate concentration required to attain half of the maximum enzyme velocity. The value of *K_m* thus obtained was 0.055 mM. Smaller *K_m* value is a representative of powerful affinity towards substrate. Lineweaver–Burk plot was attempted to endorse the results.


Figure 2 (a) Molecular marker, (b) SDS-PAGE, (c) Zymography.

Figure 3 Lineweaver–Burk plot (1/V vs 1/[S]) for amylase activity.

This calls for the inverse of the substrate concentration on the X-axis and the inverse of the enzymatic activity on the Y-axis. The equation can be expressed as

$$1/V = K_m/V_{max} \times 1/[S] + 1/V_{max} \quad (2)$$

A linear fit was obtained between these two variables.

$$1/V = 0.0025 \times 1/[S] + 0.0451 \quad (3)$$

From the Eq. (2) it is evident that the intercept at X-axis gives the value of 1/*K_m* and the intercept at Y-axis gives the value of 1/*V_{max}*. Hence, the value of *V_{max}* obtained was 22.07 U/mL and the value of *K_m* was 0.055 mM. The *V_{max}* and *k_m* of α-amylase were derived from the Lineweaver–Burk plot and found to be 2778 U/mg/min and 8.3 mg/mL, respec-

Table 4 Purification summary of amylase enzyme.

Purification	Amylase activity (U/mL)	Protein (U/mL)	Specific activity (U/mg protein)	Purification factor
Crude extract	0.03	0.36	0.0833	1
Dialysate extract	0.194	0.435	0.446	6.46

tively [17]. As the amount of starch concentration increases in the reaction medium, alpha amylase activity also increases until it reaches a maximum after which it starts decreasing. This behaviour is attributed to the fact that the enzyme becomes saturated with substrate and reaches V_{max} , the enzyme's maximum rate. If an enzyme has a small value of K_m , it achieves its maximum catalytic efficiency at low substrate concentrations. Hence, the smaller the value of K_m , the more efficient is the catalyst [16].

3.5. Effect of inhibitors/activators on amylase activity

For amylase, maximum inhibition was observed with both NaCl and KCl (0.105 U/mL, inhibition by 44.7%) and mini-

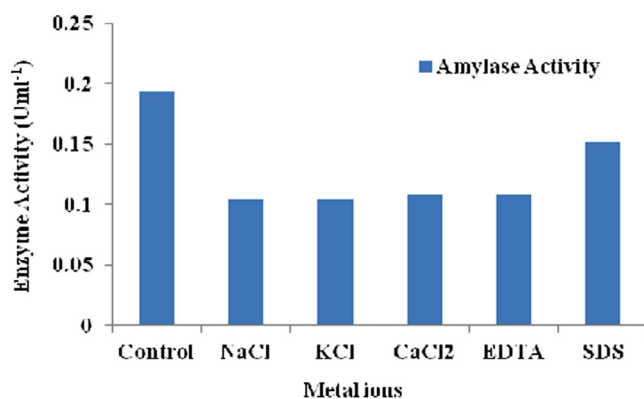


Figure 4 Influence of chemical reagents on glucoamylase and amylase activity.

um inhibition was observed with SDS (0.152 U/mL, inhibition by 18.4%) as compared to control (Fig. 4). The activity of enzymes is dependent on the requirement of a coenzyme as well as a metal. Addition of chelating agents to the reaction mixture interferes with the active site of the enzyme causing inhibition of enzyme activity. Removal of metal ion often reduces the enzymatic activity or even results in a total loss of an enzymatic activity. Metals are the common inorganic modifiers. Besides accelerating the rate of enzyme-catalysed reactions, it can also inhibit the rate of reaction [10].

3.6. Wash performance analysis of enzyme for cold washing

Analysis was carried out with a new white cotton cloth stained with two different stains. First used stain was mixture of Spinach curry and second stain was Chocolate. Alkaline amylase from *M. sanguineus* exhibited high efficiency for the removal of both stains in combination with commercial detergent (Surf excel) at 20 °C (Fig. 5). These fragments are initially removed from the fabric surface either by component of the detergent matrix, or by water alone. This combination of enzymatic-detergent makes the fabric feel softer on touch than any other cotton cloths washed with starch stains with detergent. Amylase provides improved starch hydrolysis, resulting in better stain removal and anti-deposition benefits.

Recently, fabrics and their washing requirements have been changed. Demand of washing detergent with cold water under mild conditions has increased over the decades. Previously, chemicals were common in laundry and dishwashing, which were very harsh towards cloth and hands. Due to selectiveness and washing under controlled condition, enzyme based detergent is high on demand. Hence microbial enzymes are becom-

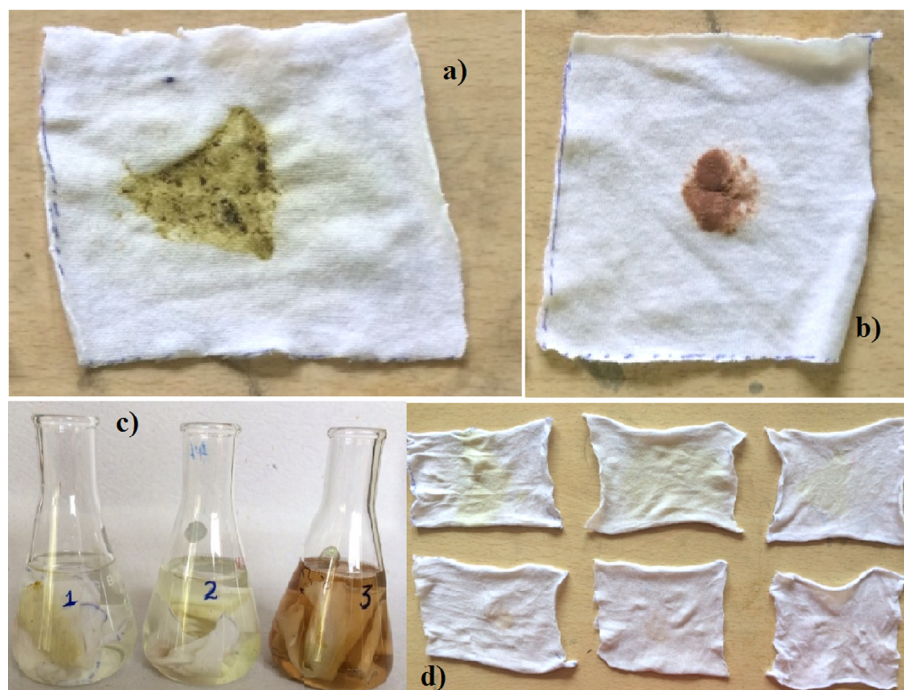


Figure 5 (a): Cloth stained with Spinach gravy. (b) Cloth stained with chocolate. (c) Flasks with (i) control; (ii) stained cloth with detergent; (iii) stained cloth with detergent and enzyme extract. (d) Cloth after wash performance (i) control; (ii) stained cloth with detergent; (iii) stained cloth with detergent and enzyme extract.

ing a better alternative source to the detergent industry [3]. The uses of amylase enzyme in various detergents are due to its ability to degrade the residues of starchy foods such as gravies, chocolate, etc. into dextrans and other moiety of oligosaccharides and monosaccharides.

4. Conclusion

This study was attempted to explore the *M. sanguineus* as an efficient producer of α -amylase enzyme and also to find out optimal parameters to get maximum enzymatic activity with the help of a widely accepted statistical tool. The results concluded that the fungus *M. sanguineus* is a good source of amylase enzyme under solid state fermentation quite similar to other *Monascus* spp. Application of amylase produced by *M. sanguineus* in detergent industry was also carried out and it was shown very effective in stain removal from the fabrics. Hence *M. sanguineus* can be considered as a potential source for amylase production, and can be explored further for its application in textile and food industries in the future.

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