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RESEARCH ARTICLE

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CONSEQUENCE OF COPPER IONS ON THERMAL STABILITY OF GLUCOAMYLASE FROM ASPERGILLUS *NIGER*

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ABSTRACT

The production of glucoamylase is carried out in the basal medium containing ammonium sulphate, magnesium sulphate, hydrated calcium chloride, and hydrated potassium dihydrogen phosphate by using medium optimization-Plackett Burman Design. *Aspergillus niger* is inoculated for the release of Glucoamylase into the surrounding medium. Then the supernatants collected by centrifugation is subjected for ammonium sulphate precipitation .Followed with this the sample is purified by using the ion exchange chromatography .The purified fractions of the sample is analyzed in SDS-PAGE. The kinetic parameters measurements for the purified sample are carried out by using starch as substrate. The purified fractions are analyzed for the effect of copper ions and it is found to have maximum activity at 5mM concentration .Then the activation energy for the native glucoamylase is 94.46 KJ/mol and the copper modified glucoamylase is 119.98 KJ/mol. The copper modified glucoamylase is found to be stable at high temperature compared to that of native enzyme.

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KEY WORDS

Plackett Burman design, Glucoamylase, ion exchange chromatography, SDS-PAGE, Thermal stability.

INTRODUCTION

Glucoamylase degrades starch to glucose in theoretically 100% yields. The reaction rate decrease with the decreasing chain length of the dextrin substrate. Glucoamylase is capable of catalyzing a reverse of the normal hydrolysis reaction to produce mainly maltose and isomaltose. Amylases are produced by various organisms including bacteria, fungi and yeast. These enzymes have found wide applications in the processed food industry, fermentation industry, textile and paper industries (Selvakumar *et al.,* 1996). Glucoamylase play an important role in the improvement of starch based food products. It is highly desirable to increase the thermal stability of glucoamylase so that starch hydrolysis could be carried out at higher temperature. Therefore, the present study was undertaken to study the effect of copper ions on the thermal stability of glucoamylase from *Aspergillus niger.*

Sources:

Glucoamylase occurs in plants and microorganisms, including bacteria, fungi and yeasts.

Fungal sources: Glucoamylase is mostly produces by molds, mainly *Aspergillus sp,Rhizoups sp* and *Endomyces sp* production is generally extracellular and enzyme can be recovered from culture filtrates. Most of the industrial processed use Glucoamylase obtained from *Aspergillus awamori*. Species of Rhizopus such as *R.oryzae*, *R.niveus*, *R.delemar*, *R. javanicus* are also potent producers of Glucoamylase. A few species of Penicillium and mucor have also been reported to produce Glucoamylase. *Monascus sp*, which generally produces pigment, can also been reported to produce Glucoamylase, namely, *T. viride*, T.reesei.Few Thermophilic fungi have also been reported as the sources of Glucoamylase. Some examples are *Thermomyces lanuginosus*, *Scytalidium thermophilum.*

Bacterial sources: A few aerobic bacteria such as *Bacillus strearothermophilus*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* are known as the sources of Glucoamylase. Some anaerobic bacteria such as *Clostridium thermosachalyticum* and *C. thermohydrosulfuricum* have also been reported as the source of Glucoamylase.

Yeast sources: Some species of yeast such as *Candida antartica*, *Saccharomycopsis fibuligera*, *Pichia subpelliculosa*, *Saccharamyces diasticus* have been described as the source of Glucoamylase.

Glucoamylase finds application in the food and fermentation industries for the saccharification of starch, brewing, distilling, etc. Fungal Glucoamylase is widely used in the manufacture of glucose and fructose syrup, and in the production of sweeteners. Enzymatic process to produce high-glucose syrups is widely used in food industry and alternatively, glucose may be an important substrate for fermentative processes to produce other products such as ethanol, amino acids or organic acids. Glucoamylase also helps in reducing the dough viscosity to improve the texture and appearance of bread. Further, they are used in the manufacture of pharmacologically active digestive aids.

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MATERIALS AND METHODS

Microbial culture and inoculums development:

The preparation of inoculums ,the fungus was grown in a liquid medium containing(g/100ml): glucose,2.0;Urea,0.3;magnesium sulphate 0.05;potassium chloride,0.015;potassium dihydrogen phosphate,0.008 and zincsulphate 0.001. The pH of the medium is adjusted at 4 and the temperature is 30^0C .After inoculation, incubation is done at 37^0C (150rev/min) for three days to get a homogenous conidial suspension.

Medium optimization:

Medium optimization involves investigation of differential combinations and sequences of process conditions, to determine the growth condition which produces the biomass. When more than 5 variables to be optimized Placket Burman design is for 7 variables is generally used. The general design is for 7 variables in which three are dummy variables.E,F and G are designed as dummy variables. These dummy variables provide an adequate estimation of error.

The procedure identifies the important variables and allows them to be rank in order of importance and to investigate in a more detailed study, to determine the optimum values to use.

The stages in analyzing the datas are

1. Determine the differences between average of H and average of low response for each independent and dummy variable.

   Difference = $\sum A(H) - \sum A(L)$

2. The effect of independent variables on the response is the difference between the average response for the four experiments for the high and low values respectively.

   Effect of A = $\frac{\sum A(H)}{4} - \frac{\sum A(L)}{4}$

   This value should be nearer to zero for dummy variables.

3. Estimate the mean square of each variable for the variable A,

   Mean square of A =($\left(\sum A(H) - \sum A(L)\right)^2$)/8

The experiment error can be calculated by averaging the mean squares of the dummy variables E, F and G

Thus mean squares for error

$= \sum \frac{\text{(mean square of dummy variables)}}{\text{Number of dummy variables}}$

4. Final stage is to identify the factors which are showing larger effects.This is done using F test

   F Test = $\frac{\text{Factor mean square}}{\text{Error mean square}}$

The factor which is having highest F test value is identified as the most important factor.

Substrate and growth conditions:

The basal medium used in this study is(g/100ml):ammonium sulphate,0.4;magnesium sulphate,0.02;calcium chloride,0.01 and potassium di hydrogen phosphate 0.05,Erlenmeyer flasks(500 ml) containing this basal medium is sterilized at 121^0C.The media is then inoculated with 5ml containing 107-108 spores/ml and the flasks were incubated at 30^0C for 72 hours.

Determination of Growth Pattern:

The different phases of the *Aspergillus niger* cells such as lag,log,stationary and decline phases are viewed by plotting time in the X-axis and optical density value (Absorbance) in the Y-axis.

Determination of Specific Growth Rate:

The rate of microbial growth is characterized by the specific growth rate defined as

$$\mu = 1/x \, (dX/dt)$$

Hence

$$\ln(X/X_0) = \mu t$$

To determine the specific growth rate (μ), natural log of biomass $\ln(X/X_0)$ was plotted against time(t).The slope of the line at any moment gives the specific growth rate of the *Aspergillus niger*.

Growth Yield:

Yield coefficient is defined by

$$Y_{x/s} = -\Delta X/\Delta S$$

$$Y_{x/s} = -(X - X_0)/(S_o - S)$$

Biomass growth yield is determined on the basis of the medium consumption. A graph is plotted between changes in dry weight and changes in the amount of the medium consumed. The slope obtained will give the value of $Y_{x/s}$.

Enzyme purification:

Ammonium sulphate (65% final concentration) is added to the supernatant. After stirring it for 4 hours at 4^0C, the precipitate is collected by centrifugation at 12000rpm.Then the pellet is dissolved in 10ml of 50mM Tris-HCL buffer (pH 8) and it is loaded into the ion exchange chromatography column. The sample is collected from the ammonium sulphate precipitation.

1. First the column is equilibrated with equilibration buffer
2. Then the sample is loaded into the column packed with carboxymethyl (CM)-cellulose(negatively charged),Now close the top and bottom of column.
3. Incubate this arrangement for 30min with gentle intermittent mixing.
4. Then the enzyme was eluted from the column using elution buffer and the sample fraction collected is estimated by using Lowry et al method.

SDS Polyacrylamide gel electrophoresis:

The 10μg sample of protein with glucoamylase activity is submitted to 12% SDS-PAGE and Bovine serum albumin (66 kDa) used as marker.

Effect of Cu2+:

The glucoamylase from *Aspergillus niger* was treated with 0.5 mM EDTA to remove the metal ions. The type of glucoamylase activation/inhibition by Cu^{2+} was determined at 1 to 10 mM concentration of Cu^{2+}.

Thermal stability:

Thermal inactivation of native and Cu2+ modified glucoamylases were determined by incubating enzymes in 50 mM acetate buffer having pH 5.5 (Haq Nawaz Bhatti *et al.,* 2005) at different temperatures (50, 55, 60 & 65°C). The data was fitted to first order plots and analyzed .The first-order rate constants for irreversible thermal denaturation (Kd) of

glucoamylases were determined and Arrhenius plots were applied to determine the activation energy for denaturation (E_a). The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory .

$K_d = (k_b T/h) \exp(-\Delta H_a /RT) .\exp(\Delta S_a/R)$

Where,

h = Planck's constant = 6.63×10^{-34} Js

k_b = Boltzmann's constant (R/N) = 1.38×10^{-23} Jk^{-1}

R = gas constant = 8.314 Jk^{-1} mol^{-1}

N = Avogadro's No. = 6.023×10^{23} mol^{-1}

T = Absolute temperature, K

ΔH_a (enthalpy of activation) = $E_a - RT$

ΔG_a (free energy of activation) = RT ln (K_d, h/k_b. T)

ΔS_a (entropy of activation) = $(\Delta H_a - \Delta G_a)/T$

RESULTS

MEDIUM OPTIMIZATION:

Plackett Burman Design

Error mean square:

1.1166X10^-3

Calculated F test values:

A = 79.3534

B = 26.9406

C = 0.24491

D = 3.91868

Effect of Cu2+:

Maximum activity was observed at 5 mM concentration.

Thermal stability:

Thermal denaturation of enzymes occurs in two steps as shown below:

$$N \leftrightarrow U \rightarrow I$$

Where N is the native enzyme, U is the unfolded inactive enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolong exposure to heat and therefore cannot be recovered upon cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996). The opening up of enzyme structure is accompanied by an increase in disorder or entropy of activation (Vieille &Zeikus, 1996). The kinetics and thermodynamics of irreversible thermal denaturation of native and copper modified glucoamylases from *A.niger* were studied at 50 to 65°C.

It is obvious from the results that coupling of copper with glucoamylase resulted into stable enzyme. Native glucoamylase was

stable up to 50°C and showed a half-life of 111.25min.With increase in temperature the half-life decreases. At 65°C, the native form displayed a half-life of 23.1 min only indicating that the enzyme is not thermostable at higher temperatures. While the copper modified form displayed a
Half-life of 36.47 min indicating that the modified form was stable at higher temperature .

In order to determine the thermodynamic parameters for irreversible thermal stability, the energy of activation for thermal denaturation was determined by applying the Arrhenius plots .

The Activation energy of native glucoamylase from *Aspergillus niger* is 94.46 KJ/mol.
The Activation energy of copper modified glucoamylase from *Aspergillus niger* is 119.98 KJ/mol.

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