Integrated Green Bioprocessing of Agro-Waste for Enhanced Industrial Enzyme Production - Kinetics and Mixed Culture Optimization

S.Kavitha[a*], G.Surendran[b],

[a*]Department of Chemical Engineering, Hindusthan College of Engineering and Technology, Coimbatore, India.
[b] Department of Chemical Engineering, KPR Institute of Engineering and Technology, Coimbatore, India.
* Corresponding author. Tel.: +91 8925148453
E-mail address: kavitha212418@gmail.com

ACCEPTED MANUSCRIPT

ABSTRACT

This study investigates the bioprocess optimization of enzyme production using agrowaste substrates and mixed fungal cultures. Kinetic studies were conducted to analyse endoglucanase production by *Trichoderma viride*, *Aspergillus niger*, and their mixed culture. The modeling covered growth, product formation, and substrate consumption. To date, limited studies have systematically compared monoculture and mixed culture systems using rice bran and wheat bran under structured kinetic modelling. This synergistic interaction contributes to improved bioconversion efficiency compared to the use of single substrates, representing a novel approach in agrowaste valorization. The mixed culture, utilizing rice bran and wheat bran, achieved a maximum endoglucanase production of 145.35 U/ml, outperforming *T. viride* and *A. niger* monocultures by approximately 46% and 27%, respectively. Maximum specific growth rates (μmax) were 0.02421 h^{-1} for *T. viride*, 0.0325 h^{-1} for *A. niger*, and 0.0375 h^{-1} for the mixed culture. The Yxs values were 0.2855, 0.2933, and 0.2601, while Ypx values were 6.5051, 5.6092, and 9.9631, respectively, highlighting the superior productivity of the mixed culture. The improved results were due to the combined action of both fungal species. They worked together, helping each other break down the material more effectively. This showed that using mixed cultures could be a low-cost and eco-friendly way to produce enzymes from agricultural waste. It also supported the development of better and more efficient methods for bioprocessing strategies.

Key words: *Kinetics, Endoglucanase, Mixed Culture, Aspergillus niger, Trichoderma viride.*

1. INTRODUCTION

The growing interest in sustainable waste management and bioresource utilization has highlighted the potential of enzymatic conversion of agricultural waste into valuable bioproducts. Recycling agrowaste into enzymes and biofuels offers a promising approach to address environmental challenges while adding economic value. Among the various biological conversion technologies, enzymatic hydrolysis of lignocellulosic biomass has gained particular prominence due to its efficiency and eco-friendly nature (Rath T et al, 2022; Sun L. et al 2020; Martín A et al 2019).

Lignocellulosic wastes such as rice straw, wheat bran, wheat straw, rice bran, corn stover, sugarcane bagasse, corn straw, and paper waste serve as abundant and low-cost substrates for microbial enzyme production, especially endoglucanase (Santos J et al 2020; Celebioglu A. & Uyar T 2021; Rana S et al 2020). Endoglucanase plays a crucial role in breaking down cellulose into fermentable sugars. Enzymatic treatment involves a two-step mechanism: initial depolymerization of cellulose into cellobiose, followed by its hydrolysis into glucose via cellulase enzymes (Xu D et al. 2020; Khalid S et al. 2021). Fungi and bacteria are the primary microbial producers of cellulases, offering potential for efficient enzyme production from lignocellulosic substrates (Korcz E. & Varga L. 2021; Gaur et al 2015). To date, there is limited data comparing mixed vs. monoculture systems using rice and wheat bran under structured kinetic modelling approaches. This gap in knowledge underscores the need for systematic studies evaluating the synergistic interactions of mixed fungal cultures for enhanced enzyme production.

The objectives of this study were to:

- Investigate endoglucanase production using *Trichoderma viride*, *Aspergillus niger*, and their mixed culture grown on rice bran and wheat bran substrates.
- Apply Response Surface Methodology (RSM) to optimize process parameters influencing enzyme production.
- Model and analyse the growth, product formation, and substrate consumption kinetics under different culture conditions.
- Compare and evaluate the performance of monocultures and mixed cultures based on the optimized conditions identified through Response Surface Methodology.

ACCEPTED MANUSCRIPT

2. EXPERIMENT

2.1. Materials and Methods

The composition of the growth medium for *Trichoderma viride*, *Aspergillus niger* and mixed culture (*T.viride* & *A.niger*) is as follows: Potato nutrient broth-20 g/L, Agar- Agar - 10 grams per liter g/L, and the medium pH was set to 5.The culture media for all strains were maintained and incubated for a time over 6 days at a temperature 30° C and then stored in a refrigerator at 4° C (Zubair A et al 2019; Khalid Sayed et al 2022).

2.1.1 Preparation of mixed culture broth

Mixed cultures were grown on special PDA slants and the spores were removed aseptically from 6 days aged PDA slants. Sterile distilled water was added to all culture agar slants (*Trichoderma viride* and *Aspergillus niger*) and mixed for preparing uniform liquid mixture which was used as fungal inoculum. A total volume of 4.0 ml of conidial suspension (*Aspergillus niger* and *Trichoderma viride*) was used to initial growth in 250 ml conical flask containing 100 ml of E and P (Eggins and Pugh) medium supplemented with (g/L) mineral salt medium (Khalid Sayed et al 2021; Sarma H et al 2021).

2.1.2 Fermentation medium

For the growth of *Aspergillus niger* and *Trichoderma viride*, the following nutrients (in g/L) were used: 0.3 g urea, 2 g ammonium sulfate ($(NH_4)_2SO_4$), 2.6 g potassium dihydrogen phosphate (KH_2PO_4), 0.5 g calcium chloride ($CaCl_2$), 1.5 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1.5 g zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 2 g cobalt chloride ($CoCl_2$), 10 g ammonium sulfate, 4 g KH_2PO_4, and 2 ml of Tween 80. For mixed culture growth (A. niger and T. viride), E and P media were used, containing (in g/L): 2 g KH_2PO_4, 1 g potassium chloride (KCl), 1 g ammonium sulfite ($(NH_4)_2SO_2$), 1 g $MgSO_2 \cdot 7H_2O$, 1.5 g L-asparagine, 0.1 g $CaCl_2$, and 1 g yeast extract.

Then the growth media were supplied with different quantities of substrate wheat bran and rice bran. All experiments were carried out in 250 ml flasks containing the prepared medium. The mixed culture medium was sterilized at 121° C under 15 psi pressure for 45 minutes, and the medium was allowed to cool afterward, all conical flasks were inoculated with the fungal mycelial inoculum prepared as mentioned above. The inoculated flasks were incubated for 0 to 144 hrs. at 30° C. The culture samples were spun at 10,000 rpm

ACCEPTED MANUSCRIPT

for 15 minutes, and the resulting clear supernatant was collected for enzymatic processing (Irfan M et al 2017; Khalid Sayed et al 2024).

2.2. Cellulosic materials

Lignocellulosic waste materials wheat bran (WB) and rice bran (RB) were used as substrates. Lignocellulosic materials were collected from the local industries.

2.2.1 Rice bran: The proximate composition of rice bran is as follows in (%): moisture – 10.1, crude fat - 11.3, crude protein-12.6, crude Fiber – 19.3, total ash – 10.2, Nitrogen Free Extract (NFE) – 36.5.

2.2.2 Wheat bran: The proximate composition of wheat bran is as follows in (%): moisture – 10, crude fat - 13.5, crude protein-2.6, crude fiber – 12.2, total ash – 3.00, Nitrogen Free Extract (NFE) – 58.7.

2.3 Pretreatment of cellulosic materials

A quantity of 500 gram of the pretreated lignocellulosic material was treated separately with 2L of 5% sodium hydroxide solution and autoclaved at 121° C for 1 hour. Then the medium was filtered and the solution was neutralized with phosphoric acid. Again, the residue material was dried at 75 °C in an oven after that the same volume of distilled water was added and heated at 75° C for 30 minutes. The suspension was filtered, and the residue was dried at 75° C. (Soeka YS. 2019; Pan M et al 2010; Manikandan K and Viruthagiri T 2009).

2.4 Analytical Techniques

Batch process was carried out on mixture of rice bran and wheat bran hydrolyzed with fermentation elements. The endoglucanase activity, concentration of substrate, pH, process temperature and biomass concentration were determined every 24 hours for 8 days. A reactive mixture of one ml of 1% (W/V) carboxymethyl cellulose solution in 0.01 M citrate buffer (pH 5.5) and one ml of fermentation culture supernatant was incubated at 65 ° C after incubation period for 30 min the reducing sugars were adding with 10 ml of DNS reagent. Samples were prepared with 15 min boiled endoglucanase enzyme and activity was expressed as the amount of endoglucanase required to release 1µ mol reducing sugars per ml under the above-mentioned conditions by using glucose as

ACCEPTED MANUSCRIPT

standard curve. For cellulose evaluation, 1ml of sample, 10 ml of 1.0% Anthrone reagent solution and 10 ml of distilled water and the solution incubated in boiling water for 45 min. The samples were analyzed using spectrophotometer technique at 620 nm and crude cellulose was used as standard (Kola S.O et al 2018; Guichuan Ye et al 2017).

2.5 Estimation of biomass

A 5 ml portion of culture broth was withdrawn and kept at 4°C for 3 hrs. The lighter mycelia were separated from the fermentation broth. A sample was centrifuged at high speed for 30 minutes. The top portion of the fermentation broth was discarded and the solid mass was dried and dry weight of the biomass were estimated.

2.6 Response Surface Methodology (RSM) for endoglucanase production

The effect of temperature, pH, fermentation time and inoculum size were studied using central composite design (CCD) for the monoculture and coculture fungal strains of *Aspergillus niger* and *Trichoderma viride*. The effects of four major key factors responsible for enzyme production were studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) by CCD. Here the independent variable X_1 is temperature (°C), X_2 is pH, X_3 is fermentation time (hrs) and X_4 is inoculum size % (v/v). The CCD contained 30 experiments to optimize the parameters for the production of endoglucanase.

A second-order model can be constructed efficiently with Central Composite Design (CCD). CCD is first-order (2N) design augmented by additional centre and axial points to allow estimation of the tuning parameters of a second-order model. The design involves 2N factorial points, 2N axial points and 1 central point. CCD presents an alternative to 3N designs in the construction of second order models because the number of experiments is reduced as compared to a full factorial design for the multi objective design of a flywheel. A full factorial design which, includes all possible factor combinations in each of the factors, is a powerful tool for understanding complex processes, the detailed mechanisms of which are not known and for describing factor interaction in multifactor systems. A combination of the levels of the factors that leads to a certain optimum response can also be identified through this approach.

The second-order model used to fit the response to the independent variable is shown in equation (1). The data obtained from RSM on endoglucanase production are subjected to analysis of variance (ANOVA) (Robert Marian et al 2018).

ACCEPTED MANUSCRIPT

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \text{.....(1)}$$

Where, Y is the predicted response k the number of factor variables, X_i and X_j are the input variables that influence the response variable Y, β_0 is the intercept, β_i represents the linear effect of X_i, βij represents the interaction between X_i ,X_j and βii represents the quadratic effects of X_i. However, in this study, the independent variables are coded as X_1, X_2, X_3 and X_4.

$$x_i = \frac{X_i - X_o}{\Delta x_i}, \; i=1,2,3.....k, \quad \text{.....(2)}$$

Where x_i is the dimensionless value of an independent variable. X_i is the real value of an independent variable, X_o is the real value of the independent variable at the center point, and ΔX_i is the step change value. The second order polynomial model equations (3) for the four factors take the following form:

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad \text{.....(3)}$$

The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-test. The significance of each coefficient was determined using student's t-test. The coefficients of the equation were determined by employing Design Expert software version 8.0.7.1. Analysis of variance (ANOVA) for the final predictive equation was done using the same software package.

2.7 Optimization using response surface methodology for mixed culture of *Trichoderma viride and Aspergillus niger.*

To optimize the combined effect of four different experimental parameters, on the production of endoglucanase, 30 trials were performed. The regression constants were calculated and the data was close-fitting to a second –order polynomial equation. The response, Y (Endoglucanase activity, U/ml) by mixed culture was stated in terms of the following regression equation (4)

ACCEPTED MANUSCRIPT

$$Y = 146.650 - 1.88125X_1 - 1.86125X_2 - 1.49042X_3 - 0.584583X_4 - 1.51740X_1^2 - 2.45365X_2^2 - 2.05990X_3^2 - 3.81865X_4^2 - 3.38062X_1X_2 + 1.87813X_1X_3 + 1.4688X_1X_4 + 1.61812X_1X_3 + 0.750625X_2X_4 + 0.499375X_3X_4 \quad \ldots\ldots..(4)$$

The response value Y and X_1, X_2, X_3 and X_4 were the coded levels of temperature, pH, fermentation time and inoculum size respectively. Statistical testing of the model was executed for analysis of variance. The ANOVA result of quadratic regression model for Y is described in Table 3. ANOVA of the regression model for Y proved that the model was significant due to an *F*-value and low probability value. Values of "*Prob* > *F*" less than 0.0500 indicate model terms were significant. The *P*-values were used to check the significance of each of the coefficients.

To fix the optimum levels of variables for maximum endoglucanase production, isoresponse contour plots and three-dimensional response surface plots were built by plotting the response, Y (Endoglucanase activity) on the Z axis against two variables, while keeping other variables at their optimum levels. Fig 1 show the isoresponse contour and response surface plots of the endoglucanase production with the interactive result of incubation temperature, initial pH, fermentation time and inoculum size respectively.

The figure of the corresponding contour plots showed whether the mutual interactions between the independent variables were significant or not. From the response surface plots, the optimal values of the independent variables were observed and the interaction between each independent variable pair was described.

The isoresponse contour plots of RSM as a function of two variables at a time, holding all other factors at fixed level, were helpful interaction between the two variables (fermentation time and temperature) showed that the endoglucanase production was complex even when inoculum size and temperature were subject to minor alterations. Under certain conditions, a maximal contour (endoglucanase production 146.65 U/ml) were determined. The endoglucanase production was significantly affected by fermentation time, pH, inoculum size and temperature. Constructed on the model, the optimal working conditions were obtained to achieve high percentage conversion of wheat bran and rice bran. The optimum values of the parameters X_1, X_2, X_3, X_4 were found to be 36 °C, pH 5.4, 97.38 hrs and 3.78 ml respectively.

ACCEPTED MANUSCRIPT

2.7.1 Validation of the Model for *Trichoderma viride* and *Aspergillus niger*

The values of process variables were obtained. The (CCD) central composite design and the response yielded maximum endoglucanase production. There were very minor differences in the predicted response (146.65 U/ml) and the observed value (147.37 U/ml) during the experiments when carried out at optimal conditions. The comparison of predicted and experimental values for the endoglucanase production was shown in the Fig. 2. The linear line in the figure showed that the predicted data of the response have an agreement with the observed values.

2.8 Preparation of SDS-PAGE

Acrylamide/Bis: (30 % T, 2.6 % C): Dissolve 87.5 g of acrylamide and 3.0 g of N'Nbis-methylene-acrylamide in 300 ml. The solution was stored in the dark and stable for maximum of 30 days.

10 % Ammonium per sulphate (APS) (w/v): 0.1 g of APS was liquified in 1 ml of ddH_2O. The solution was prepared fresh daily.

10 % SDS (w/v): Liquify 10 g of SDS in 90 ml ddH_2O with gentle stirring and make up to 100 ml with ddH2O.0.5 % (w/v) Bromophenol blue: Liquify 1.0 g of bromophenol blue in 100 ml ddH_2O.

Gel Buffer: Tris base (27.23g) was liquified in80 ml of ddH_2O; the pH was adjusted to 8.8 with 6 M HCl.

Stacking Buffer: Tris base (5 g) was dissolved in 60 ml of ddH_2O and adjusted to pH 6.5 with 6 M HCl. The total volume was carried up to 100 ml with ddH_2O and stored at 4 °C.

Sample Buffer: Double deionised water (3.55 ml), 1.25 ml of stacking gel buffer, 2.0 ml of glycerol, 2.5 ml of SDS and 0.5 ml of bromophenol blue were mixed to make solution A. Beta-mercaptoethanol (5 µl) was added to 950 µl solution A prior to use. Dilute the sample at least 1:2 with sample buffer.

10 × Electrode (Running) Buffer, pH 8.3: Dissolve 30.3 g of Tris base, 144 g of glycine and 10 g of SDS in ddH_2O and make up to 1 l with ddH_2O. Store at 4 °C. Dilute (1:10) before use. Staining Solution: Brilliant blue R-staining solution (Sigma, B6529).

Coomassie Gel Destain: Mix 100 ml of methanol and glacial acetic acid and make the total volume up to one liter with dH_2O.

ACCEPTED MANUSCRIPT

2.9 Online monitored batch fermenter

Experiments were carried out in a pilot plant scale batch fermenter (APPLICON BIOCONSOLE ADI 1025 controller, Holland) for endoglucanase production, with 2L volume, furnished with flat blade impeller, oxygen, and pH electrodes, temperature and DO_2 probe. All operating parameters were online monitored with Bio expert Lite 1.00 software. 1000 ml of production medium was taken in the fermenter and sterilized in the autoclave at 121^{o} C for 30 minutes. The sterilized medium was inoculated with the required inoculums and fermentation was carried out for 6 days at controlled condition of temperature, pH and agitation speed. The samples were drawn at every 24 hrs and the supernatants are analyzed for endoglucanase concentration.

2.10. Kinetics and modeling of endoglucanase production

Endoglucanase fermentation kinetics is one important research area. It was a mathematical model of the metabolic processes of *Trichoderma viride* and *Aspergillus niger,* which are contained in a batch reactor. Bio catalyst reaction engineering was wide area, which includes the influences of reaction kinetics, and its mode of operation process and kinetic models were used for the design and operation of bioreactor. In modeling bioreactor system, however. In these case unstructured models have been used to describe only the quantity of the biological species. In these situations, cell population biological phase and composition changes significantly influence in kinetics parameters and structured models. (Khalid Sayed et al 2024; Hamouda R A et al 2016; Lakhundi S et al 2015).

The optimum operating conditions to obtain maximum endoglucanase production for batch processes having different of operating variables and extended fermentation time per cycle particularly for complex systems like endoglucanase production. Generally different interacting systems were involved in the process, the biological phase and growth medium (Douglas E et al 2009; Marko Božinović et al 2023). The growth medium is a multicomponent system which must contain all the required nutrients for fungal growth, and which will accumulate, as the fungal grows, different end products of cellular metabolism. During biological reactions, both temperature, pH, ionic and all process

ACCEPTED MANUSCRIPT

properties many change with time. (Chukwuemeka U et al 2017; Karunakaran S et al 2022)

2.10.1. Representations of rates in terms of equations

Mathematical modeling and simulation are an important method in the design of production process for its implementation and control. Modeling means arriving at equations for the experimental data. A typical bioprocess has three terms namely, substrate (Input), Microbes (Output), Product (Output).

The balance equations for the substrate, biomass and product are given as

Substrate consumption (s): $\frac{ds}{dt} = f(s, x, p)$ ……(5)

Cell growth rate (x): $\frac{dx}{dt} = g(s, x, p)$ ……..(6)

Product formation (p): $\frac{dp}{dt} = h(s, x, p)$ ……..(7)

The functions f, g, h depends purely on the mechanism of the bioprocess which was very complex in nature. In this process, the mass transfer property was lumped together with the biological reactions and the expressions were represented in terms of the properties of the organism. Thus, the concentrations of the system represent the conditions at the bulk only. Mathematical models are described to represent change in concentrations of biomass concentration(x), mixture of wheat and rice bran concentration (s) and endoglucanase concentration (p) in the bulk as a function of process time.

3. Kinetics of microbial growth

Microbial growth kinetics can be represented using various models namely Monod model, Logistic model, Andrew model, Moser model, Tessier model, etc (Muthuvelayudham R and Viruthagiri T 2006; Narasimha G et al 2006).

3.1 Monod model

Most fungal fermentation studies were performed using a growth limiting substrate to control the microbe. A limiting substrate material which was used to change concentration, affects the growth of organism, substrate consumption, and formation of endoglucanase. Mathematical models such as Monod's explained the limiting substrate concentration, which has detailed explanation on the specific growth rate. Monod model described the growth of two parameters. Since growth of fungal species was an effect of catabolic and anabolic enzyme behavior, these processes i.e., substrate utilization process

ACCEPTED MANUSCRIPT

or growth associated product formation process, can also be quantitatively described based on cell growth models.

$$\frac{dx}{dt} = \frac{\mu_{max} s x}{k_s + s} \qquad \text{.....(8)}$$

3.2 Logistic model

The growth kinetics of microorganisms in the logarithmic phase which attains stationary phase was explained by the logistic model and the population growth with the assumption that inhibition is proportional to the square of the microbial concentration (x). This was given by.

$$\frac{dx}{dt} = kx(1 - \beta x) \qquad \text{.....(9)}$$

The constant terms k and β were logistic constants. The sigmoidal logistic curve leads a stationary population of size x = 1/β. Hence, it can be given that, higher the value of β, low value of the stationary phase concentration obtained. Mathematical modeling and simulation studies from experimental data based on the logistic models find an important tool in the design of kinetic studies for its implementation and control (Pandey A et al 2000; Marulasiddaiah R et al 2012). In fermentation processes involving various microbial populations, the performance and behavior of the tool depend not only on the cell growth characteristics of the individual microbial species but also on the interaction patterns among them. Kinetic studies of mixed microbial fermentation in batch mode of operation and process schemes can be developed only through better understanding of the interaction of species and development of suitable microbial models (Kavitha S et al, 2020; Sasikumar E and Viruthagiri T, 2008; Dhanasekar R and Viruthagiri T, 2005). During mixed culture operations, the growth of all fungal species depends on not only the environmental surroundings and the available nutrients for each fungal species but also on the interaction among them.

logistic curve was explained by using Riccati equation.

$$X = \frac{X_0 \, e^{kt}}{1 - \beta' X_0 \, (1 - e^{kt})} \qquad \text{.....(10)}$$

ACCEPTED MANUSCRIPT

3.3 Other models

Andrew $$\frac{dx}{dt} = \frac{\mu_{max} s x}{k_s + s + s^2 / k_I} \quad \text{……..(11)}$$

Tessier $$\mu = \mu_{max} (1 - e^{-S/Ks}) \quad \text{……..(12)}$$

Moser $$\mu = \mu_{max}(1+K_S^{-1}) \quad \text{.....(13)}$$

4. Model evaluation of growth kinetics

Monod model, Logistic model are tried for representing the experimental data obtained with *Trichoderma viride*, *Aspergillus niger* and mixed culture of *Trichoderma viride*, *Aspergillus niger* using mixed substrate (Kamaluddeen, S.Y and Madika 2023; Nawaz S et al 2006). A better prediction of biomass concentration in the log phase regime is obtained using the logistic model. The logistic constants were estimated using MATLAB 7.1 and the values were given in Table.1. The comparisons between experimental and predicted data for the three models were portrayed in Fig.6. And the values shown in Table 7.

Percentage Error is calculated using the following equation.

$$Error = \frac{\sqrt{(Predicted - Experimental)^2}}{Experimental} \quad \text{……..(14)}$$

The average error was found to be minimum for mixed culture compared to monocultures. The average errors for the *Trichoderma viride, Aspergillus niger* and mixed culture were found to be 11.9103, 8.800425 and 5.2285 percentages respectively. It is evident from the Table1 that the value of k is maximum and the value of β is minimum for the mixed culture when compared to the monocultures. This reflects the experimental observation that the mixed culture growth was more than the single. The maximum specific growth rate of *Trichoderma viride, Aspergillus niger* and mixed culture were found to be 0.02421, 0.0325 and 0.0375 h^{-1} respectively. Also, they are shown in Fig 5.

4.1. Substrate utilization kinetics of endoglucanase production

Fungal growth involves consumption of mixed substrates, which supply energy and raw materials required for the synthesis of additional biomass (Immanuel G et al 2006; Robert M R et al 2018; Elumalai S and Thangavelu V 2010). The general purpose in making a culture medium was to maintain growth and high production rates. In this process excessive concentration of a nutrient can inhibit or even poison microbial growth and

ACCEPTED MANUSCRIPT

disrupt the normal biochemical processes of the cells. The cell growth was controlled by limiting the amount of one nutrient in the medium.

A mass balance equation on limiting substrate that couples to the cell mass balance since substrate concentration depends on substrate concentration. In the substrate balance, the yield factor $Y_{x/s}$ equation becomes,

$$Y_{x/s} = \frac{Weight\ of\ Cells\ formed}{Weight\ of\ Substrate\ consumed} \qquad \ldots\ldots\ldots\ldots\ldots(15)$$

It has been obtained often that the total amount of biomass formed by growth is proportional to the weight of substrate (carbon source, energy source or oxygen) used. The yield factor $Y_{x/s}$ was defined as

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \qquad \ldots\ldots\ldots\ldots\ldots(16)$$

It should be noted that yield factor has dimensional units implied by the units used for cell amount and substrate amount (Sudhakar P and Nagarajan P 2010).

The substrate balance for batch culture is given as

$$\frac{ds}{dt} = -\frac{1}{Y_{XS}}\frac{dx}{dt} \qquad \ldots\ldots\ldots(17)$$

The Y_{xs} values of *Trichoderma viride, Aspergillus niger* and mixed culture were calculated using the plot of x vs s for the three cases are found to be 0.2855, 0.2933 and 0.2601 respectively. The theoretical substrate concentrations are found to be in close approximation with the experimental values and the average error was found to be minimum (%) for mixed culture compared to monocultures. Experimental and predicted values of substrate concentration were presented in Table 3,4 and 5.

4.2. Product formation kinetics on endoglucanase production

The simplest types of product formation kinetics arise when there was simple stoichiometric connection between product formation and substrate utilization of cell growth. The number of products formed by the cell growth was often proportional to the amount of consumption of substrate (Karmakar M and Ray RR 2011; Zhang Y et al 2010; Jahangeer S et al 2005).

ACCEPTED MANUSCRIPT

In this case, product formation occurs late in the log phase (approaching the stationary phase). Leudeking- Piret kinetic model was used to combine both cell growth associated (α) and non-cello growth associated (β) contributions:

$$r_{fp} = \alpha r_{fx} + \beta x \qquad \ldots\ldots\ldots\ldots\ldots\ldots (18)$$

This two-parameter kinetic model has proved in fitting of endoglucanase product formation data of different fermentation processes (Xu P 2020; Chellapandi P and Jani HM 2008; Duncan SM 2006). The equation represents the energy used for the growth and maintenance. The above equation can be written as

$$\frac{dp}{dt} = \propto_{LP} \frac{dx}{dt} + \beta_{LP} x \qquad \ldots\ldots\ldots\ldots\ldots (19)$$

Since the endoglucanase production is growth associated and produced during growth phase, β value is negligible, it almost zero. Hence the equation becomes,

$$\frac{dp}{dt} = \propto_{LP} \frac{dx}{dt} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (20)$$

In such case α becomes the yield coefficient Y_{PX}

$$Y_{PX} = \frac{\text{weight of product formed}}{\text{Increase in weight of cell mass}} \qquad \ldots\ldots\ldots\ldots\ldots (21)$$

The Y_{px} values of *Trichoderma viride, Aspergillus niger* and mixed culture were calculated using the plot of p vs x for the three cases is found to be 6.5051, 5.6092 and 9.9631 respectively and the predicted and experimental error values were found to be 10.3383, 13.3510 and 8.6234 respectively. The average error is minimum for mixed culture compared to monocultures (Dror TW et al 2005; Parry N J et al 2002). Experimental and predicted values of product concentration for single and mixed cultures are shown in Table3, Table4 and Fig2. This indicates Leudeking Piret model is more suitable for predicting the endoglucanase production using mixed culture. Since the above equations are nonlinear and difficult to solve using simple integration, they are solved using the numerical integration applying Runge – Kutta's method. The predicted values of the substrate, microbial and product are simulated using this integration with ODE solver in MATLAB14 software (Muhammad I et al 2016; David H 2016). The

ACCEPTED MANUSCRIPT

comparisons between the experimental and simulated values for each of the three cases were presented in Table 7 and Fig 6 (a, b, c).

5.SDS – PAGE analysis of the purified endoglucanase from Trichoderma *viride*, *Aspergillus niger* and mixed cultures

(SDS-PAGE) method for determining the molecular weight (MW) of an unknown protein. The gel was then analyzed to obtain the R_f values for all band. The distance should be measured from the top of the resolving gel to the band. A plot of log MW versus R_f was generated from the bands in the gel shown in Appendix -IV to determine the MW of the unknown protein. The simplest method for this is to base the MW determination on a standard curve. Analysis of SDS –PAGE for endoglucanase showed that the molecular weight from *Trichoderma viride* was found to be 27 kDa, *Aspergillus niger* was found to be 31 kDa and mixed culture was found to be 32 kDa.

6. CONCLUSIONS

In conclusion, our study revealed significant insights into endoglucanase production by the mixed culture of *Aspergillus niger* and *Trichoderma viride*. The utilization of 5% rice bran in the medium led to an impressive production level of 142.21 U/ml, while a similar concentration of wheat bran yielded slightly lower at 140.65 U/ml. Remarkably, the highest endoglucanase activity of 145.35 U/ml was achieved when utilizing a mixed substrate of rice bran and wheat bran in a 4:1 ratio (RB: WB), under optimized conditions of 35°C temperature, pH 5.0, 2 ml inoculum size, 200 rpm agitation speed, and a 96-hour fermentation period. These findings underscored the superior potential of mixed substrate combinations over single substrates for endoglucanase production. Furthermore, the utilization of both rice bran and wheat bran in conjunction with a mixed culture demonstrated enhanced enzyme activity compared to monoculture scenarios. The kinetics of endoglucanase production for both *Aspergillus niger* and *Trichoderma viride*, along with the mixed culture, were thoroughly examined. Modelling of endoglucanase production using single and mixed cultures provided valuable insights into growth kinetics, product formation kinetics, and substrate utilization kinetics, paving the way for future research endeavours in this domain.

ACCEPTED MANUSCRIPT

Table 1 Experimental design of central composite for four factors and the production of endoglucanase from mixed culture.

Run	Coded Levels				Real variables				Endoglucanase activity (U/ml)	
	X_1	X_2	X_3	X_4	[a]X1	[b]X2	[c]X3	[d]X4	Exp	Pred
1	1	-1	-1	-1	40	4.5	72	3	128.45	126.79
2	-1	1	-1	1	25	5.5	72	5	185.05	191.63
3	1	-1	1	1	40	4.5	120	5	135.25	133.56
4	-1	1	1	-1	25	5.5	120	3	141.02	140.69
5	1	1	-1	1	40	5.5	72	5	135.26	134.80
6	0	0	0	0	35	5	96	4	146.65	147.37
7	0	2	0	0	35	6	96	4	141.09	146.52
8	2	0	0	0	45	4.5	96	4	134.85	136.81
9	-1	-1	-1	-1	30	5	72	3	144.25	144.00
10	0	0	0	0	35	5	96	5	146.65	147.37
11	0	0	0	0	35	5	96	5	146.65	147.37
12	0	0	0	2	35	5.5	96	6	129.65	130.20
13	-1	1	-1	-1	30	5	72	3	138.12	139.23
14	0	0	0	-2	35	5.5	96	2	132.05	132.54
15	1	1	1	-1	40	5	120	5	145.25	144.51
16	-2	0	0	0	25	5	96	4	145.26	144.34
17	0	0	0	0	35	5	96	4	146.65	147.37
18	0	0	0	0	35	4.5	96	4	146.65	147.37
19	1	-1	1	-1	40	5.5	120	3	129.35	129.29
20	-1	1	1	1	30	5	120	5	135.01	136.09
21	0	0	0	0	35	5	96	4	146.65	147.37
22	0	0	2	0	35	5.5	144	4	140.65	141.39
23	1	1	-1	-1	40	4.5	72	3	135.56	135.54
24	-1	-1	1	-1	30	5	120	3	139.12	138.97
25	0	0	-2	0	35	4.5	48	4	135.12	135.43
26	-1	-1	1	1	30	5.5	120	5	137.85	137.39
27	1	1	1	1	40	4.5	120	5	146.65	147.37
28	-1	-1	-1	1	30	5.5	72	5	140.25	140.40
29	1	-1	-1	1	40	4	72	5	129.21	129.06
30	0	-2	0	0	35	5	96	4	131.53	133.11

ACCEPTED MANUSCRIPT

Table 2 CCD coefficients values for endoglucanase production from mixes culture

Term constant	Coefficient	Estimation Coefficient	t-statistics	P-value
Constant	146.650	0.4022	364.605	0.000
X_1	-1.881	0.2172	-8.661	0.000
X_2	1.861	0.2172	8.568	0.000
X_3	1.490	0.2172	6.861	0.000
X_4	-0.585	0.2172	-2.691	0.016
X_1*X_1	-1.517	0.1990	-7.625	0.000
X_2*X_2	-2.454	0.1990	12.330	0.000
X_3*X_3	-2.060	0.1990	-10.351	0.000
X_4*X_4	-3.819	0.1990	-19.189	0.000
X_1*X_2	3.381	0.2660	12.707	0.000
X_1*X_3	1.878	0.2660	7.060	0.000
X_1*X_4	1.467	0.2660	5.514	0.000
X_2*X_3	1.618	0.2660	6.082	0.000
X_2*X_4	-0.751	0.2660	-2.821	0.012
X_3*X_4	0.499	0.2660	1.877	0.079

R^2 = 0.984, Adjusted R^2 =0. 971

Table 3. ANOVA analysis for the quadratic polynomial model of endoglucanase production from mixed culture.

Source of variation	Degrees of freedom(DF)	Sum of squares(SS)	Mean square(MS)	F-value	P-value
Regression	14	1176.60	84.043	74.21	<0.001
Linear	4	229.59	57.399	50.69	<0.001
Square	4	618.38	154.595	136.51	<0.001
Interaction	6	328.62	54.770	48.36	0.004
Error	16	18.12	1.132	-	-
Lack-of-Fit	10	18.12	1.812	-	-
Pure Error	6	0.00	0.000	-	-
Total	30	1194.71	-	-	-

ACCEPTED MANUSCRIPT

Table 4. Logistic constant for *Trichoderma viride, Aspergillus niger* and mixed culture.

Microorganisms	Logistic constants		
	k (h^{-1})	β (ml/mg)	R^2
Trichoderma viride	0.0303	0.0495	0.9191
Aspergillus niger	0.0315	0.0448	0.9583
Mixed culture	0.0312	0.0476	0.97521

Table 5. Substrate utilization kinetics constants for *Trichoderma viride, Aspergillus niger* and mixed culture.

Microorganisms	Substrate utilization kinetics constants	
	Y_{xs}	% Error
Trichoderma viride	0.2855	6.5415
Aspergillus niger	0.2933	9.3681
Mixed culture ***(T.viride* and *A.niger)***	0.2601	8.8004

Table 6. Leudeking Piret model constants for *Trichoderma viride, Aspergillus niger* and mixed culture

Microorganisms	Leudeking Piret constants	
	Y_{px}	% Error
Trichoderma viride	6.5051	10.3383
Aspergillus niger	5.6092	13.3510
Mixed culture *(T.viride* and *A.niger)*	9.9631	8.6234

ACCEPTED MANUSCRIPT

Table 7. Experimental and predicted values for cell concentration(x), substrate concentration(s), and endoglucanase concentration (p) for *Aspergillus niger. Trichoderma viride* and mixed culture

	Time	cell concentration(x)		substrate concentration(s)		Product (Endoglucanase) concentration(p)	
	(Hrs)	**x exp**	**x pred**	**s exp**	**s pred**	**p exp**	**p pred**
T.viride	0	4.7	4.7	57	57	0	0
	24	6	8.3858	53	44.0931	21	24.9375
	48	9.3	12.5847	39.3	29.3898	48	53.3456
	72	14.7	15.9928	20	17.4555	79	76.4038
	96	18.5	18.0771	10.4	10.1565	95	90.5062
	120	20	19.1381	5.5	6.4412	97.5	97.6846
	144	20	19.6254	4.7	4.7349	97.5	100.9813
A.niger	**Time(hrs.)**	**x exp**	**x pred**	**s exp**	**s pred**	**p exp**	**p pred**
	0	5.02	5.02	60	60	0	0
	24	8.9	8.3659	56.8	48.5884	17	18.7676
	48	11.3	12.3485	37.2	35.0051	47	41.1067
	72	16.5	16.0442	19.7	22.4003	72	61.8367
	96	18.9	18.7623	13.6	13.1299	85	77.0829
	120	22.3	20.4383	5.2	7.4137	91	86.4837
	144	22.3	21.3601	4.8	4.299	91	91.654
Mixed culture *(T.viride* and *A.niger)*	**Time(hrs.)**	**x exp**	**x pred**	**s exp**	**s pred**	**p exp**	**p pred**
	0	5.7	5.7	60	60	0	0
	24	6.9	9.1491	55	46.7417	33	34.3633
	48	12.6	12.9224	35.9	32.2366	78	71.9579
	72	16.5	16.1341	20	19.891	115	103.9557
	96	19.2	18.3339	15.5	11.4348	125	125.8729
	120	21	19.6255	6.3	6.47	145	138.7408
	144	21	20.3146	5.5	3.8209	145	145.6085

ACCEPTED MANUSCRIPT

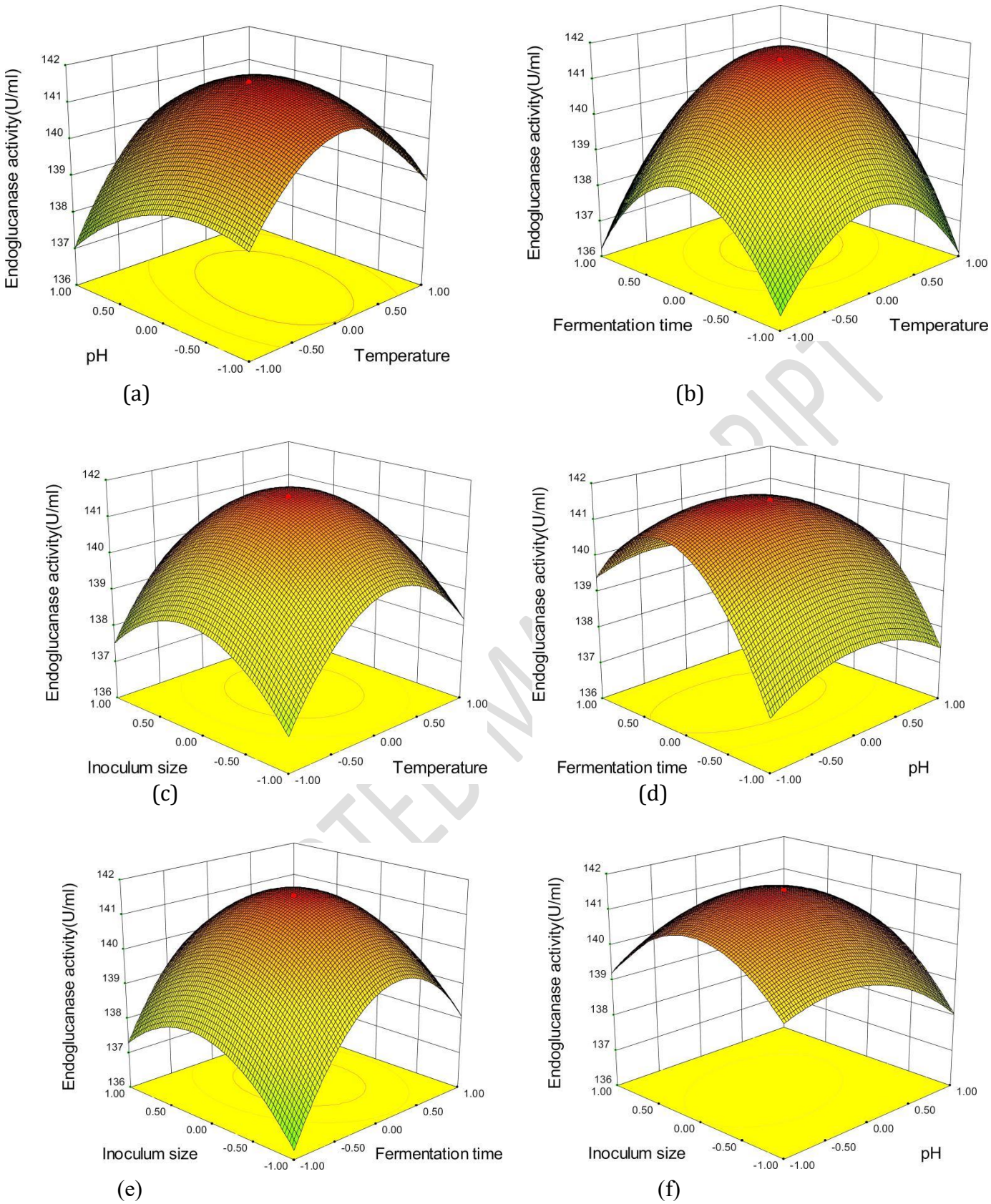


Fig 1 (a,b,c,d,e,f)Three dimensional response surface plot and isoresponse contour plot on endoglucanase production by mixed culture

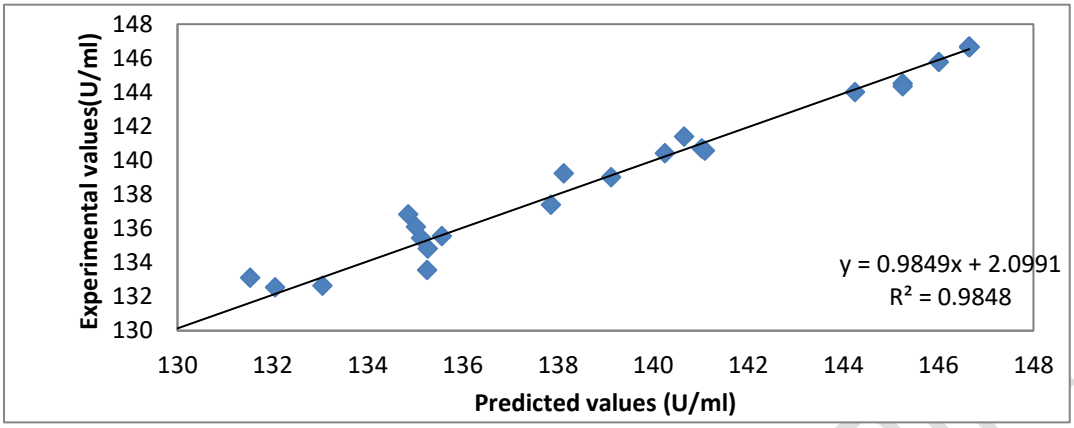


Fig.2 Comparison of experimental and predicted values for the endoglucanase production

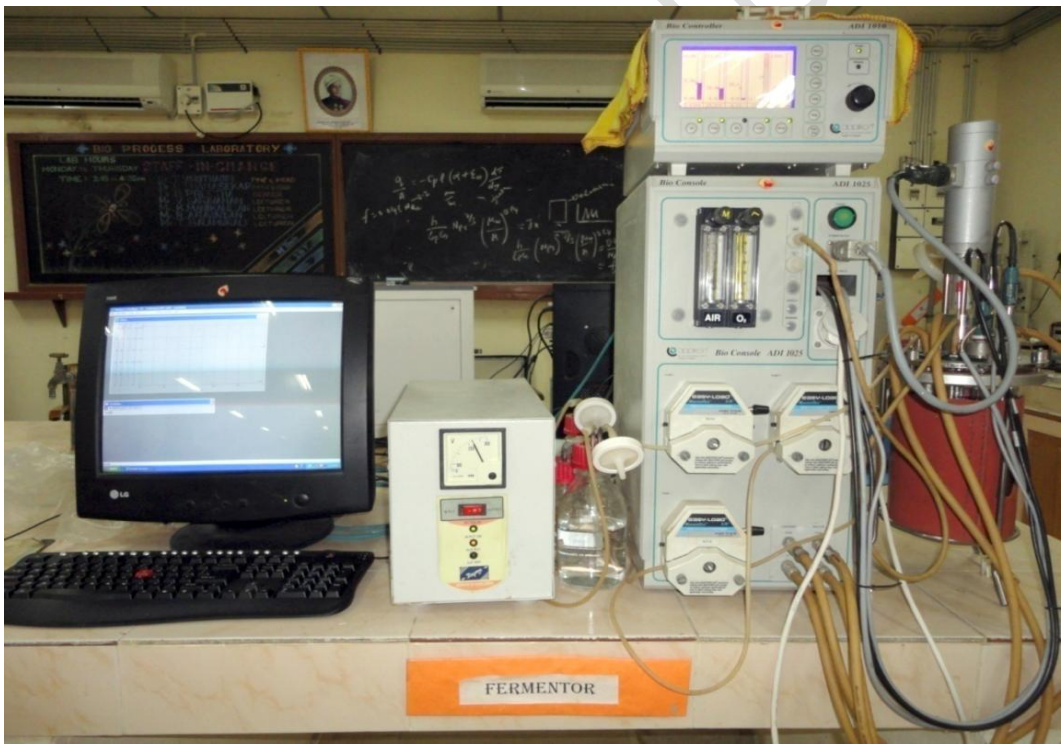


Fig.3 Online monitored batch fermenter (APPLICON Biotech, Holland)

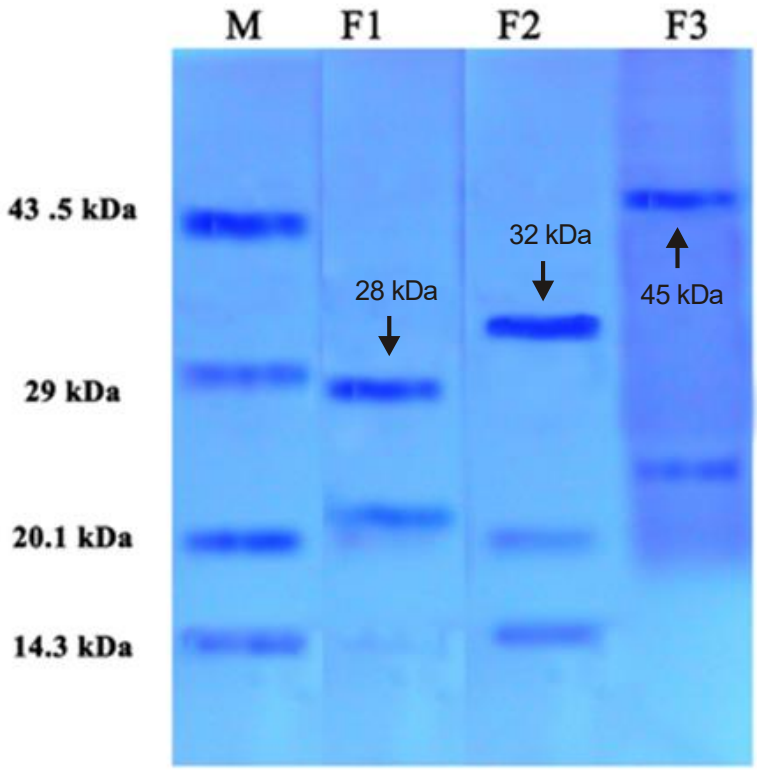


Fig 4. SDS – PAGE Analysis of the purified endoglucanase

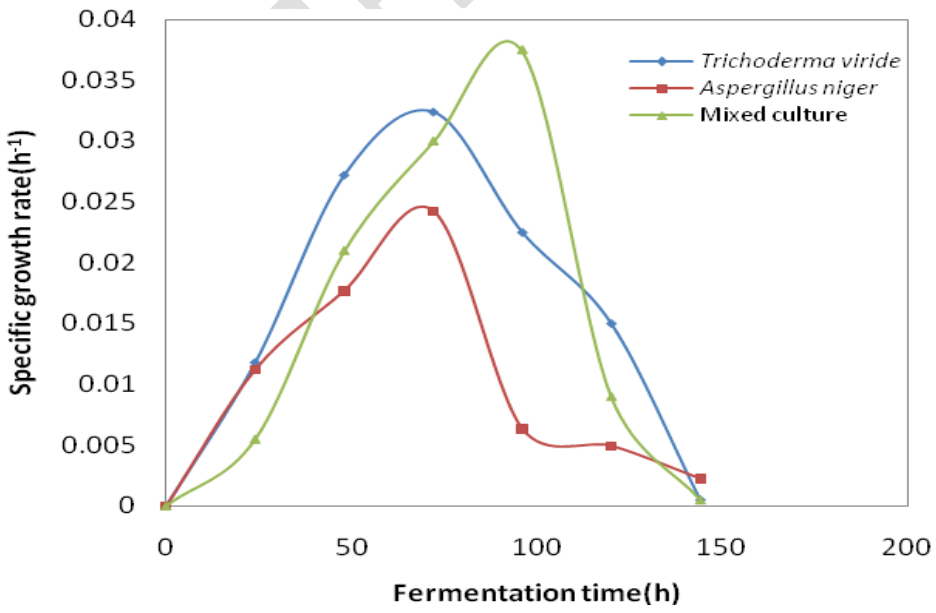
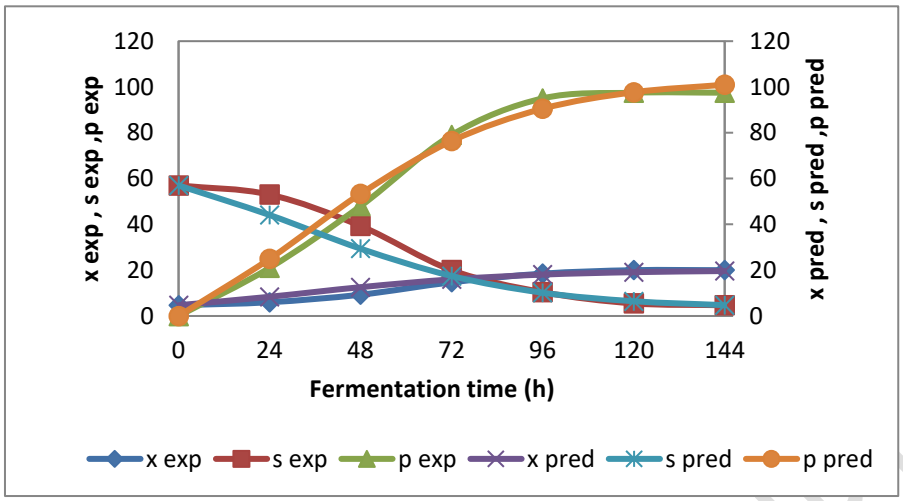
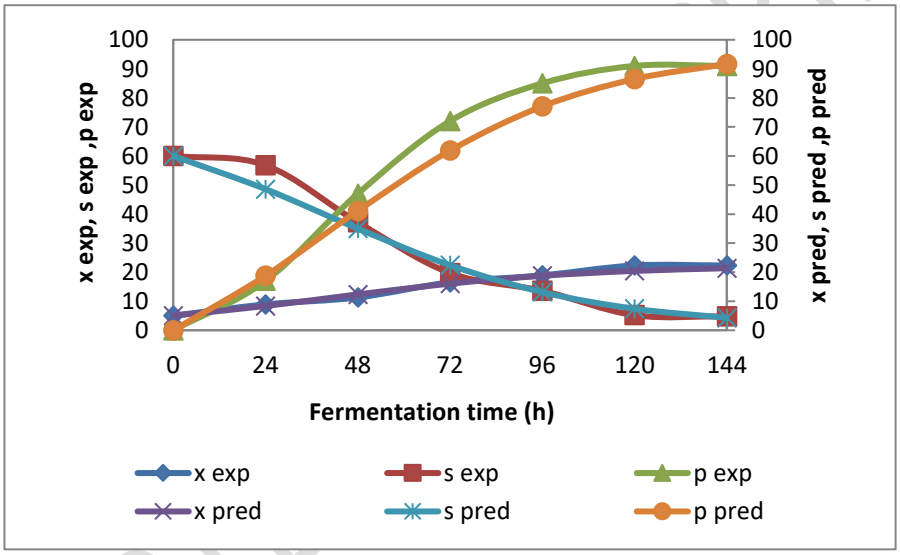


Fig 5. Specific Growth rate for Mixed and Monocultures

(a)



(b)



(c)

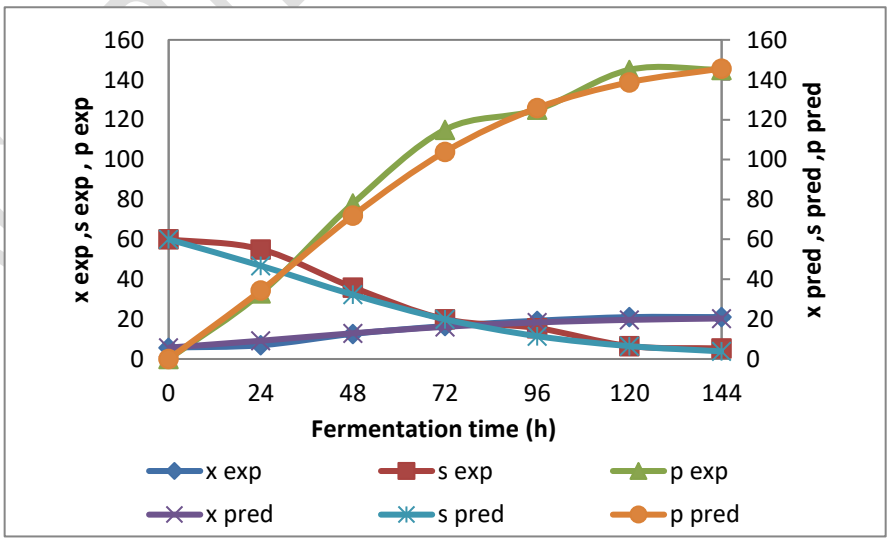


Fig 6 (a,b,c). The comparative study between the experimental and predicted values of concentration(x), substrate concentration(s), and product (endoglucanase) concentration(p).

NOMENCLATURE

K	-	Constant
K_s	-	Monod constant
P	-	Product concentration (U/mL)
S	-	Cellulose concentration, (mg/mL)
T	-	Time (hrs)
X	-	Cellmass concentration, (mg/mL)
X_0	-	Initial concentration, (mg/mL)
X_m	-	Maximum concentration, (mg/mL)
X_s	-	Concentration in the Stationary phase, (mg/mL)
$Y_{x/s}$	-	Yield coefficient (cell mass)
$Y_{p/s}$	-	Yield coefficient (product)

Greek symbols

α	-	Cell growth associated parameter (mg_p/mg_x)
β	-	non-cell growth associated parameter (mg_p/mg_xh)
μ	-	Specific growth rate, (h^{-1})
μ_0	-	initial growth rate, (h^{-1})
μ_m	-	Maximum growth rate, (h^{-1})

Abbreviations

X – Cell mass concentration, (mg/mL)

P - Concentration of endoglucanase (U/mL)

C - Concentration of Substrate, (mg/mL)

CMC – Carboxyl Methyl Cellulose

ACCEPTED MANUSCRIPT

7. REFERENCES

1. Rath, T., Rühmann, B., Schmid, J. & Sieber, V. J. C. P. (2022). Systematic optimization of exopolysaccharide production by *Gluconacetobacter sp*. and use of (crude) glycerol as carbon source. *carbohydrate polymers* . **276**, 118769.

2. Sun, L. *et al.* (2020) Effects of exopolysaccharide derived from *Pantoea alhagi NX-11* on drought resistance of rice and its efficient fermentation preparation. *International Journal of Biological Macromolecules.* **162**, 946–955.

3. Martín A. Palazzolo., Pablo D. Postemsky & Marcela Kurina-Sanz. (2019) From agro-waste to tool: biotechnological characterization and application of Ganoderma lucidum E47 laccase in dye decolorization. *3Biotech*. 9, 434**.**

4. Santos, J., Alcaide-González, M., Trujillo-Cayado, L., Carrillo, F. & Alfaro-Rodríguez, M. C. (2020). Development of food-grade Pickering emulsions stabilized by a biological macromolecule (xanthan gum) and zein. *International Journal of Biological Macromolecules.* **153**, 747–754.

5. Celebioglu, A. & Uyar, T. J. F. H. (2021). Electrohydrodynamic encapsulation of eugenol-cyclodextrin complexes in pullulan nanofibers. *Food Hydrocoll.* **111**, 106264.

6. Rana, S. & Upadhyay, L. S. B. (2020). Microbial exopolysaccharides: Synthesis pathways, types and their commercial applications. *International Journal of Biological Macromolecules.* **157**, 577–583.

7. Xu, D. *et al.* (2020). Comparison of the functionality of exopolysaccharides produced by sourdough lactic acid bacteria in bread and steamed bread. *Journal of Agricultural and Food Chemistry* . **68**, 8907–8914. https://doi.org/10.1021/acs.jafc.0c02703.

8. Khalid S., Lavania B and Naresh K Sharma. (2021) Bioremediation of Total Petroleum Hydrocarbons (TPH) by Bioaugmentation and Bio-stimulation in Water with Floating Oil Spill Containment Booms as Bioreactor Basin. International Journal of Environmental Research and Public Health., ***18*(5)** 2226**.** https://www.mdpi.com/1660-4601/18/5/2226

9. Korcz, E. & Varga, L. (2021). Exopolysaccharides from lactic acid bacteria: Techno-functional application in the food industry. Trends in Food Science & Technology. **110**, 375– 384. https://doi.org/10.1016/j.tifs.2021.02.014 .

10. Gaur, R., Tiwari, S. (2015). Isolation, production, purification and characterization of an organic solvent-thermostable alkalophilic cellulase from Bacillus vallismortis RG-07. BMC *Biotechnology*, **15**, 1-12.

11. Zubair A., Nadeem M., Shah AA., Nelofer R. (2019). Statistical optimization, production and characterization of CMCase from mutant Bacillus Subtilis ML-1UVb. *Journal of Multidisciplinary Applied Natural Science* . **11(1)**, 18–37.

12. Khalid Sayed., Lavania Baloo., Shamsul R.B.M. Kutty., Wesam Al Madhoun., Mubarak Usman Kankia., Ahmad Hussaini Jagaba and Pradeep Kumar Singa. (2022). Optimization of palm oil mill effluent final discharge as bio-stimulant for biodegradation of tapis light crude petroleum oil in seawater. *Journal of Sea Research.* **188**. 102268.

13. Khalid Sayed., Lavania Baloo., Shamsul R.B.M. Kutty., Farhann Makba. (2021). Potential biodegradation of Tapis Light Crude Petroleum Oil, using palm oil mill effluent final

discharge as biostimulant for isolated halotolerant *Bacillus* strains. *Marine Pollution Bulletin.* **172.** 112863

14. Sarma, H., Islam, N. F., Prasad, M. N. V., Renganathan, P., & Walther, C. (2021). Microbial adaptation and enzymatic degradation of agricultural and industrial waste for sustainable bioeconomy. Journal of Basic Microbiology, 61(5), 409–425. https://doi.org/10.1002/jobm.202100225

15. Irfan M., Mushtaq Q., Tabssum F., Shakir HA., Qazi JI. (2017). Carboxymethyl cellulase production optimization from newly isolated thermophilic Bacillus subtilis K-18 for saccharification using response surface methodology. *AMB Express*. **7(1)**, 1–9. doi: 10.1186/s13568-017-0331-3. [PMC free article] [PubMed] [Google Scholar]

16. Khalid Sayed., Wan Hanna Melini Wan Mohtara., Zarimah Mohd Hanafiah , Aziza Sultana Bithi., Wan Abd Al Qadr Imad Wan-Mohtar. (2024). Removal Of Pharmaceuticals from Municipal Wastewater Using Malaysian Ganoderma Lucidum Fungal Strain. Jurnal Kejuruteraan. **36(4).** 1467–1476. https://doi.org/10.17576/jkukm-2024-36(4)-12.

17. Soeka YS. (2019). Production and characterization of cellulase from the newly isolated Bacillus subtilis A8 on rice bran and corncob. *IOP Conference Series: Earth and Environmental Science.* **308(1)**, 012033. [Google Scholar].

18. Pan M., Zhu L., Chen L., Qiu Y., Wang J. (2010). Detection techniques for extracellular polymeric substances in biofilms: *A review. BioResources* **11**, 8092–8115. [Google Scholar]

19. Manikandan K., Viruthagiri T. (2009). Simultaneous saccharification and fermentation of wheat bran flour into ethanol using coculture of amylotic *Aspergillus niger* and thermotolerant *Kluyveromyces marxianus*. *Frontiers of Chemical Engineering in China***. 3,** 240-249.

20. Kola S. O, Parveen J and Md Zahangir A. (2018). Growth, substrate consumption, and product formation kinetics of *Phanerochaete chrysosporium* and *Schizophyllum commune* mixed culture under solid-state fermentation of fruit peels. *biotechnology*. **8(10)**. PMID: 30305998

21. Guichuan Ye., Liqiang Ma., Lu Li., Junli Liu., Shuangjun Yuan & Gen Huang. (2017). Application of Box–Behnken design and response surface methodology for modeling and optimization of batch flotation of coal. *International Journal of Coal Preparation and Utilization.* 40(1), 1-15 https://doi.org/10.1080/19392699.2017.1350657 .

22. Robert Marian Ruginescu, Roxana Cojoc, Mădălin Enache, Veronica Lazăr. (2018). Preliminary Characterization of a Cellulase Producing Bacterial Strain Isolated from a Romanian Hypersaline Lake. *Journal of Environmental Protection*. **9(10)**, 1066-1081. DOI: 10.4236/jep.2018.910066.

23. Khalid Sayed., Wan Hanna Melini Wan Mohtar., Zarimah Mohd Hanafiah., Wan Abd Al Qadr Imad Wan-Mohtar., Teh Sabariah Binti Abd Manan., Sarah Athirah Binti Mohamad Sharif. (2024). Simultaneous enhanced removal of pharmaceuticals and hormone from wastewaters using series combinations of ultra-violet irradiation, bioremediation, and adsorption technologies. *Journal of Water Process Engineering*, **57,** 104589

24. Hamouda RA, Sherif SA, Dawoud GT, Ghareeb MM. (2016). Enhancement of bioethanol production from Ulva fasciata by biological and chemical saccharification. *Rendiconti Lincei*. **27**, 665-672.

ACCEPTED MANUSCRIPT

25. Lakhundi S, Siddiqui R, Khan NA. (2015). Cellulose degradation: a therapeutic strategy in the improved treatment of Acanthamoeba infections. *Parasit Vectors.* **14**, 8:23 doi: 10.1186/s13071-015-0642-7

26. Douglas E Eveleigh, Mary Mandels, Raymond Andreotti and Charles Roche. (2009). Measurement of saccharifying cellulase. *Biotechnology for biofuels.* **2,** 21 *doi: 10.1186/1754-6834-2-21*

27. Marko Božinović, Tea Sokač, Anita Šalić , Ana-Marija Dukarić , Marina Tišma , Mirela Planinić, Bruno Zelić. (2023). Standardization of 3,5-dinitrosalicylic acid (DNS) assay for measuring xylanase activity: detecting and solving problems. *Croatian Journal of Food Science and Technology*. **15 (2)**, 151-162. DOI: 10.17508/CJFST.2023.15.2.03.

28. Chukwuemeka Uzukwu, Davide Dionisi. (2017). Comparison of the Biodegradation of n-alkanes and Readily Biodegradable Substrates Using Open Mixed Culture under Aerobic, Anoxic and Anaerobic Conditions. *International Journal of Environmental Bioremediation & Biodegradation*. **5(2)**, 65-76, DOI: 10.12691/ijebb-5-2-5.

29. Karunakaran S, Surendran G, Kavitha S, Jayakumar C, Reddy Prasad Donipathi Mogili, Saroj Sundar Baral. (2022). Process technology for the removal of Cr (VI) from wastewater using pig iron sludge, *Chemical Engineering & Technology*. **45(3),** 543-551**.**

30. Muthuvelayudham R, Viruthagiri T. (2006). Fermentative production and kinetics of cellulase protein on Trichoderma reesei using sugarcane bagasse and rice straw. *African Journal of Biotechnology*. **5(20**).

31. Narasimha G, Sridevi A, Buddolla V, Subhosh CM, Rajasekhar RB. (2006). Nutrient effects on production of cellulolytic enzymes by Aspergillus niger. . *African Journal of Biotechnology* . **5(5)**, 472-476.

32. Pandey A, Soccol CR, Nigam P, Soccol VT. (2000). Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource Technology.* **74(1)**, 69-80.

33. Marulasiddaiah R, Rajesh G. Kalkhambkar, Manohar V. Kulkarni. (2012).Synthesis and Biological Evaluation of Cyclic Imides with Coumarins and Azacoumarins. *Open journal of medicinal chemistry*. **2(3)**, 89-97 Doi: 10.4236/ojmc.2012.23011.

34. Kavitha M, Surendran G and Saroj Sundar Baral. (2020). Selection of suitable adsorbent for the removal of Cr(VI) by using objective based multiple attribute decision making method. *Journal Preparative Biochemistry & Biotechnology.* **51,** 69-75.

35. Sasikumar E, Viruthagiri T. (2008). Optimization of process conditions using response surface methodology (RSM) for ethanol production from pretreated sugarcane bagasse: kinetics and modeling. *BioEnergy Research*. **1,** 239-247.

36. Dhanasekar R, Viruthagiri T. (2005). Batch kinetics and modeling of poly--hydroxy butyrate synthesis from Azotobacter vinelandii using different carbon sources. *Indian Journal of Chemical Technology*. **12(3)**, 322-326.

37. Kamaluddeen, S.Y., Madika. (2023). Production of Cellulase by Trichoderma viride via Submerged Fermentation using Corncob as Substrate. *Nig. Journal of Pure & Applied Sciences.* **36 (1**). https://doi.org/10.48198/NJPAS/22.B12.

ACCEPTED MANUSCRIPT

38. Nawaz S, Malana MA, Ikram N, Hafeez S, Ghori MI, Jamil A. (2006). Kinetic study of carboxymethyl cellulase from Trichoderma harzianum. *Pakistan Journal of Life and Social Sciences*. **4 (1-2),** 15-19.

39. Immanuel G, Dhanusha R, Prema P, Palavesam AJ. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology*. **3**, 25-34.

40. Robert Marian Ruginescu, Roxana Cojoc, Mădălin Enache, Veronica Lazăr. (2018) Preliminary Characterization of a Cellulase Producing Bacterial Strain Isolated from a Romanian Hypersaline Lake. *Journal of Environmental Protection.* **9 (10**), 1066-1081. DOI: 10.4236/jep.2018.910066.

41. Elumalai S, Thangavelu V. (2010). Simultaneous saccharification and fermentation (SSF) of pretreated sugarcane bagasse using cellulose and Saccharomyces cerevisiae-kinetics and modeling. *Chemical Engineering Research Bulletin* . **14(1).** 29-35.

42. Sudhakar P, Nagarajan P. (2010) Production of chitinase by solid state fermentation from rice bran. *International Journal of Environmental Science and Development*.**1(5**), 435-40.

43. Karmakar M, Ray RR. (2011). Optimization of saccharification conditions and sugar production by endoglucanase from Rhizopus oryzae PR7 MTCC 9642 using response surface methodology. *International Journal of Pharmaceutical and Biological Research (IJPBR)*. **2(3)**, 72-80.

44. Zhang Y, Xu JL, Xu HJ, Yuan ZH, Guo Y. (2010). Cellulase deactivation based kinetic modeling of enzymatic hydrolysis of steam-exploded wheat straw. *Bioresource Technology*. 101(21), 8261-8266.

45. Jahangeer S, Khan N, Jahangeer S, Sohail M, Shahzad S, Ahmad A, Khan SA. (2005). Screening and characterization of fungal cellulases isolated from the native environmental source. *Pakistan journal of botany***. 37(3**), 739.

46. David H. Miller, Xiangsheng Xia, Wei-Chuang Huang, Ronald Rossmann. (2016). Distribution of Sediment Measurements in Lake Michigan as a Case Study: Implications for Estimating Sediment and Water Interactions in Eutrophication and Bioaccumulation Models. *Applied Mathematics*, **7(15)**, 1846-1867. DOI: 10.4236/am.2016.715153.

ACCEPTED MANUSCRIPT