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Optimization of Cellulase (E.C. 3.2.1: 4) Production Using *Penicillium citrinum* MTCC 9620 in Solid State Fermentation

G. Ghoshal[1*], U. C. Banerjee[2] and U. S. Shivhare[3]

[1]Dr. S.S. Bhatnagar University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh - 160014, India.
[2]Department of Pharmaceutical Technology, National Institute of Pharmaceutical Education and Research, SAS Nagar-160062, Punjab, India.
[3]Central Institute of Post Harvest Engineering and Technology, Ludhiana, Punjab, India.

Authors' contributions

Authors may use the following wordings for this section: This work was carried out in collaboration between all authors. Author GG designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors UCB and USS managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: The objective of the present work was to optimize the environmental parameters for Cellulase (1,4 β-endoglucanase, E.C.3.2.1:4) production using *Penicillium citrinum* MTCC 9620 in Solid State Fermentation.
Study Design: One unit of Cellulase (1,4 β-endoglucanase, E.C.3.2.1:4) activity is defined as the amount of enzyme producing 1µmole of glucose equivalent/min measured using UV visible spectrophotometer at 540 nm.
Place and Duration of Study: Food Technology laboratory of Dr. S. S. Bhatnagar University Institute of Chemical Engineering & Technology, Panjab University, Chandigarh between January and June 2011.
Methodology: *Penicillium citrinum* MTCC 9620 was maintained on potato dextrose agar (PDA) at 4°C. For Cellulase production Czapek Yeast Extract medium was used as moistening medium. Incubation temperature, pH, incubation time and other parameters like suitable substrate, pre-treatment of the substrate on production of Cellulase in Solid State Fermentation (SSF) was optimized using agricultural residues by *Penicillium*

**Corresponding author: Email: gghoshal@yahoo.com;*

citrinum MTCC 9620. Microscopic and Spectral properties of substrates were determined to detect the structural changes after pre-treatment.
Results: Production of extracellular Cellulase was greatly affected by variation in substrates, pre-treatments of substrate and variation in pH and incubation temperature. Cellulase activity was significantly ($p < 0.05$) higher when alkali treated wheat bran was used as substrate than untreated substrate. Among three substrates and their three pre-treatment conditions, It has been observed that alkali treated wheat bran was the most suitable substrate for maximum cellulase production (12.56± 0.097U/mL) at pH 5.5 and 30ºC without any extraneous nitrogen source by *Penicillium citrinum* MTCC 9620 after 120 h of fermentation time. SEM study revealed that during alkali treatment the solid surface become rough which results growth of fungus eventually maximum cellulase production.
Conclusion: *P. citrinum* MTCC 9620 is one of the potential cellulase producing fungal strain. Optimum condition of cellulase (1,4 β-endoglucanase, E.C.3.2.1:4) production by *P. citrinum* MTCC 9620 was 30°C temperature, 5.5 pH when alkali treated wheat bran was used as substrate. Growth kinetics of *P. citrinum* MTCC 9620 was studied and it showed adequacy of fit to Monod Model to describe the growth pattern of *P. citrinum* MTCC 9620 in SSF at 30°C for 120 h incubation period.

Keywords: Cellulase; solid state fermentation; Penicillium citrinum; wheat bran; optimization.

1. INTRODUCTION

Cellulase is one of the commercially important group of enzymes. Cellulase complex is a multi enzyme system comprised of the following major components; Carboxymethyl cellualase (CMCases) or Endo-ß-glucanase (EC 3.2.1.4), Exo-ß-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Fig.1) [1,2]. Cellulose, the principle component of all plant materials, is considered as one of the most abundant renewable resources in nature [3]. Cellulose is made up of glucose molecules connected by β-1, 4 bonds. Cellulose is chemically simple, but structurally a complex polymer and several enzymes are needed for complete degradation of this molecule [4]. Cellulose is regarded as a valuable resources largely because it can be decomposed into soluble cellobiose, cellotriose, cellotetrose and finally to glucose when β- bonds are broken. And the bioconversion of cellulose to soluble sugar and glucose is catalyzed by a group of enzymes called cellulases [5,6]. Cellulose hydrolysis occurs naturally in soils, sediments, aquatic environments, and in the digestive tracts of animals by microorganisms capable of producing cellulase enzymes [7]. Cellulases are widely used in paper pulp industry to enhance the bleachability of softwood kraft paper. In textile industry cellulases are used for biopolishing of cotton and other cellulosic fabrics and in detergent industry to improve color, brightness, feel, and dirt removal from the cotton blend garments. Most popular application of cellulases are in bio-fuel and bio-ethanol industries for enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corn cobs, rice straw etc. Cellulases are used in clarification of fruit juices, to improve filterability of alcoholic beverages, to extract oil and pigments like carotenoids etc [8,9,10]. It is also used in animal feed industry to improve the feed value and performance of animal [11]. Cellulolytic enzymes are produced by a wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles [12,13]. However, relatively few fungi and bacteria produce high levels of extracellular cellulase.

Agricultural wastes are generated round the year all over the world. Solid State Fermentation is one of the potential technologies to transform this type of heterogeneous polymeric agricultural residues to value added products to reduce environmental pollution and to widen the economic growth. Cellulose present in these renewable lignocellulosic materials are considered to be the most abundant organic substrate on earth for the production of glucose, for fuel and as chemical feed stock. Cellulase plays the most important role in the enzymatic hydrolysis of cellulose to glucose. From the literature it is found that the use of cellulase in food processing is lacking therefore isolation of thermo stable cellulase producing organism is most important. There is an ever present need for novel strains for the production of enzyme in high titers and the development of low cost industrial media formulations. To get the improvement in productivity in optimization of fermentation media of the microbial metabolite is achieved by manipulating the nutritional and physical parameters and sometime by mutation of strain.

Cellulase production was studied in submerged and solid state fermentation initially. Lower amount of cellulase activity was obtained in submerged fermentation compared to solid state fermentation. Not much litertature is available on application of cellulase in food processing therefore our purpose was to isolate one thermostable cellulase producing organism that can be safely used in food processing.

The aim of the present study was to investigate i) cellulase (1,4 β-endoglucanase, E.C.3.2.1:4) production using selected agricultural wastes such as wheat bran, rice husk or rice bran as substrate using *Penicillium citrinum* MTCC 9620 during Solid State Fermentation and ii) to optimize cellulase activity with respect to temperatures, pH of the medium, substrates, and nitrogen sources.

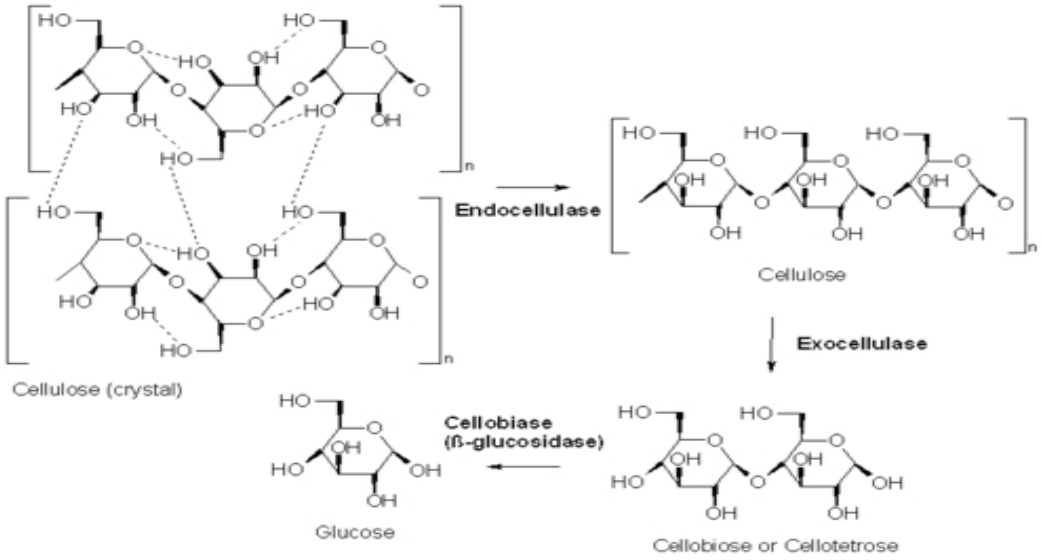

Fig. 1. Bio conversion of cellulose (http://en.wikipedia.org/wiji/cellulase)

2. MATERIALS AND METHODS

2.1 Materials

Agar powder, Potato dextrose agar (PDA), 3-5 dinitrosalicylic acid and peptone were procured from Himedia (Mumbai, India). All the reagents used were of analytical grade. Three different solid substrate rice husk, rice bran and wheat bran were gifted by Sohan Lal Rice mill Dera Bassi, Punjab.

2.2 Micro-Organism and Culture Condition

Penicillium citrinum MTCC 9620 was isolated from Panjab University soil and isolation work was carried out in Food Technology Laboratory, Dr. S. S. Bhatnagar University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh. It was identified by Institute of Microbial Technology (IMTECH), Chandigarh and was maintained on PDA and stored at 4°C.

2.3 Treatment of Substrate

Three different substrates (rice husk, rice bran, and wheat bran) were treated with 1N HCL and 0.1N NaOH, and kept for overnight in dark place individually, after that substrate those were treated with acid were neutralized with alkali and the substrate those were treated with alkali were neutralized with acid. After neutralization the treated substrates were washed with distilled water, then substrates were dried in the tray dryer (Basic technology private limited, Kolkata, India) till constant weight at 80°C. Substrates were ground in the mixer (Phillips, India) and passed through screen to ≤ 0.5mm particle size and stored in plastic jars for subsequent in Solid State Fermentation.

2.4 Scanning Electron Microscopy (SEM) Study

Dried substrates were mounted on aluminium stubs with double face tape and coated with a thin layer of gold and palladium alloy using a fine coat iron sputter (JFC-1100, JEOL, Tokyo, Japan) to impart electrical conductivity to the sample. The samples were viewed and photographed at an accelerating voltage of 10 kV and magnification in the range of 500–1500 X. Images were taken with Kodak Plus X 620 roll film and later scanned in a HP scanner.

2.5 Fourier Transform Infrared Spectroscopy (FTIR) Study

FTIR spectra were recorded at room temperature (28± 2^0C) with a Tensor-27 spectrophotometer (Bruker, Germany) in the range of 600-4000 cm^{-1} by accumulating 16 scans at 4 cm^{-1} resolution. For measuring the peak intensity of samples platinum ATR from Bruker was used. About a pinch of wheat bran sample (treated and untreated) was placed on ATR for measurement.

2.6 Solid State Fermentation Process (SSF)

Five gram of substrate of ≤ 0.5 mm particle size was taken in 250 ml Erlenmeyer flask and Czapek yeast extract medium (Sucrose 30g/L, K_2HPO_4 1 g/L, yeast extract 5 g/L, $NaNO_3$,

$7H_2O$ 3.0 g/L, KCl 0.5 g/L, $MgSO_4$, $7H_2O$ 0.5 g/L, $FeSO_4$, $7H_2O$, 10 mg/L) as a moistening media of suitable pH was added in such a way that the final solid to moisture ratio was 1:5. Then the flask containing the mixture of substrate and moistening media were autoclaved at 15 psig pressure and 121°C for 15 minutes. Then one loop full of organism (*Penicillium citrinum* MTCC 9620) was suspended in 10 ml sterile distilled water. Flasks were inoculated using 2ml of suspension in each to maintain $4x10^{-2}$ spore/ml. And incubation was carried out at different temperatures for 5 days in the incubator (Innova 4230, New brunswick, NJ, U.S.A). After 5 days incubation, enzyme was extracted using buffer of different pH at 200 rpm for 1 hour. Cell mass and the residual substrate were separated from the filtrate, and then the filtrates were centrifuged at 10,000 rpm for 10 min. The supernatants were used for the estimation of cellulase activity (U/ml) and protein content (mg/ml). Experiments were carried out in triplicate and the results of mean of triplicate experiments were reported.

2.7 Optimization of Solid Substrate

Untreated and pre-treated wheat bran, rice husk and rice bran substrates of ≤ 0.5mm particle size were used for cellulase production in SSF. 5 g of each substrate was taken in 250 ml Erlenmeyer flask and Czapek yeast extract medium as a moistening medium (pH 7) was added in such a way that the final solid to moisture ratio was 1:5. The substrate giving the maximum cellulase activity was used for the optimization of pH, temperature, and nitrogen source.

2.8 Optimization of pH

For the optimization of suitable pH, 5 g of alkali treated wheat bran of ≤ 0.5mm particle size were taken in different Erlenmeyer flasks (250 ml) and Czapek yeast extract media as a moistening media of different pH, ranging from 3 to 9 using the following buffers: M/20 citrate buffer for pH 3 to 5.5, phosphate buffer for pH 6 to 7.5, and glycine buffer for pH 8 to 9 were added in such a way that the final solid to moisture ratio was 1:5.

2.9 Optimization of Suitable Temperature

For the optimization of suitable temperature, incubation was carried out at different temperatures ranging from 20 to 40°C for 5 days in the incubator (Innova 4230, Newbrunswick, NJ, U.S.A).

2.10 Selection of Suitable Nitrogen Source

For the optimization of suitable nitrogen source, 5 g of alkali treated wheat bran of ≤0.5mm particle size was taken in four different Erlenmeyer flasks (250 ml) and Czapek yeast extract media as a moistening media of above optimized pH and 0.5 g of different nitrogen sources such as Ammonium sulphate, Ammonium chloride, Yeast extract, Peptone and Beef extract were added.

2.11 Enzyme Assay

All the enzyme assays were carried out using M/20 citrate buffer (pH 5.5). 1,4 β-endoglucanase activity was determined in accordance with the International Union of Pure and Applied Chemistry recommendation, using 1% Carboxy Methyl cellulose (CMC) as the substrate. The release of reducing sugar was determined after incubation for 30 min at 50°C,

and the reaction was terminated by adding dinitrosalicylic acid reagent followed by 10 min boiling in boiling water bath [14] and the absorbance of the mixture was measured at 540 nm in UV visible spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan). One unit of enzyme activity is defined as the amount of enzyme producing 1µmole of glucose equivalent/mL/min [15] under the given conditions.

1,4 β- Exoglucanase assay was carried out using Whatmann No.1. filter paper in 0.5 ml acetate buffer (pH 5.0) and 0.5 ml crude enzyme source. Reaction mixtures were separately incubated for 4 h at 60°C and reducing sugar released were measured by Nelson method [16]. One unit of enzyme activity is defined as the amount of enzyme producing 1µmole of glucose equivalent/mL/min under given conditions.

β- Glucosidase assay was carried out with reaction mixture containing 1.5 mL p-nitrophenyl D glucopyranoside (60.26 mg were dissolved in 100 mL sodium acetate / acetic acid buffer, pH 5.0) and 0.5 mL of enzyme extract by incubating at 60°C for 4h. Reaction was quenched by adding 2 mL of 1M Na_2CO_3 and absorbance was measured at 420 nm. One unit of enzyme activity in each case is defined as the amount of enzyme which producing 1µmole of p-nitro phenol /mL /min [17].

2.12 Protein Estimation

Total protein in the crude enzyme extract was determined by Lowry's method [18] using Bovin Serum Albumin (BSA) solution at 680 nm using Folin Cio-calteau reagent.

2.13 Dry Cell Weight

After filtration fungal biomass and the residual substrates were separated using a 0.5 mm mesh size screen. After separation of fungal cell, it was washed with distilled water and dried in the tray dryer (Basic technology private limited, Kolkata, India) at 80ºC till constant weight. After drying the dry cell weight was calculated.

2.14 Kinetic Model

Dry cell weight at different interval of fermentation was measured and Monod equation and Malthus' Law were used for modeling of the cell growth.

Malthus' Law: $\ln x = \ln x_o + \mu t$ (1)

Monod equation: $\mu = \mu_m s/(k_s + s)$ (2)

Where x is the weight of the fungal cell per unit volume, μ_m is the maximum growth rate achievable, k_s is the limiting substrate concentration.

2.15 Statistical Analysis

Multiple (triplicate) analyses were performed on each sample taken from the fermentation experiments. Data were statistically analyzed by single factor ANOVA test for significance ($p < 0.05$) using Microsoft Excel.

3. RESULT AND DISCUSSION

3.1 Production of Cellulase

Penicillium citrinum MTCC 9620 was used for the production of cellulase and the effect of environmental factors like pH, temperature and effect of other factors such as substrate, nitrogen sources were studied. Cellulase activity (U/mL), protein content (mg/mL) and dry cell weight (mg/mL) were determined to optimize the factors for maximum cellulase production. Though activity of three enzymes was determined, production of exoglucanase and β-glucosidase were negligible therefore 1, 4 β-Endoglucanase activity was reported.

3.2 Effect of Different Substrates (Agricultural Residues) and Their Pre-Treatment for Cellulase Production

Cellulase was produced by *Penicillium citrinum* MTCC 9620 on untreated, acid treated and alkali treated substrates such as wheat bran, rice bran and rice husk at 30ºC temperature, 5.5 pH, 1:5 solid to moisture ratio over a period of 120 h.

It is apparent from the Fig. 2A that the cellulase production was influenced by the type of substrate used and the pre-treatment of the substrate. In solid state fermentation fungal growth depends on few factors. Most important factor is the solid substrate, its composition and its particle size eventually that depends oxygen transfer. Another important factor is solid to moisture ratio. Water affects the physical properties of the solid support by swelling of the solid and facilitates absorption of nutrient which results growth of the fungus. Different treatments of substrate cause change in structure of solid substrate due to removal of some component or by conversion of one component to another. After 120 h of fermentation it was observed that out of three untreated and six pre-treated substrates alkali treated wheat bran at pH 5.5 and 30°C gave maximum activity (6.07 ± 0.075) U/mL and protein 7.34 mg/mL respectively. Untreated rice husk gave minimum activity (1.07 ± 0.56) U/mL and alkali treated rice bran gave minimum protein content (0.9 ± 0.76) mg/mL. And therefore alkali treated wheat bran was selected as a suitable substrate for further study for the optimization of different parameters for cellulase production.

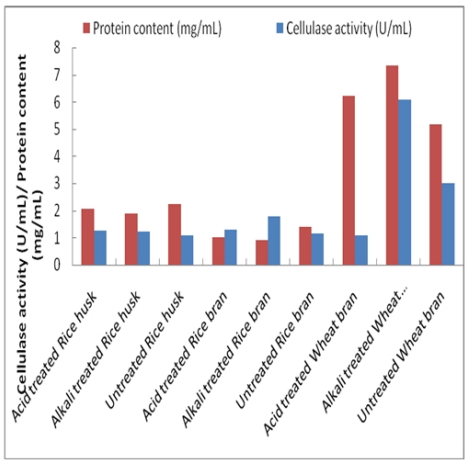

Fig. 2A. Effect of different substrates on the production of cellulase by *P. citrinum* MTCC 9620

3.3 FTIR Studies of Alkali Treated Wheat Bran and Untreated Wheat Bran

Using FTIR, spectral bands were obtained for alkali treated wheat bran and untreated wheat bran. Table 1 lists the vibrational groups and modes present in alkali treated wheat bran and untreated wheat bran samples. As shown in Fig. 2B, the spectra appeared rather similar, but due to stretching of bonds remarkable difference in intensities were observed, which suggested that during alkali treatment the micro structure of the substrate (untreated wheat bran) altered. From Fig. 2B, it has been observed that due to stretching of C-O, C-C and sp^3 C-H, O-H bonds, peak intensity decreased in case of alkali treated wheat bran. Significant decrease in intensity is observed in the region of 1640 cm^{-1} due to removal of free COO^- group. This may be due to washing of organic acids, proteins and free fatty acid in lipid fraction of bran.

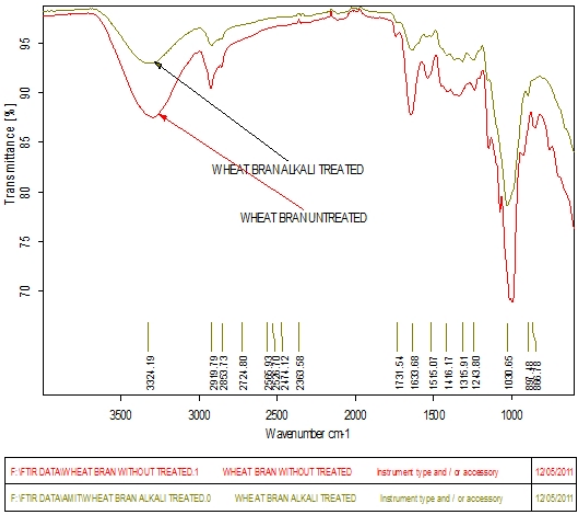

Fig. 2B. FTIR spectra for alkali treated cellulase and untreated wheat bran

Table 1. Vibrational mode in the FTIR spectra

Wave number (cm^{-1})	Vibrational group and mode
900-1106	C-C and C-O stretching
1200-1416	Bending of O-C-H, C-C-H and C-O-H
1640	Free COO^-
2930	sp^3 C-H stretching
3000-5000	O-H stretching

And the largest vibration in spectra of wheat bran for both the substrates was found from 900-1106 cm^{-1}. There may be stretching of C-C and C-H bond. Bending vibration in the range of 900-1106 cm^{-1} indicated that after alkali treatment the bran structure changed to more solid like compact structure. Band intensities decreased in alkali treated wheat bran; might be due to solubilization of non-starch polysaccharides. Therefore it can be inferred that readily utilizable sugar contents for the use of microorganisms as nutrient source increased therefore maximum growth might be obtained in alkali treated wheat bran.

3.4 Scanning Electron Microscopy (SEM) for Wheat Bran (Alkali Treated and Untreated)

Fig. 2C and 2D showed the Scanning electron micrograph of untreated wheat bran and alkali treated wheat bran. From the Figs. 2C and 2D it is clear that outer surface of alkali treated wheat bran was rough as compared to untreated wheat bran. It can be inferred that alkali treatment might cause removal of lipids and proteins from the surface of the wheat bran. As lipids and proteins are acidic in nature, alkali treatment might have removed acidic moiety, leaving readily utilizable carbohydrates on the surface. The surface of the wheat bran became rough, and therefore it is suitable for microorganism to grow on the surface of alkali treated wheat bran.

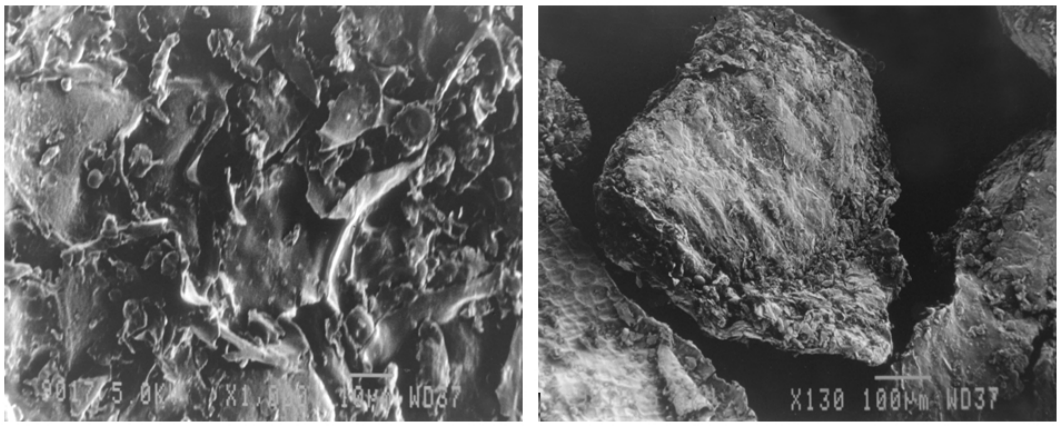

Fig. 2C. SEM for untreated wheat bran

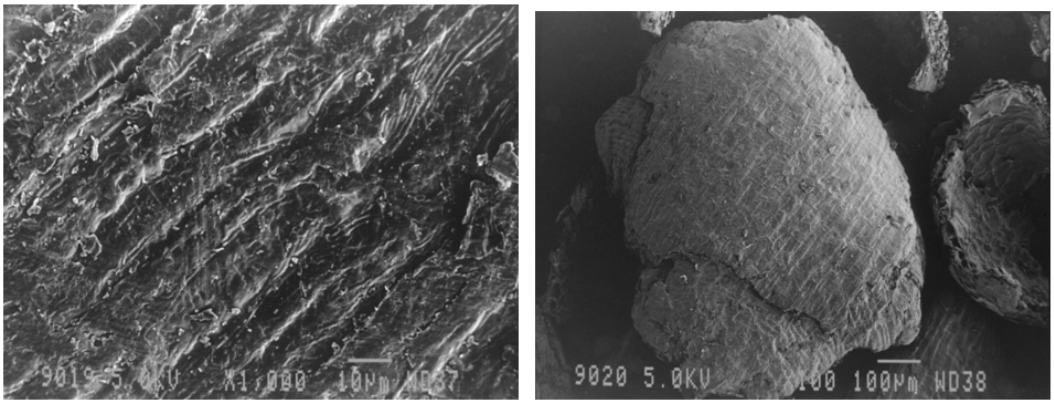

Fig. 2D. SEM for alkali treated wheat bran

From Fig. 2A It is clear that alkali treated wheat bran is the most suitable substrate for cellulase production and the results of FTIR and SEM support the same for the production of cellulase. Therefore alkali treated wheat bran was selected as the most suitable substrate for further study of optimization of different parameters such as pH, temperature and suitable nitrogen source for cellulase production.

3.5 Optimization of pH for the Production of Cellulase

Effect of pH on cellulase production was studied by *Penicillium citrinum* MTCC 9620 using alkali treated wheat bran as substrate at 30ºC, solid to moisture ratio 1:5 for 120 h varying pH from 3 to 9 using 1N HCl and 0.1N NaOH. Czapek Yeast extract media was used as moistening agent. Fig. 3A describes significant variations in cellulase production. From the data it is clear that pH levels affected cellulase production drastically. It is also clearly seen that at pH 5.5 cellulase activity and protein content were maximum. At highly acidic pH, the production was quite lower, and it was progressively increased from pH 3 to 5 and exhibited maximum cellulase activity (10 U/mL) at pH 5.5 after 120 h of fermentation. There was a decrease in enzyme production at all pH levels beyond pH 5.5. It is clear that highly acidic pH and alkaline pHs are not suitable for the growth of *P.citrinium* they might exhibit some adverse effect. Therefore decrease in the production of cellulase was observed. pH 5.5 was selected as the most suitable pH for the production of maximum cellulase when alkali treated wheat bran was used as solid substrate. At pH 3 to 3.5 protein content was found almost constant then a sharp increase in the protein content was observed from pH 4 to 5.5 then gradual decrease of protein content from pH 5.5 to 9 was observed. It can be concluded that though cellulase activities were very less in the pH range 6 to 9 but substantial amount of proteins were present. Therefore it is inferred that except cellulase other undesirable proteins were present in the media. The maximum cellulase activity and protein was observed at pH 5.5 (10 ±0.67) U/mL and (5.17 ± 0.06) mg/mL respectively. Previous reports showed that cellulase show optimum activity at slightly acidic range of pH [19] which supported our findings also.

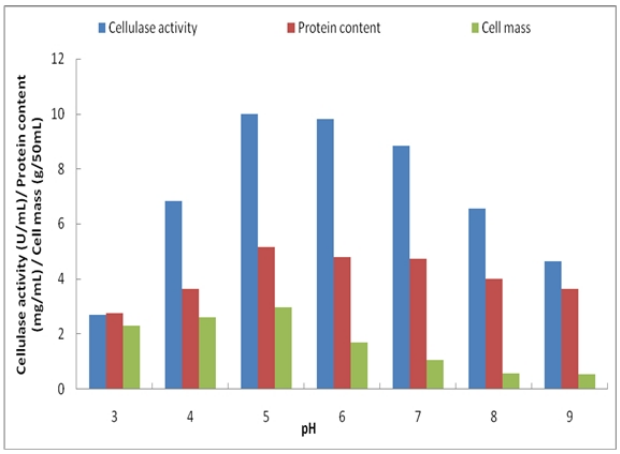

Fig. 3A. Effect of pH on Cellulase activity, protein content and cell mass using *P. citrinum* MTCC 9620

3.6 Optimization of Temperature for the Production of Cellulase

Solid State Fermentation was carried out for the production of cellulase by *Penicillium citrinum* MTCC 9620 on alkali treated wheat bran at 5.5 pH, using 1:5 solid to moisture ratio varying incubation temperatures (20-40°C) for 120 h. Czapek Yeast extract medium was used as a moistening media. Fig. 3B represented an increasing trend in cellulase activity from 20 to 30ºC and then showed a decline in cellulase activity. It is clear that when the incubation temperature for the production of cellulase was kept at 30ºC the organism

produced maximum cellulase (12.56 ± 0.09) U/mL at 120 h which simultaneously decreased to (3.9± 0.64) U/mL at 40°C. Protein content gradually increased from 20 to 30°C and started decreasing above 30ºC. Reduction in enzyme production at higher temperature can be caused due to denaturation of enzyme or its inactivation at higher temperature.

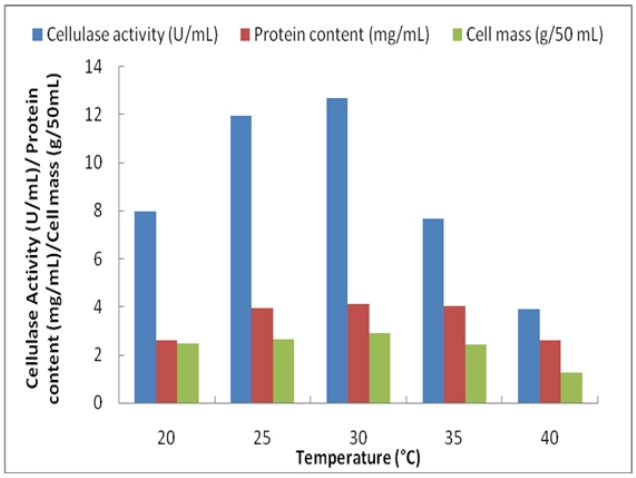

Fig. 3B. Effect of different incubation temperature on Cellulase activity, Protein content, Cell mass using *P. citrinum* MTCC 9620 at pH 5.5

Fig. 3B also represented the production of cell mass at different pH levels. It is clear that from temperature 20-30°C cell mass was steadily increased and above temperature 30°C cell mass decreased progressively. It is clear that at temperature 30°C cell mass was maximum (2.89 ± 0.89) g/mL. Therefore enzyme production and cell mass content can be correlated, it showed that at temperature 30°C both were maximum.

3.7 Optimization of Nitrogen Source for the Production of Cellulase

Fig. 3C indicates the effects of addition of different types of nitrogen sources on the cellulase production by *Penicillium citrinum* MTCC 9620 on alkali treated wheat bran. The variation in nitrogen sources (sodium nitrate, peptone, urea, beef extract) showed significant effect on cellulase production at pH 5.5 and incubation temperature 30ºC for 120 h fermentation period. It is apparent from the data that cellulase has given the maximum activity when no other extraneous nitrogen source was added. Fig. 3C depicted the production of cellulase by *Penicillium citrinum* MTCC 9620 on alkali treated wheat bran at 30ºC incubation temperature and pH 5.5. The graphical representation indicates addition of nitrogen source has no effect on cellulase activity. The fungus exhibited maximum protein 4.7 mg/mL in control sample where no extraneous nitrogen source was added. It can be concluded that there was no need to add extra amount of nitrogen source for the production of cellulase using *Penicillium citrinum* MTCC 9620. It is clear from Fig. 3C that cell mass was maximum when no extraneous nitrogen source was added.

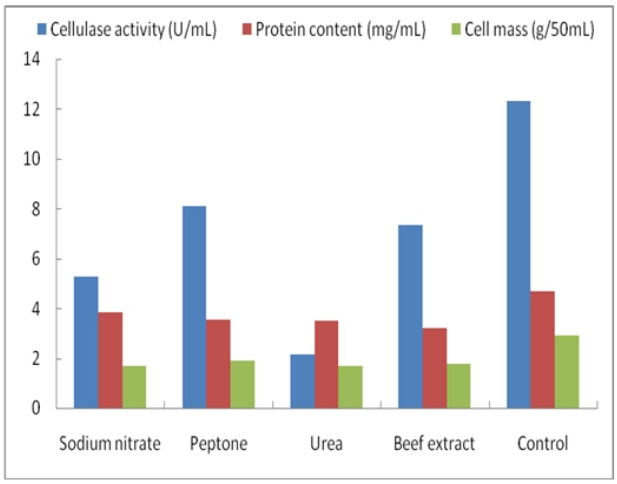

Fig. 3C Effect of different nitrogen sources on Cellulase activity, Protein content and Cell mass using *P. citrinum* MTCC 9620 at pH 5.5 and 30°C

3.8 Course of Growth of *P. citrinum* and Cellulase Production during Incubation at 30ºC

Cellulase production by *Penicillium citrinum* MTCC 9620 was studied using alkali treated wheat bran as substrate at pH 5.5, 30ºC, 1:5 solid to moisture ratio during fermentation in SSF. From Fig. 4A it is apparent that cellulase activity was progressively increased from 0 to 120 h and exhibited maximum cellulase activity at 120 h. It was observed that there was a decrease in the cellulase activity after 120 h. After 120 h, stationary phase started and followed by death phase, eventually cellulase activity decreased in the media. It can be inferred that optimum time for the growth of microorganism in the optimized media was 120 h. From Fig. 4A it is clear that there was a sudden increase in the protein content from 12 to 24 h incubation time. After 24 h protein content was gradually increased till 120 h incubation time. And maximum protein content was obtained after 120 h incubation, after that protein content was gradually decreased. There was an increase in the production of the cell mass on the surface of the substrate. Constant increase in the production of the cell mass from 0 to 120 h was observed. At 120 h the cell growth was maximum (2.43 g/mL), after that microbial growth declined to almost constant (2.42 g/mL) might be due to lack of substrate. Fig. 4A described that residual substrate was gradually declined from 0 h to 84 h due to consumption of the substrate by the organism, after 84 h the consumption of the substrate by the organism was almost constant because substrate concentration decreased to limiting substrate concentration which reflected in the cellulase production, protein content as well as cell mass production pattern also. Therefore specific growth rate decreased after 120h of fermentation.

3.9 Kinetic Model

Based on measured data a straight line was found between cell mass weight (x, mg/mL), and incubation time (t, hour) between the incubation period 36 h to 120 h. Exponential phase was found at that period of fermentation. The linear fit was found between increase of cell

weight with time. R^2 for this equation was 0.9744 and SE was 0.041152, which prove the adequacy of fitting. Therefore equation (1) can be written as

$$\ln x = 0.008t + 2.9759 \quad (1)$$

Specific growth rate for exponential phase was 28.8 s^{-1}. And initial cell weight was 19.60 mg/mL.

From equation (1) cell weight was calculated at different interval of fermentation time in exponential phase. Fig. 4B represents the plot of experimental and calculated values of cell weight (mg/mL) with respect to fermentation time (h). From Fig. 4B it was found that calculated values of cell weight was almost similar to experimental cell weight values. The present study established that Malthus Law is adequate to predict correctly the cell weight.

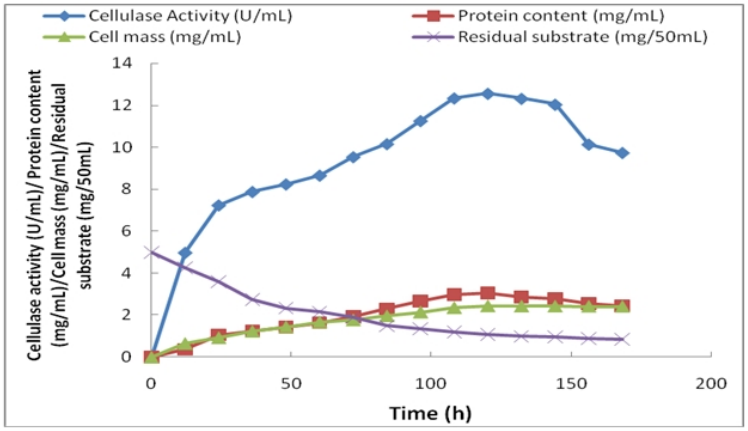

Fig. 4A. Growth curve of *P. citrinum*

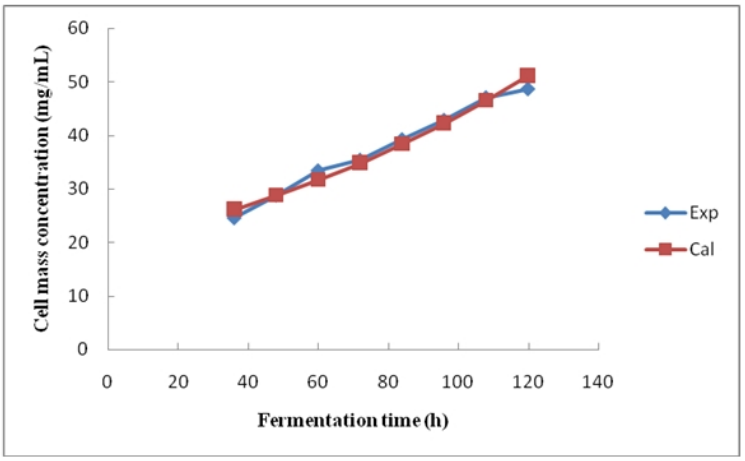

Fig. 4B. Plot of cell mass vs fermentation time during exponential phase

Specific growth rate (μ) at different period was determined and 1/μ was plotted with respect to inverse of residual substrate concentration (1/s). A straight line was found with R^2 value of

0.8292. Slope was 261.74 and intercept was found 10.45. Therefore we can write equation (2).

$$1/\mu = 261.74(1/s) + (-10.45) \quad (3)$$

From the above equation $1/\mu_m = 10.45$, therefore $\mu_m = 0.0956$ (h^{-1}) And $K_s/\mu_m = 261.7$. Therefore $K_s = 25.02$ mg/mL

Above data shows the adequacy of fit to Monod Model to describe the growth pattern of *P. citrinum* in SSF at 30ºC for 120 h incubation period.

4. CONCLUSION

The present work was done to optimize the different environmental parameters for the production of cellulase (1,4 β-endoglucanase, E.C.3.2.1:4) in Solid State Fermentation using agricultural residue. Optimization of cellulase was carried out in terms cellulase activity, protein content, cell weight, residual substrate, microscopic and spectral characteristics. It was observed that production of cellulase was greatly affected by variation of substrate and their pretreatment, variation in pH and temperature range. It was found that alkali treated wheat bran was the most suitable substrate for cellulase production. FTIR and SEM study also confirmed the results. The optimized condition for cellulase production was 30°C, pH 5.5 on alkali treated wheat bran without any extraneous nitrogen source, maximum cellulase activity was obtained (12.56 ± 0.09) U/mL after 120 h incubation period.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

REFERENCES

1. Wood TM, McCrae SI. The cellulase of *Penicillium pinophilum*. Synergism between enzyme components in solubilizing cellulose with special reference to the involvement of two immunologically distinct cellobiohydrolases. Biochemical Engineering Journal. 1986;234:93-99.
2. Available: http://en.wikipedia.org/wiji/cellulase.
3. Cen P, Xia L. Production of cellulase by solid-state fermentation. Advances in Biochemical Engineering Biotechnology. Edition: T. Scheper and G. T. Tsao. Springer, New York. 1999;69-92.
4. Wood TM, Carcia- Campayo V. Enzymology of cellulose degradation. Biodegradation 1990;1:147-161.
5. McHale A, Coughlan MP. Synergistic hydrolysis of cellulose by components of the extracellular cellulase system of *Talaromyces emersonii.* Federation of European Biological Societies Letters. 1980;117:319-322.

6. Bhat MK, Bhat S. Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advances. 1997;15:583-620.
7. Leschine SB. Cellulose degradation in anaerobic environments. Annual Review of Microbiology. 1995;49:399-426.
8. Mandel M, Andreotti R, Roche C. Measurement of saccharifying cellulase. Biotechnology and Bioengineering Symposium. 1976;6:21-33.
9. Sandhu DK, Kalra MK. Production of cellulase, xylanase, pectinase by *Trichoderma longibrachiatum* on different substrate. Transaction of the British Mycological Society. 1982;79:409-413.
10. Spiro RG. In methods of Enzymology, 8 edited by S.P. Colowick and N.O. Kaplan (Academic Press, New York) 1966;3-26.
11. Kuhad RC, Gupta R, Singh A. Microbial cellulases and their industrial applications. Enzyme Research. 2011; doi: 10.4061/2011/280696.
12. Coughlan MP, Ljungdahl LG. Comparative biochemistry of fungal and bacterial cellulolytic enzyme system. In: biochemistry and genetics of cellulose degradation. Fedaration of European Microbiological Societies Symposium. 1988; 43:11-30.
13. Ljungdahl LG, Eriksson KE. Ecology of microbial cellulose degradation. Advance in Microbial Ecology. 1985;8:237-299.
14. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959;31:426.
15. Ghosh T. Measurement of cellulose activities. In Commision on Biotechnology. IUPAC. 1994;1-12.
16. Nelson NA. Photometric adaptation of somogyi method for determination of glucose. Journal of Bio-Chemistry. 1944;15:375.
17. Ahlawat OP, Vijay B. potential of thermophillic bacteria as microbial Inoculants for commercial scale white button mushroom (*Agaricus bisporus*) compost production. Journal of Scientific & Industrial Research. 2010;69:948-955.
18. Lowry OH, Roseborough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951;193:265-275.
19. Dutta T, Sahoo R, Sengupta R. Novel cellulase from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. Microbial Biotechnology. 2008;35:275-282.

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