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Statistical optimization of xylanase and alkaline protease co-production by *Bacillus spp* using Box-Behnken Design under submerged fermentation using wheat bran as a substrate

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ABSTRACT

This study explores the co-production of xylanase and alkaline protease from *Bacillus licheniformis* NRRL 14209 in single step using agricultural waste as a substrate. The aim of the present study was to screen the low cost and easily available agricultural lignocellulosic wastes as a substrate to substitute commercially available xylan. Five agricultural wastes were screened as a substrate and wheat bran was found to be most suitable for co-production of xylanase and alkaline protease. Optimization of various fermentation parameters affecting enzyme co-production by *B. licheniformis* was investigated. Maximum enzyme productivity of xylanase 4.31 U/mL and alkaline protease 3.66 U/mL was obtained at 24 h of incubation period, initial media pH 8.5 with 0.5% w/v wheat bran and 4% (v/v) inoculum concentration at 30 °C temperature. Box-Behnken Design was applied to obtain optimal concentrations of significant variables such as substrate concentration (0.25% w/v), inoculum concentration (4%v/v) and pH of 7.5. The maximum xylanase and alkaline protease production after Box-Behnken Design was 5.49 U/mL and 4.87 U/mL respectively, which are 1.27 and 1.33 fold higher than initial one factor at a time media optimization. Xylanase and alkaline protease isolated in this study can be useful in pulp pre-bleaching process to remove the hemicelluloses and in detergent industry respectively.

1. Introduction

The most abundantly available and renewable biomass on earth is lignocellulose, consisting of three major groups of polymers; lignin, hemicellulose, and cellulose. Cellulose is the dominant structural polysaccharide of plant cell walls (35–50%), which is followed by hemicellulose (20–35%) and then lignin (10–25%) (Saha, 2003). Hemicelluloses contain heteropolymers like xylan, mannan, galactan and arabinan. D-xylose, D-mannose, D-galactose and L-arabinose are the principle monomers present in the most hemicelluloses. Xylans are heteropolymers containing D-xylose as its main monomeric structural unit and traces of L-arabinose (Beg et al., 2001).

Xylanases (endo-1, 4-β-d-xylanohydrolase; EC 3.2.1.8) are glycosidases (O-glycoside hydrolases) which catalyze the endohydrolysis of 1, 4-β-D-xylosidic linkages in xylan (Sanghi et al., 2008) that leads to the formation of a sugar hemiacetal moieties and the corresponding free aglycone (Motta et al., 2013). Xylanases are extensively used in pulp and paper industries. Other applications of xylanases include, bioconversion of agricultural wastes and lignocellulosic material to fermentative products, clarification of juices, improve consistency of beer and the overall digestibility of animal feed stock (Polizeli et al., 2005). Xylanase has been produced mainly from bacteria (Talamantes et al., 2016), fungi (Polizeli et al., 2005), yeast (Otero et al., 2015) and actinomycetes (Saini et al., 2015).

Purified xylan can be used as a substrate for the production of xylanase but the process becomes uneconomical. Use of low cost and abundantly available agricultural wastes in place of commercially available xylan reduces the overall process cost (Namvar et al., 2014). Various agricultural wastes such as rice bran, corn stover, corn cobs, wheat bran, mustard seed bagasse, sugarcane bagasse can be used as a substrate for the cost effective production of xylanase (Mussatto and Teixeira, 2010).

Microbial proteases are amongst the most important proteolytic enzymes that hydrolyse peptide bonds in aqueous environments and able to synthesize peptide bonds in micro aqueous environments (Tunga et al., 2003). Proteases play an important role in the cellular metabolic processes and also gained considerable attention in the industrial sector (Gupta et al., 2002). Alkaline proteases have optimum pH in the range of 7–11 and can be produced from various sources like plants (Domsalla and Melzig, 2008) and microorganisms.

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Microorganisms like bacteria (Zhou et al., 2009) and fungi (Rizzello et al., 2007) produce various types of proteases which are extracellular and/or intracellular in nature. Intracellular proteases play an important role in cellular as well as metabolic processes. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments thereby assisting the cells to absorb and utilize hydrolytic products (Kumar et al., 2016). Proteases produced from microorganisms are preferred over plant and animal sources as they possess almost all the characteristics required for their biotechnological applications (Kumar and Takagi, 1999). Proteases have wide range of application in various industries like food and detergent industries, leather industry, pharmaceutical, silk industries and for recovery of silver from used X-ray films (Virupakshi et al., 2005; Gessesse and Gashe, 1997; Manachini and Fortina, 1998).

The growing awareness about utilization of agricultural waste as a substrate for value added chemicals in last decade has made industrial enzymes such as xylanase and cellulase most popular. These enzymes are in great demand as they effectively degrade agricultural biomass to simple sugars which can be utilized for the production of healthy, environmentally friendly and value added chemicals. So there is a need of novel and cost effective bioprocess for the production of high titres of xylanases that meet the industrial demand. Additionally, alkaline protease which accounts for roughly 55% of total market share is another most important enzyme of detergent industry (Mamun et al., 2015). Thus, present report coins a novel and cost effective process for the production of xylanase as well as alkaline protease simultaneously using industrially important microbe *Bacillus licheniformis* NRRL 14209. Different fermentation parameters were optimized using one factor at a time approach. The classical method, “one factor at a time” design of bioprocess does not consider the interaction of variables and fails to give combined effect of factors on overall enzyme production. Response surface methodology (RSM) is a statistical and mathematical tool useful to develop, improve and optimize the processes in which response of interest is influenced by several variables to optimize the response (Bas, 2007). Present study aims to develop a process by which xylanase and alkaline protease can be produced simultaneously in single step with statistical optimization of significant parameters such as substrate concentration, inoculum concentration and pH for maximum co-production of enzymes using Box Behnken Design (BBD).

2. Materials and methods

2.1. Materials

Beechwood xylan for xylanase assay and casein for protease assay were purchased from Hi-media Laboratories Pvt. Ltd., India. All the other media components and chemicals used were of highest purity grade made available commercially from Hi-media and SD Fine Chemicals Limited; Mumbai. Rice bran, Wheat bran, Corn cobs, Mustard seed bran and Sugarcane bagasse were collected from local farmers of Pune, Maharashtra, India.

2.2. Microbial strain

The bacterial strain used in this study was acquired from ARS culture collection centre (NRRL). This strain was isolated from ground black pepper and was identified as *Bacillus licheniformis* by ARS culture collection centre, with the accession number NRRL B 14209. The optimum growth temperature of this strain was 28 °C. The culture was maintained on nutrient agar slants, stored at 4 °C and sub-cultured routinely every three-four weeks.

2.3. Inoculum preparation

For inoculum preparation, the culture was grown in a sterile nutrient broth at 28 °C ± 2 °C for 8–12 h to obtain seed culture in a log phase. The seed culture was then used to inoculate 50 mL production media at 2% (v/v) concentration and incubation is continued for enzyme co-production at 30 °C.

2.4. Media preparation

Production media for enzyme production was prepared as different media formulations varying in their content of carbon to nitrogen ratio. The different media formulations studied are **Media 1**- Beef extract 10 g/L, peptone 10 g/L, NaCl 1 g/L, KH_2PO_4 1 g/L, Na_2CO_3 5 g/L, xylan 10 g/L. **Media 2**- glucose 10 g/L, yeast extract 5 g/L, tryptone 5 g/L, K_2HPO_4 1 g/L, xylan 10 g/L. **Media 3**- yeast extract 10 g/L, NH_4NO_3 10 g/L, NH_2PO_4 5 g/L, $MgSO_4$ $7H_2O$ 1 g/L, $CaCl_2$ $2H_2O$ 0.1 g/L, $MnSO_4$ H_2O 0.1 g/L, xylan 10 g/L. **Media 4**- yeast extract 5 g/L, peptone 5 g/L, $MgSO_4$ $7H_2O$ 0.2 g/L, K_2HPO_4 1 g/L, xylan 5 g/L. In all cases, 50 mL of production media was prepared in 250 mL Erlenmeyer flask, autoclaved at 121 °C for 20 min and inoculated with log phase seed culture for enzyme co-production.

2.5. Xylanase production on various substrates

Various agricultural wastes such as rice bran, wheat bran, corn cobs, mustard seed bran and sugarcane bagasse were screened as a substrate for the production of xylanase by *B. licheniformis*. These agricultural wastes were milled and sieved to particle size of 0.5 mm ± 0.1 mm. Fifty millilitres of media 2 containing (%w/v): glucose 0.10, yeast extract 0.50, tryptone 0.50, K_2HPO_4 0.10 and substrate 1.0 was placed in 250 mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min at 15 psi. The flasks were then cooled and inoculated with 4% (v/v) of 12 h old seed culture of the bacterial strain. Initial cell density of production media was maintained at 0.05 absorbance units measured spectrophotometrically at 600 nm. The, flasks were incubated at 30 °C at 180 rpm till a stationary phase was reached. During the fermentation, samples were collected at regular time interval of 4 h and harvested by centrifugation at 12000 × *g* for 10 min at 4 °C. The cell-free supernatant obtained which is a crude enzyme extract was used for the enzyme activity assays. All the experiments were performed in duplicates and average of the enzyme activities was recorded. Enzyme activities against time were plotted to determine the optimum incubation time for the co-production of xylanase and alkaline protease.

2.6. Xylanase assay

Xylanase activity was measured using 3,5-dinitrosalicylic acid (DNS) assay with xylan as a substrate (Miller, 1959). Beechwood xylan (1%w/v) was used as substrate and dissolved in 50 mM Sodium phosphate buffer of pH 7.0. For the enzyme reaction, 0.1 mL of the cell-free supernatant was added to 0.9 mL of substrate solution in a test tube and incubated at 50 °C for 5 min. Termination of reaction was done by the addition of 1 mL of DNS and the mixture was incubated at 95 °C for 5 min and allowed to cool to room temperature. An enzyme blank was run simultaneously which contained all the reagents except an enzyme extract. The amount of D-xylose released was determined by measuring its absorbance at 540 nm. The activity of xylanase was measured according to the D-xylose standard curve. One unit (U) of enzyme activity on the substrate is defined as the amount of enzyme required to release 1 μmol of D-xylose per minute under the assay conditions.

2.7. Protease assay

Protease activity was measured by Anson-Hagiwara's method as mentioned by Shimogaki et al. (1991). One unit of alkaline protease activity (1 APU) was defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the standard assay conditions as mentioned in the above mentioned literature.

2.8. Protein content

Total protein content was measured by Bradford assay as described by Bradford (1976) using Bovine Serum Albumin (BSA) as a standard. The color formation was measured against blank at 595 nm using spectrophotometer.

2.9. Statistical analysis

One factor ANOVA in Microsoft excel was used to analyse the experimental results. Statistical analysis is considered as an important tool to interpret and summarise the experimental data. All the experiments were carried out in duplicate and data obtained is represented as mean ± SD in this paper. P-values of the experimental data are reported in respective figures. The P-value less than 0.05 is considered to be statistically significant.

3. Results and discussion

3.1. Effect of media

Media composition is one of the most important parameters that affects the growth as well as enzyme production in bacterial cells. Effect of different media composition on co-production of xylanase and alkaline protease from *B. licheniformis* was studied. Different media compositions of varying carbon to nitrogen ration (C/N ratio) are studied as described in Section 2.4. Fig. 1 demonstrates the effect of different media formulation on enzyme co-production. Due to high growth rate in media 2, it recorded the highest optical density which resulted in higher protein content. Higher protein content can be attributed to higher enzyme production and thus higher the enzyme activity in media 2. While, media 4 and media 3 has lower growth rates as compared to media 2 which resulted in lower enzyme production and lower enzyme activity. Media 1 seems to be not suitable for the growth and production of enzymes by *B. licheniformis*. Thus it can be concluded that media 2 is the optimum mixture of growth components and optimum carbon to nitrogen (C/N) ratio that can result in maximum enzyme co-production at standard fermentation conditions. Further studies are carried out with media 2 while pure xylan is replaced with different agricultural wastes for cost effective production of both the enzymes.

3.2. Effect of incubation time on screening of substrate

Most of the enzymes are produced in the log phase of cell growth as they act as primary metabolites whereas secondary metabolites are produced in stationary phase of cell growth (Hesketh et al., 2002). To optimize the growth phase at which xylanase and alkaline protease enzymes are produced maximally, the fermentation broth was harvested at a regular time interval of 4 h till a stationary phase and analyzed for respective enzyme activities. Here xylan of media 2 was replaced with agricultural wastes such as wheat bran, rice bran, corn cobs, mustard seed bagasse and sugarcane bagasse as these are cheaper substrates than xylan. It is observed from the Figs. 2(a) and 2(b) that both xylanase (5.89 U/mL) and alkaline protease (3.87 U/mL) production was maximum at 24 h incubation period when the cells of *B. licheniformis* are in late log phase. Wheat bran was found to be the superior substrate over other substrates as they require longer time than wheat bran to give maximum productivity of the enzymes. This can be attributed to the fact that wheat bran contains maximum amount (43% of dry matter) of hemicelluloses than other substrates as seen in Table 1. Thus wheat bran is considered as a suitable substrate for further optimization. This is in accordance with maximum xylanase production at 24 h in an alkalophilic *Bacillus pumilus* strain MK001 (Kapoor et al., 2008) and 26 h in *Bacillus pumilus* (Battan et al., 2007) in submerged fermentation (SmF). Optimized incubation time for protease production was 72 h in bacteria *Bacillus subtilis* (Yang et al., 2000) and 120 h in *Streptomyces* sp. in SmF (Azeredo et al., 2004). Wheat bran was also used as a substrate for production of xylanases by *Aspergillus niger* in 72 h (Ahmad et al., 2009) and alkaline proteases by *Pseudomonas aeruginosa* (Meena et al., 2013) in 24 h.

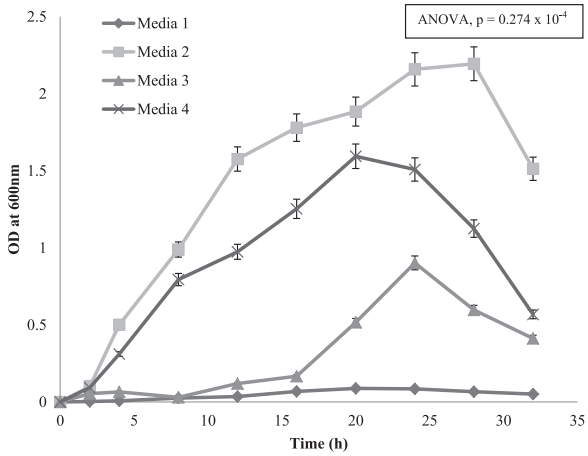

Fig. 1. Growth rate of *Bacillus licheniformis* on different media compositions at 30 °C and 180 rpm. (p value < 0.05, It is statistically significant).

3.3. Effect of inoculum concentration

Effect of different inoculum concentrations within a range of 1–10% (v/v) was studied for xylanase and alkaline protease enzyme co-production. The results are shown in Fig. 3 and it is observed that the inoculum concentration of 4% v/v gives maximum xylanase as well as alkaline protease activity followed by 1% and 8% inoculum concentration. Xylanase and alkaline protease enzyme activities were lower for inoculum concentration 2%, 6% and 10%. Optimum inoculum concentration is necessary as higher than optimum inoculum concentration affects the substrate availability and lower than optimum inoculum concentration increases the fermentation time respectively. This in turn decreases the overall productivity of the process. In earlier reports 2% v/v inoculum was used for xylanase production by *Bacillus subtilis* (Irfan et al., 2015) and *Aureobasidium pullulans* (Nasr et al., 2013). For protease production, in *Streptomyces sp*. 594 (De Azeredo et al., 2006), 2% (v/v) inoculum was used in SmF.

3.4. Effect of substrate concentration

Production of xylanase and alkaline protease at different substrate concentrations was studied. Wheat bran concentration of 0.25%, 0.5%, 1%, 1.5%, 2%, (w/v) was studied with respect to xylanase and alkaline protease enzyme production and activity. As the substrate concentration increased there was higher substrate availability to microorganisms so that respective enzymes are induced for utilization of that substrate. Whereas beyond an optimal substrate concentration no further increase in enzyme production was observed though an excess of substrate was available to the microbial load. The results are depicted in Fig. 4(a) and Fig. 4(b). From the concentrations under study, the wheat bran concentration of 0.5% w/v gives little higher xylanase productivity of 4.96 U/mL than other concentrations. However, there is a marginal difference in the activities of alkaline protease at different substrate concentrations. Highest protease activity of 4.3 U/mL was obtained at 0.25% substrate concentration followed by 0.5% substrate concentration with activity of 3.95 U/mL at 24 h of incubation. Thus, substrate concentration of 0.5% w/v was considered the optimum for co-production of enzymes at 24 h of fermentation time as there is highest co-production of both the enzymes. Substrate concentration of 1% w/v was used to study the xylanase production in *Clostridium absonum* CFR-702 (Rani and Nand, 2000). Similarly 1% w/v substrate was used to study protease production in *Bacillus sp*. L21 (Tari et al., 2006).

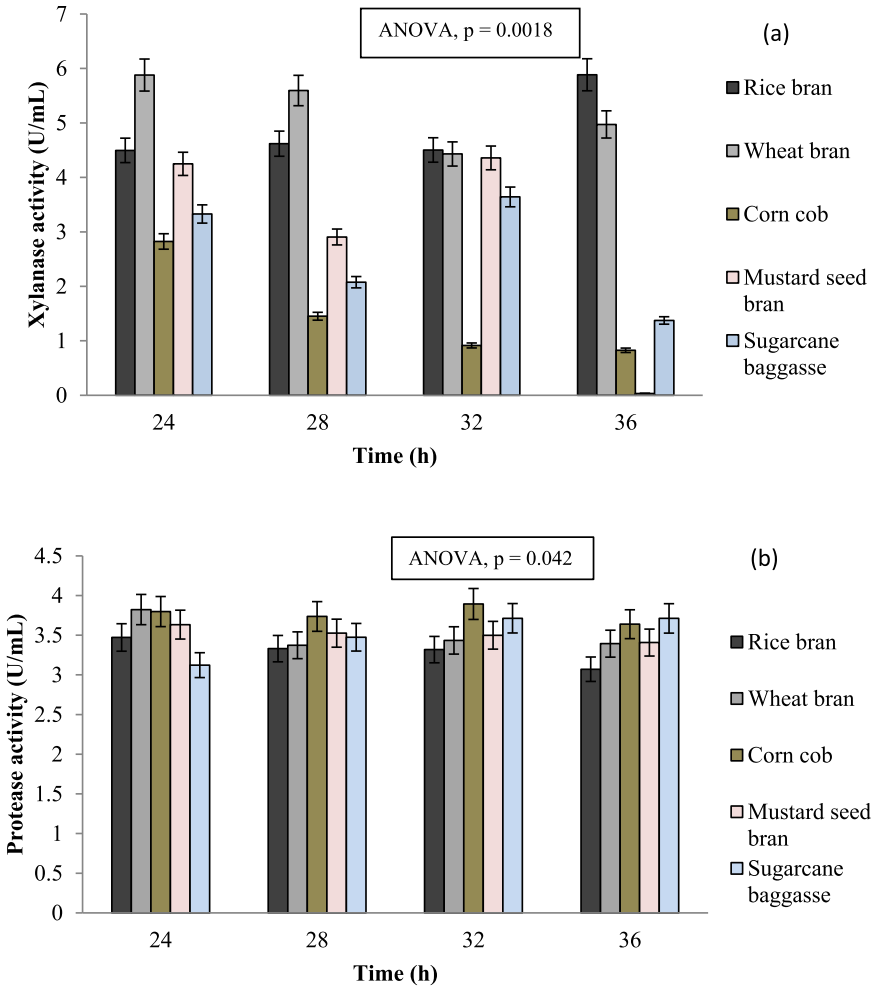

Fig. 2. Enzyme activities on different low cost waste substrates at different fermentation time using media 2, 30 ° C temperature and 180 rpm (a) xylanase activity (b) protease activity. (p values < 0.05, bothe the enzyme activities are statistically significant).

Table 1
Hemicellulose content of wheat bran, rice bran, corn cobs, sugarcane bagasse and mustard seed bagasse expressed as % dry matter of substrate.

Sr. No.	Agricultural waste	Hemicelluloses content (% dry matter of waste)	Reference
1	Wheat bran	43 ± 5	(Saunders et al., 1969; Sehwag et al., 2015)
2	Rice bran	37	(Baig et al., n.d.)
3	Corn cobs	34.7 ± 1.1	(Ruzene et al., 2008)
4	Sugarcane bagasse	27.89 ± 2.68	(Guilherme et al., 2015)
5	Mustard seed bran	3 ± 2	(Sehwag et al., 2015)

3.5. Effect of pH

Enzyme productions are highly dependent on the external or media pH as it strongly influences ionization and the transport of nutrient components across the cell membrane. This in turn reduces their availability to the organism and enzymatic processes which further affects the cell growth and product formation. Likewise every enzyme has optimum pH at which it gives maximum production. Thus, the production of xylanase and alkaline protease was studied at different initial media pH within a range of 6–9. The xylanase and alkaline protease enzyme activities at different initial pH are shown in Figs. 5(a) and 5(b) respectively. The results show that pH of 8.5 gives maximum xylanase and alkaline protease activity of 3.79 U/mL and 3.66 U/mL respectively. After 8.5, pH 9 gives the higher xylanase enzyme yield whereas in case of alkaline protease pH 7.5 gives the higher enzyme yield as compared to other pH. Optimum pH is desirable for maximum utilization of organism's capacity for enzyme productivities. Optimum pH is the pH at which the ionic and hydrogen bonds within the enzyme are influenced by both H^+ and OH^- ions in a way that the shape of the active site of enzyme is utmost complimentary to the shape of its substrate. Enzymes exist in their most active form at optimum pH. However extremely acidic or basic environments results in enzyme denaturation and inactivation (Bergamasco et al., 2000). In previous work, maximum production of protease was obtained at pH of 7.6 in *B. licheniformis* (Maghsoodi et al., 2013) whereas xylanase was optimally produced at pH 5.5 by *Aspergillus niger* (Ahmad et al., 2009).

3.6. Effect of temperature

Different organisms have different optimum temperature for enzyme production (Sharma et al., 2016). The effect of temperature on fermentative co-production of xylanase and alkaline protease was

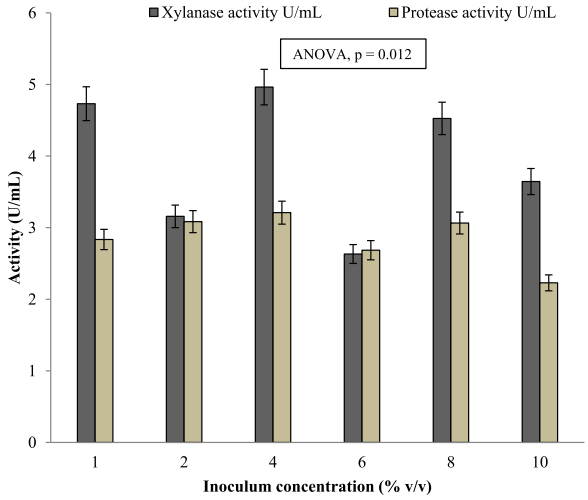

Fig. 3. Effect of inoculum concentration on enzyme co-production using media 2, at 24 h fermentation time, 30 ° C temperature, 180 rpm and with wheat bran as a substrate. (p values < 0.05, results are statistically significant).

studied at different temperatures like 25, 28, 30, 33, 37 °C. From Fig., 6 it is observed that maximum co-production of xylanase and alkaline protease was obtained at 30 °C with productivities i.e. 3.64 U/mL and 3.71 U/mL respectively. Nonetheless, at 25 °C and 33 °C, xylanase production was higher than alkaline protease production. This confirms a report that intracellular and extracellular enzyme synthesis at transcriptional and translational level is regulated by temperature (Rahman et al., 2005). Thus it is utmost important to study optimum temperature for enzyme production as it is different for every single organism. For instance, xylanase production was found to be optimum in *Bacillus circulans* D1 at 45 °C (Bocchini et al., 2002), *Penicillium oxalicum* ZH-30 at 31.1 °C (Li et al., 2007), *Bacillus pumilus* SV-85S at 37 °C (Nagar et al., 2010), in SmF. Alkaline protease production was found to be optimum in *Bacillus clausii* I-52 at 37 °C (Joo and Chang, 2006) and in *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus megaterium* and *Bacillus licheniformis* at 50 °C (Boominadhan et al., 2009).

3.7. Statistical analysis

The statistical analysis software, design expert 10, Stat-Ease, Inc., Minneapolis, USA was used for regression analysis of experimental data. Response surface plots were plotted and ANOVA was used to estimate the statistical parameters.

3.8. Statistical optimization of co-production of xylanase and alkaline protease by Box Behnken Design (BBD)

A Box Behnken Design (BBD) for three factors with replicates at the centre points was used for statistical optimization for the co-production of xylanase and alkaline protease. The variables that significantly affect the enzyme production are considered for further optimization using statistical model of response surface methodology. On the basis of p

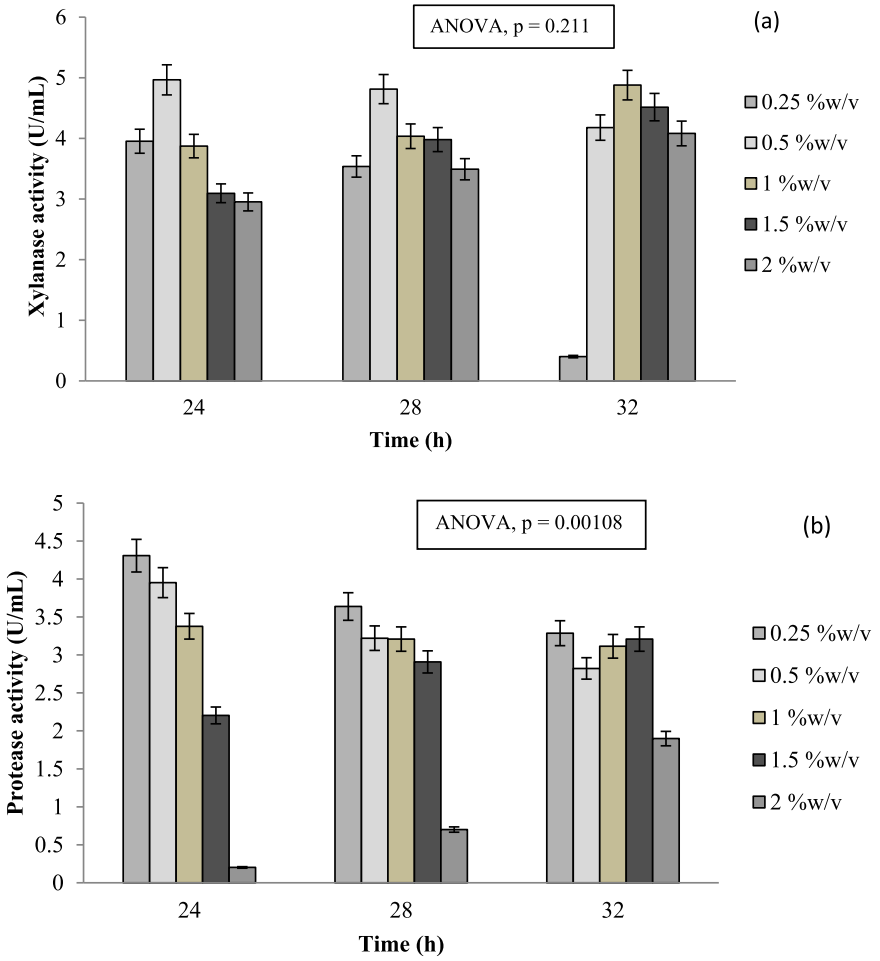

Fig. 4. Enzyme activity at different wheat bran concentrations using media 2, at 24 h fermentation time, 4% (v/v) inoculum concentration, 30° C temperature and 180 rpm (a) xylanase activity (b) alkaline protease activity. (p value of xylanase activity is > 0.05 so it is not statistically significant and protease activity p value is < 0.05 so it is statistically significant.).

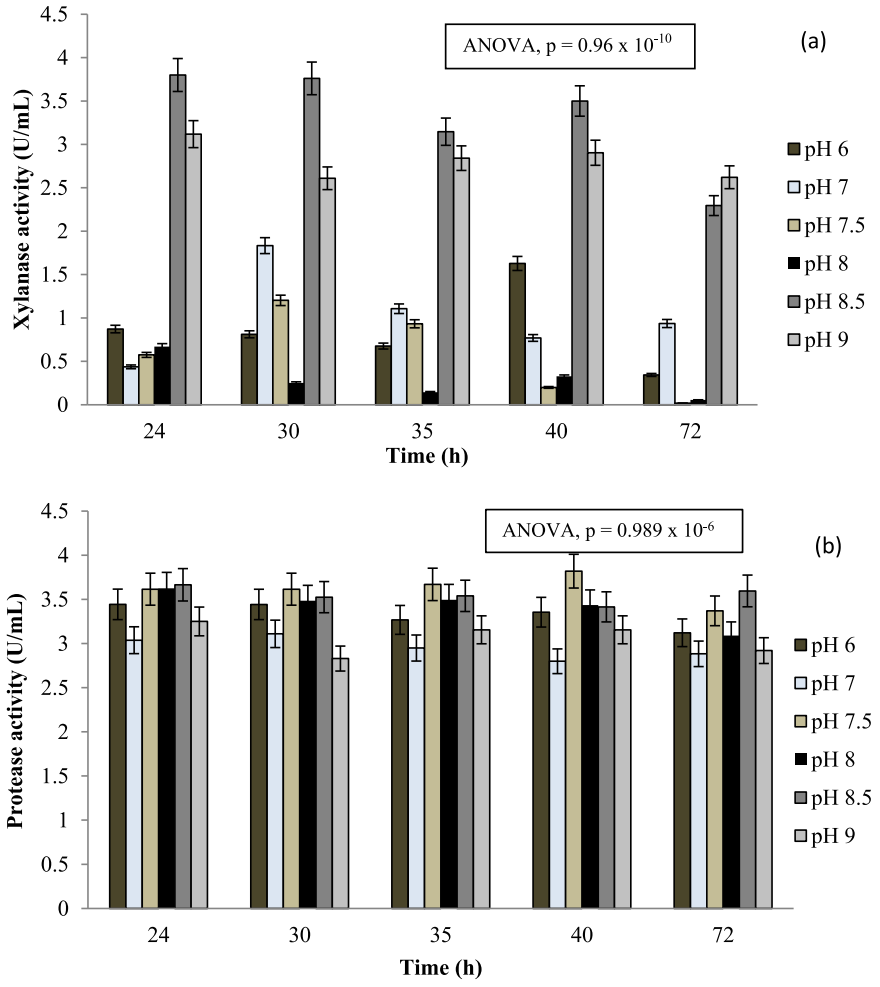

Fig. 5. Enzyme activity at different pH using media 2, at 24 h fermentation time, 4% (v/v) inoculum concentration, 30 ° C temperature, 180 rpm and 0.5% wheat bran concentration (a) xylanase activity (b) alkaline protease activity. (p values < 0.05, results are statistically significant).

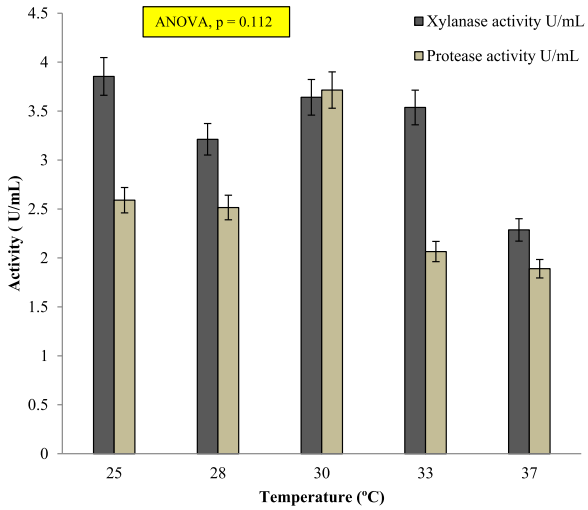

Fig. 6. Effect of temperature on enzyme co-production using media 2, at 24 h fermentation time, 4% (v/v) inoculums concentration, 0.5% wheat bran concentration, 180 rpm and pH 8.5. (p values > 0.05, results are statistically not significant).

value obtained by applying one way ANOVA to all the factors, substrate concentration, inoculum concentration and pH were selected as significant variables (p value ≤ 0.05) each at three coded levels (-1, 0, +1) as shown in Table 2. The actual levels of these independent variables for Box Behnken experiments were selected from the composition of culture medium optimized by one factor at a time approach. The Box Behnken involves total of 17 experimental trails that include 12 trails for design and 5 trails for replication of centre points (CP).

3.9. Analysis of variance

Analysis of variance (ANOVA) is a method used primarily for the determination of variety of responses as a function of independent variables. Regression Eqs. (1), (2), and (3) were obtained after standard ANOVA that indicated protein content, xylanase activity and alkaline protease activity as a function of independent variables. A multiple regression analysis method was used in order to study the data and second-order polynomial equations of independent variables are shown as follows.

$$\text{Protein content} = +0.10 + 8.000E{-}003*A - 1.625E{-}003*B + 3.750E{-}004*C - 3.500E{-}003*AB + 7.000E{-}003*AC + 8.250E{-}003*BC - 6.750E{-}004*A^2 - 5.925E{-}003*B^2 - 4.925E{-}003*C^2 \quad (1)$$

$$\text{Xylanase activity} = +4.31 - 0.30*A - 1.250E{-}004*B - 1.05*C - 0.19*AB + 0.72*AC - 0.23*BC - 0.64*A^2 - 0.88*B^2 - 0.21*C^2. \quad (2)$$

Table 2
Box-Behnken design to study the effects of Substrate concentration, inoculum concentration and pH on xylanase and alkaline protease activity in U/mL.

Run	Substrate concentration (%w/v)	Inoculum concentration (%v/v)	pH	Protein content (mg/mL)	Xylanase activity (U/mL)	Protease activity (U/mL)
1	0.5	4	8.5	0.1	4.187	3.68
2	0.75	2	8.5	0.105	2.507	3.18
3	0.5	4	8.5	0.102	4.507	3.78
4	0.25	6	8.5	0.092	3.439	3.41
5	0.75	4	7.5	0.097	3.52	3.09
6	0.5	6	9.5	0.095	1.813	2.52
7	0.5	4	8.5	0.101	4.193	3.5
8	0.5	2	9.5	0.087	2.533	2.18
9	0.5	4	8.5	0.1	4.527	3.42
10	0.75	4	9.5	0.112	2.867	2.32
11	0.25	4	7.5	0.094	5.493	4.87
12	0.75	6	8.5	0.1	2.399	2.09
13	0.25	4	9.5	0.081	1.947	2.85
14	0.5	2	7.5	0.103	4.16	3.89
15	0.25	2	8.5	0.083	2.799	3.78
16	0.5	4	8.5	0.105	4.133	3.91
17	0.5	6	7.5	0.078	4.347	3.47

Footnote: Standard deviation for centre points; protein content = 0.1010 ± 0.0020, xylanase activity = 4.3094 ± 0.191, protease activity = 3.658 ± 0.2.

$$\text{Alkaline protease activity} = +3.66 - 0.53*A - 0.19*B - 0.68*C - 0.18*AB + 0.31*AC + 0.19*BC - 0.14*A^2 - 0.41*B^2 - 0.24*C^2. \quad (3)$$

To check the statistical significance of these equations, F test was applied to evaluate the coefficient of determination (R^2). The R^2 values for protein content, xylanase activity and alkaline protease activity were found to be 0.95, 0.98 and 0.95 respectively. The adjusted R^2 values were 0.88, 0.96, and 0.89 for protein content, xylanase activity and alkaline protease activity, respectively. These values indicates that there was a significant correlation between experimental and predicted values. Centre point data vary from 0.10–0.105 for protein content, 4.133–4.527 for xylanase, and 3.42–3.91 for protease respectively.

Model Eqs. (1), (2) and (3) demonstrated 94%, 98% and 95% of variation in response protein content, xylanase activity and alkaline protease activity, respectively. Therefore, Eqs. (1), (2) and (3) were found to be the appropriate models for measuring protein content, xylanase activity and alkaline protease activity, respectively.

Thus, the quadratic model equations obtained after ANOVA indicated satisfactory adjustment with experimental value. Adequate precision is the measure of signal-to-noise ratio and a ratio greater than 4 is desirable. In this case, signal-to-noise ratio in model Eqs. (1), (2), and (3) were protein content (12.3), xylanase activity (22.6), and alkaline protease activity (13.8), respectively, which indicated an adequate signal.

3.10. Model analysis

Accuracy of the model was ensured by employing ANOVA; which was tested by F test for protein content, xylanase activity and alkaline protease activity. Here, F values for the regression model of protein content, xylanase activity and alkaline protease activity were obtained as 13.81, 45.81, and 14.85, respectively. For all three responses these model F values were found to be significant. The p values are generally used as a tool to ensure significance of each coefficient and interaction strength between each independent variable. P Values lesser than 0.05 signifies that model terms are significant and values greater than 0.1 indicate that the model terms are not significant. The p values for protein content, xylanase activity and alkaline protease activity are 0.0011, 0.0001, and 0.0009 respectively.

The regression analysis of the experimental data for protein content demonstrates that the linear model term A, quadratic model terms (B^2, C^2) and interactive model terms (AC, BC) were significant for the protein content. For xylanase activity, the linear model terms (A, C), quadratic model terms (A^2, B^2) and interactive model term AC were significant for xylanase activity. Similarly, for alkaline protease activity, the linear model terms (A, C), quadratic model term B^2, and interactive model term AC were significant for alkaline protease activity. Statistically significant terms indicate that the results are unlikely to be due to by chance. Parameters of the model need to be significant as they are assessed for the goddess-of-fit of the model and contribute to the model fitting.

3.11. Response surface plot

Response surface plot represents an optimum level of independent variables and also provides a method to envisage the relationship between responses and test variable. For pair-wise combination of two factors 3D plots were generated while keeping the other factor at the central level.

The effect of pH and substrate concentration on protein content is shown in the Figs. 7a. pH 8.5 and substrate concentration 0.5% w/v showed maximum protein content of 0.112 mg/mL. Initially increase in pH and substrate concentration showed increase in protein content. Protein content was low at pH around neutrality and reached peak value at pH of 8.5, indicating that *B. licheniformis* is an obligate alkalophile. Alkalophiles are reported to be the rich sources of alkaline active enzymes (Horikoshi, 1999). There is a linear increase in protein content with respect to substrate concentration. Thus, both pH and substrate concentration have significant effect on total protein production.

The effect of pH and inoculum concentration on protein content is shown in the Fig. 7b. Variations in pH and inoculum concentration result in linear increase in total protein content as shown in Fig. 7b. Moreover, after increasing pH and inoculum concentration above 8.5% and 4% v/v, respectively, there is a decrease in total protein content. At higher inoculum concentration with available dissolved oxygen in the medium, there is reduced mass transfer which will result in the decreased cell efficiency and enzyme productivity. On the other hand, at low inoculum concentration, lag phase increases, which results in increased process time and decreased overall productivity.

The effect of inoculum concentration and substrate concentration on xylanase activity is shown in the Fig. 8a. Inoculum concentration of 4% v/v and substrate concentration of 0.5% w/v shows maximum xylanase activity of 5.49 U/mL. Initially, xylanase activity increases with an increase in inoculum and substrate concentration upto 4% v/v and 0.5% w/v respectively and then decreases thereafter (Fig. 8a). This may be

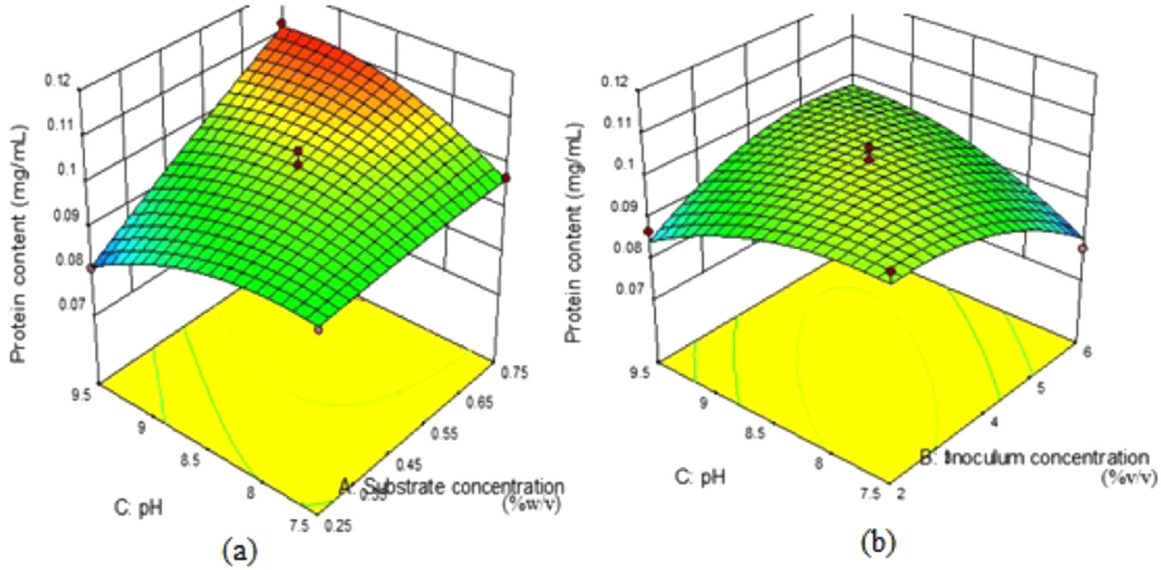

Fig. 7. Response surface plots showing interactive effects of (a) pH and substrate concentration (%w/v), and (b) pH and inoculum concentration (%v/v), on protein content. Std Dev. = 0.21, Cumulative variance (C.V.)% = 5.91, R^2 = 0.9833, Adj R^2 = 0.9618, Ade. Precision = 22.64, PRESS = 2.66.

due to the fact that, at increased substrate concentration beyond 0.5% w/v, bacteria may not be able to utilize the complete substrate with 4% inoculum concentration. This results in equllibrium between substrate utilization by microbe at 4% inoculum concentration so there is no further increase in protein content and subsequent xylanase activity at increased substrate concentration.

Counter plot Fig. 8b represents the effect of pH and substrate concentration on xylanase activity. Plot 8b depicts that, xylanase activity attains its optimum value at pH 8.5 and substrate of 0.5% w/v. Wheat bran as a substrate serves as both carbon and nitrogen source. The high xylanase production in the presence of wheat bran may be due to the synergistic effect of other hemicellulolytic enzymes released by the bacteria in the presence of wheat bran (Sharma et al., 2016).

Fig. 9a shows the effect of pH and substrate concentration on alkaline protease activity. As bacteria behaves as alkalophile, alkaline protease activity was low at pH near 7 and higher at alkaline side of pH 8.5. Substrate concentration of 0.5% w/v and pH 8.5 gives maximum protease activity up to 4.87 U/mL. Fig. 9b indicates the effect of interaction between pH and inoculum concentration on alkaline protease activity. Further, higher protease activity was found to be at inoculum concentration of 4% v/v. In literature, a wide range of applications has been described related to applicability of RSM in various chemical and biochemical processes. Central composite design has been applied for improving protease production from *Bacillus mojavensis* in a bioreactor (Khalil et al., 2003). Box-Behnken design was used for the optimization of α-amylase production by *Aspergillus oryzae* NRRL 6270 in solid-state fermentation and about 20% increase in enzyme yield was observed (Febe et al., 2003).

3.12. ValidatIon of model

The model validation was carried out by performing the experiments under the predicted conditions. The validation of the model was performed using substrate concentration 0.25% w/v, inoculum concentration 4% v/v and pH 7.5. Experimental values for protein content, xylanase activity and alkaline protease activity were 0.0913 mg/mL, 5.352 U/mL and 4.624 U/mL respectively. The predicted values for protein content, xylanase activity and alkaline protease activity were

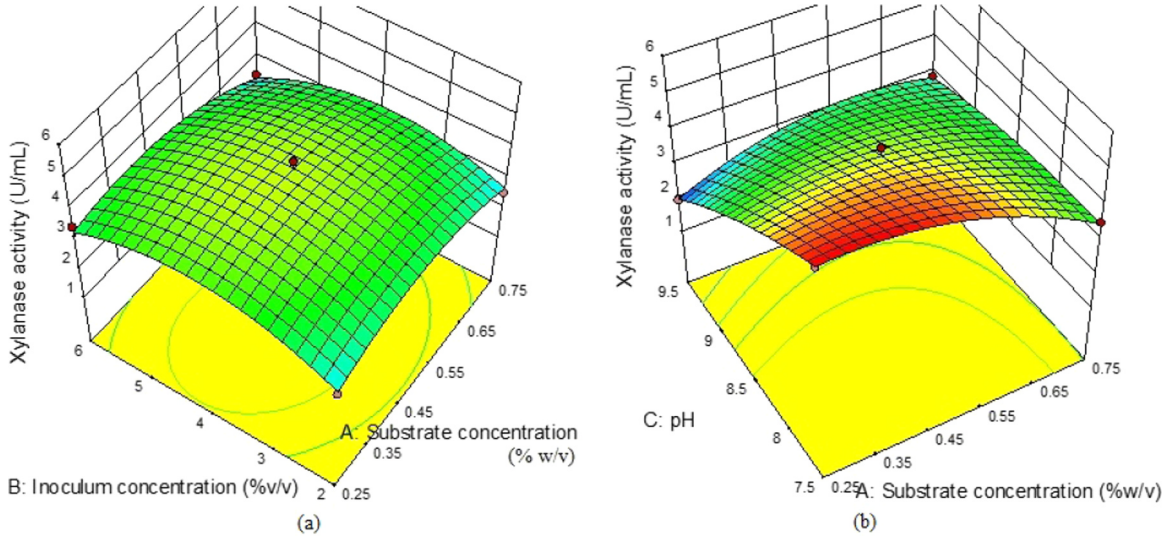

Fig. 8. Response surface plots showing interactive effects of (a) inoculum concentration (%v/v) and substrate concentration (%w/v), and (b) pH and substrate concentration (%w/v), on xylanase activity. Std Dev. = 0.24, C.V.% = 7.44, R^2 = 0.9502, Adj R^2 = 0.8862, Ade. Precision = 13.799, PRESS = 4.40.

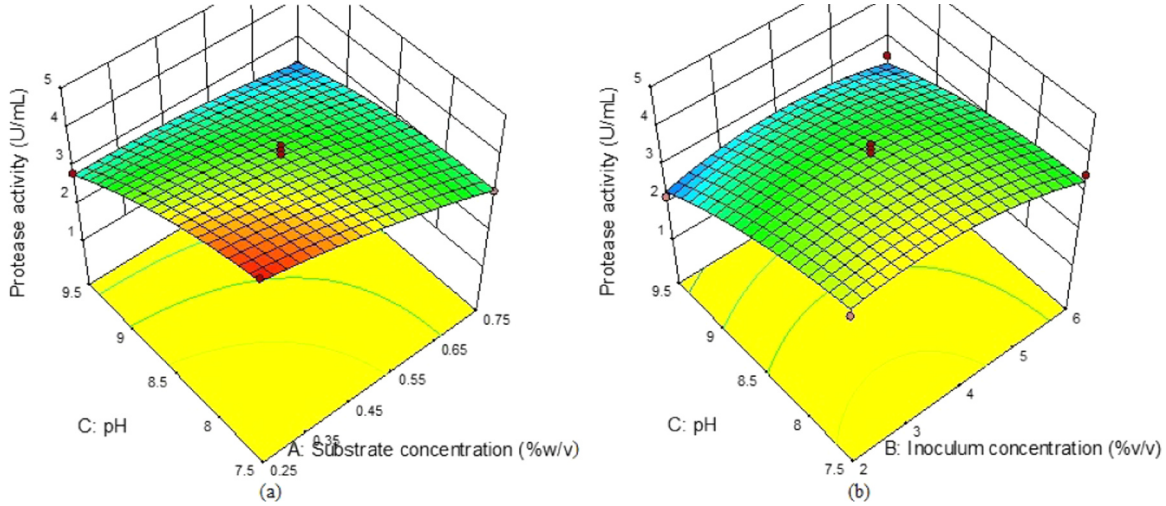

Fig. 9. Response surface plots showing interactive effects of (a) pH and substrate concentration %w/v, and (b) pH and inoculum concentration %v/v, on alkaline protease activity. Std Dev. = 0.0032, C.V.% = 3.39, R^2 = 0.9467, Adj R^2 = 0.8781, Adeq. Precision = 12.34, PRESS = 0.00094.

0.0946 mg/mL, 5.523 U/mL and 4.805 U/mL respectively. Here, it can be concluded that the model has been successfully validated as there is a marginal difference between experimental and predicted values.

4. Conclusion

The study demonstrates a novel approach about single step co-production of xylanase and alkaline protease using *Bacillus licheniformis*. Different agricultural wastes have been screened, amongst them wheat bran was found to be the optimized substrate having highest co-production rate. The productivity of enzymes obtained at optimized fermentation parameters, using single factor at a time approach, is 4.309 U/mL for xylanase and 3.658 U/mL for alkaline protease. Optimum concentrations of significant variables such as substrate concentration 0.25% (w/v), inoculum concentration 4% (v/v), and pH 7.5 were determined using Box-Behnken Design. Xylanase and alkaline protease activities were found to be enhanced by 1.27 and 1.33 fold respectively, in Box Behnken model as compared to one factor at a time model. The present study can be valuable to optimize the parameters for further scale up studies at bioreactor level as well as to know the economic feasibility of this enzyme co-production process.

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