Utility of acidic xylanase of *Bacillus subtilis* subsp. *subtilis* JJBS250 in improving the nutritional value of poultry feed

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Received: 20 July 2018 / Accepted: 22 November 2018 / Published online: 28 November 2018
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Abstract

Cane molasses has been employed as a cost-effective medium for enhanced xylanase production in submerged fermentation. *Bacillus subtilis* subsp. *subtilis* JJBS250 produced xylanase (15.16 U/ml) at pH 4.0, 35 °C and 200 rpm after 54 h using optimized basal medium by ‘one variable at a time approach’. Addition of Tween 80 and PEG 4000 also enhanced xylanase production in cane molasses medium. Combined effect of yeast extract, incubation time and PEG 4000 using statistical optimization enhanced xylanase production to 38.60 U/ml, which was 2.54-fold higher than the ‘one variable at a time approach’. The efficacy of xylanase from *Bacillus subtilis* subsp. *subtilis* JJBS 250 was evaluated in the improvement of poultry feed nutrition. Xylanase addition (10 IU/g feed) enhanced liberation of reducing sugars (95.540 mg/g substrate) after 48 h at 60 °C. Optimization has resulted in enhanced production of xylanase that improved the nutritional quality of poultry feed.

Keywords Xylanase · Cane molasses · Submerged fermentation · *Bacillus subtilis* subsp. *subtilis* JJBS250 · Response surface methodology · Poultry feed

Introduction

Lignocellulosic biomasses are ubiquitous, abundant and renewable carbon sources and due to their huge quantities have been observed as a reasonable source for the production of paper and pulp, chemicals, biofuels and other products with the help of microorganisms (Bala and Singh 2016; Singh et al. 2016). The main constituents of lignocellulosic biomasses are 23% lignin, 33% hemicelluloses (non-cellulosic polysaccharides including glucans, mannans and xylans) and 40% cellulose on the basis of dry weight. Xylan

is the most abundant renewable polysaccharide in nature after cellulose and found in large quantities in softwoods (7–10%), hardwoods (15–30% of the cell wall content) and in annual plants (<30%) (Kaseke et al. 2016; Walia et al. 2017).

Xylanase is an extracellular enzyme which is responsible for xylan hydrolysis forming usable products such as xylo-oligosaccharides (xylose, xylobiose) (Ahmed et al. 2016). It has been reported from various bacterial and fungal genera including *Bacillus*, *Staphylococcus*, *Cellulomonas*, *Micrococcus*, *Streptomyces*, *Aspergillus*, *Penicillium*, *Fusarium*, *Pseudomonas*, *Rhizopus*, *Clostridium*, *Trichoderma* (Burlacu et al. 2016). Xylanases have attracted because of their applications in textile, food industry, pulp bleaching, animal feed, waste treating, baking and brewing, bioenergy converting, etc. (Arabi et al. 2011; Goluguri et al. 2016). Xylanases have also been used as an alternate of chemicals, which are expensive and cause pollution (Kamble and Jadhav 2012).

Mostly industrial enzyme production cost is related with the cost of the medium components used (Ahmed et al. 2016). For cost-effective enzyme production, economical substrates have been required. Cane-molasses is an important relic of the sugar industry and a cost-effective carbon source for the growth and metabolite production by microorganisms. It consists of approximately 50% (w/v) total sugars,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-018-1526-2>) contains supplementary material, which is available to authorized users.

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vitamins, water, heavy metals, crude protein, fat and other nutrients (He et al. 2014; Bala and Singh 2016). Due to its low price and more carbohydrate level, it is an excellent substrate for the production of microbial enzymes (Kumar and Satyanarayana 2014). The classical 'one variable at a time, approach has been used for optimization studies, but is not able to study the interaction and combined effect of selected variables. The statistical analysis using RSM is an important tool to check the effect of combined variables. The use of software-based technique for this analysis, makes this process faster and allows to understand the effect and product yield of variables (Bala and Singh 2016; Kumar and Satyanarayana 2014; Khusro et al. 2016). Among all bacteria, *Bacillus* genus is widely used in industrial fermentations for the production of a variety of enzymes. Among all enzymes, xylanase is a more industrially important enzyme produced by microorganisms (Battan et al. 2007; Nagar et al. 2010). In the present study, cane molasses has been used for the optimization of culture conditions for xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250 followed by its applicability in improving the nutrition of poultry feed.

Materials and methods

Microbial culture and growth conditions

Bacillus subtilis subsp. *subtilis* JJBS250 isolated from soil sample was routinely grown in nutrient agar (pH 7.0) medium at 37 °C (Jain and Singh 2016). Bacterial culture was maintained at 4 °C in nutrient agar plates and at – 20 °C in glycerol stocks (Jain and Singh 2016). The nucleotide sequence for 16S rDNA has been submitted in GenBank under accession no. KC609372.1.

Enzyme production

Cane molasses (10%) medium (pH 4.0) supplemented with yeast extract (0.5%) was used for xylanase production. The Erlenmeyer flasks containing 50 ml medium were inoculated with 1% inoculum and incubated at 35 °C and 200 rpm. The bacterial culture was harvested after 24 h by centrifugation at 10,000 rpm for 10 min at 4 °C and the cell-free culture filtrate was used in enzyme assays.

Xylanase assay

Xylanase assay was carried out as described earlier (Bala and Singh 2016, 2019). Briefly, xylanase activity was measured at 60 °C and pH 7.0 (0.1 M potassium phosphate buffer) using beechwood xylan (0.5%) [Sigma–Aldrich Chemical Co, St Louis, MO, USA]. The reducing sugars released were quantified as described by Miller (1959). One enzyme unit

(IU) is defined as the amount of enzyme required to liberate 1 μmole of xylose per min under the assay conditions.

Optimization of process parameters for xylanase production

Optimization of xylanase production was carried out in two steps, first, with 'one variable at a time' approach followed by a statistical approach using response surface methodology. Effect of different parameters such as different concentrations of cane molasses (6–12%), pH (3.0–7.0), temperatures (25–40 °C), nitrogen sources (peptone, tryptone, beef extract, yeast extract, ammonium sulphate, ammonium nitrate, potassium nitrate), incubation time (24–96 h), polyethylene glycols (PEG 400, 4000, 8000, 20,000), surfactants (Tween 20, Tween 80, SDS, Triton X-100) and agitation speeds (0–250 rpm) were studied. Furthermore, the effect of each selected parameter was also studied at different levels.

Three variables viz. yeast extract (A), incubation time (B) and PEG 4000 (C) were selected as the most significant factors affecting the xylanase production on the basis of results of 'one variable at a time' approach. The basal medium contained cane molasses (10%) with pH 4.0 with five different levels of each variable (Table 1) optimized in the design was studied. The statistical software (Design Expert® 6.0.7, Stat-Ease, Minneapolis, MN, USA) generated a 2³ experimental design with a set of 20 different experiments. The following quadratic equation explained the behavior of the system:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

where Y denotes predicted response (Enzyme production), β_0 as intercept, $\beta_1, \beta_2, \beta_3$ as linear coefficient, $\beta_{11}, \beta_{22}, \beta_{33}$ as squared coefficient, $\beta_{12}, \beta_{13}, \beta_{23}$ as interaction coefficients and $A, B, C, A^2, B^2, C^2, AB, AC, BC$ as factors.

Validation of the process and large-scale enzyme production

The practicality of xylanase production was tested in shake flasks of varied volumes (0.25–1.01), under the optimized

Table 1 Range of variables used for the response surface methodology

Independent variables	Levels				
	– α	– 1	0	+ 1	+ α
(A) Yeast extract (%)	0.25	0.81	1.63	2.44	3.00
(B) Incubation period (h)	24	48	72	96	120
(C) PEG 4000 (%)	0.10	0.50	1.05	1.61	2.00

conditions. The Erlenmeyer flasks containing optimized medium were incubated at 35 °C and 200 rpm. Fermentation was carried out for 54 h and culture filtrates were used for enzyme assays.

Applicability of bacterial xylanase in improvement poultry feed nutrition

Bacterial xylanase was employed in the hydrolysis of poultry feed for the improvement of its nutrition quality. Poultry feed (commercial feed) was purchased from the poultry feed shop from local market. For enzymatic hydrolysis, 150 ml Erlenmeyer flasks containing 0.5 g poultry feed and 20 ml of 0.1 M potassium phosphate buffer was inoculated with xylanase of *Bacillus subtilis* subsp. *subtilis* JJBS 250 and kept under shaking at 150 rpm at 60 °C (Kumari et al. 2015). The samples were drawn at definite time intervals followed by centrifugation at 10,000 rpm for 10 min to remove insoluble solids and then the amount of reducing sugars was estimated as described earlier. Effect of different temperatures (37 and 60 °C), different enzyme doses (5–25 IU) and different incubation time (0–48 h) were also studied for improving the nutrition of poultry feed. Both enzyme and substrate (poultry feed) controls were also run separately and their values were subtracted from test values.

Results and discussion

Optimization of physical and chemical conditions for xylanase production

Cane molasses is a byproduct of sugar industry available in large quantities and is an economical source of sugar containing crude protein and fat, approximately 50% total sugars (mainly glucose, sucrose and fructose), nitrogenous substances (0.4–1.5%), heavy metals, vitamins like biotin, thiamine, folic acid, pyridoxine, pantothenic acid, amino nitrogen, water and trace elements (Kumar and Satyanarayana 2014). It has been used as economical medium for the production of different microbial enzymes (Singh and Satyanarayana 2008; He et al. 2014; Kumar and Satyanarayana 2014; Bala and Singh 2016; Irfan et al. 2016). Results showed that the supplementation of 10% cane molasses favoured high xylanase production (15.16 U/ml) by *Bacillus subtilis* subsp. *subtilis* JJBS 250 (Fig. S1) followed by a decline at higher levels. Similar findings were observed for concomitant xylanase and cellulase production by *Trichoderma reesei* using 10% cane molasses (He et al. 2014). Bala and Singh (2016) reported maximum xylanase production by *S. thermophile* at 8% cane molasses. Kumar and Satyanarayana (2014) reported high xylanase production at 7% cane molasses by *Bacillus halodurans* TSEV1. Singh

and Satyanarayana (2008) observed highest phytase production at 7% cane molasses by *Sporotrichum thermophile* while Kumar and Satyanarayana (2007) attained high glucoamylase titre using 8% cane molasses in the medium. Xylanase production was supported by cane molasses medium using *Bacillus subtilis* subsp. *subtilis* JJBS 250.

Medium pH is one of the important factors affecting the production of microbial enzymes (Nagar et al. 2010). Medium's pH affected various enzymatic processes and transfer of many components across the cell membrane (Kapoor et al. 2008). Bacterium produced high xylanase (13.86 U/ml) at pH 4.0 that declined under alkaline range (Fig. S2). He et al. (2014) observed more xylanase production by *Trichoderma reesei* at pH 5.0 cane molasses medium. Nagar et al. (2010) reported the same at pH 6.0 from *Bacillus pumilus* SV-85S. Some other researchers reported more xylanase production at pH 8.0 by two *Bacillus* strains (*Bacillus megaterium* BM07 and *Bacillus subtilis* BS04) (Irfan et al. 2016) and *B. mojavensis* AG137 (Sepahy et al. 2011).

Optimum temperature is necessary for growth and high enzyme production. Results indicated that *Bacillus subtilis* subsp. *subtilis* JJBS 250 produced high xylanase (15.08 U/ml) at 35 °C followed by decline afterwards (Fig. S3). Similarly, *Bacillus megaterium* BM07 and *Bacillus subtilis* BS04 also produced maximum xylanase at 40 °C and 35 °C, respectively (Irfan et al. 2016). High xylanase production in submerged fermentation was observed at 37 °C by *Bacillus pumilus* AJK (Kaur et al. 2017), *Bacillus pumilus* VLK-1 (Kumar et al. 2014), *Bacillus mojavensis* AG137 (Sepahy et al. 2011) and *Bacillus pumilus* SV-85S (Nagar et al. 2010).

Among all the nitrogen sources, high xylanase production (15.30 U/ml) was observed in medium supplemented with 0.5% yeast extract (Fig. 1a) as compared to other organic and inorganic nitrogen sources. Kumar et al. (2014) also reported highest xylanase production at 0.6% yeast extract by *Bacillus pumilus* VLK-1. In certain cases, highest xylanase production has been reported in the medium containing more than one nitrogen sources. A combination of 0.5% peptone and yeast extract supported high xylanase production by *Bacillus subtilis* ASH (Sanghi et al. 2009), while a combination of yeast extract and tryptone supported high xylanase production by *Gracilibacillus* sp. TSCPVG (Giridhar and Chandra 2010) and *Bacillus mojavensis* AG137 (Sepahy et al. 2011). The maximum titer of xylanase was reported with 0.25% each of peptone and yeast extract from *Bacillus* sp. SSP-34 (Subramanian et al. 2001). Nagar et al. (2010) attained high xylanase production using combination of peptone, yeast extract and KNO₃ using *Bacillus pumilus* SV-85S.

Among all the PEGs tested, high xylanase production (15.04 U/ml) was attained with PEG 4000 as compared to others (Fig. 1b). High xylanase production (15.89 U/ml) was attained at 1% PEG4000 followed by decline at higher and lower concentrations (data not shown Kapoor et al.

Fig. 1 **a** Effect of different nitrogen sources on xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS 250. **b** Effect of different PEGs on xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS 250

(2008) attained that among all PEG (400, 600, 1450, 3330, 8000, 7000–9000) PEG 3330 enhanced xylanase production from *Bacillus pumilus* strain MK001. Sharma et al. (2005) reported that except PEG 6000 and 8000, all tested PEG's (400, 600, 1450, 3350, 4000, 6000, 7000–9000, 8000, 10,000, 20,000) repressed production of laccase from *Ganoderma* sp. kk-02 under submerged fermentation. Polyethylene glycols have been known to change the cell membrane by interacting with phospholipid bilayers (Sharma et al. 2005). PEGs have been reported to cause alterations in the phospholipid bilayers of the plasma membrane that facilitated the enhanced secretion of enzymes (Sharma et al. 2005).

Xylanase production (16.14 U/ml) increased with increase in incubation time upto 54 h followed by decline afterwards (Fig. S4). Kumar et al. (2014) observed high xylanase production after 56 h by *Bacillus pumilus* VLK-1. Kumar and Satyanarayana (2014) reported that *Bacillus halodurans* produced high xylanase after 48 h in cane molasses medium. Some other researchers reported

Fig. 2 Effect of different surfactants on xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS 250

maximum xylanase production after 48 h from *Bacillus pumilus* SV-85S (Kaur et al. 2017) and *Bacillus* sp. (Irfan et al. 2016). High xylanase production by *Bacillus pumilus* SV-85S was attained after 36 h in submerged fermentation (Nagar et al. 2010).

Xylanase production was enhanced (19.71 U/ml) by the addition of Tween 80 (1.0%) as compared to other surfactants (Fig. 2). Similarly, production of xylanases was enhanced by Tween 80 in *Bacillus pumilus* VLK-1 (Kumar et al. 2014), *Bacillus mojavensis* AG137 (Sephay et al. 2011), *Gracilibacillus* sp. TSCPVG (Giridhar and Chandra 2010) and *Bacillus pumilus* SV-85S (Nagar et al. 2010). Surfactants are known to affect the growth and enzyme production in microbes, and therefore, have been used for improving the yield of a number of microbial enzymes (Kumar and Satyanarayana 2014). Surfactants have been reported for enhanced secretion of microbial enzymes due to increase in the permeability of cell membranes (Sapna, Singh 2017; Sharma et al. 2005).

There is need of proper mixing of nutrients in fermentation which is fulfilled by aeration and agitation by providing proper oxygen to the medium. The maximum xylanase production (19.54 U/ml) was attained at an agitation speed of 200 rpm. However, lower amount of xylanase production (13.0 U/ml) was attained under static conditions. Less agitation speed adversely affected enzyme production due to less oxygen supply to the medium. Similarly, agitation speed of 200 rpm supported maximum xylanase production by *Bacillus pumilus* AJK (Kaur et al. 2017). Similarly, agitation speed of 200 rpm supported maximum production of xylanase by *Bacillus pumilus* AJK (Kaur et al. 2017), *Bacillus pumilus* VLK-1 (Kumar et al. 2014) and *Bacillus subtilis* ASH (Sanghi et al. 2009). Nagar et al. (2010) observed high xylanase production by *Bacillus pumilus* SV-85S at 150 rpm.

Optimization of medium components for improved xylanase production using statistical design

Using optimized basal medium by ‘one variable at a time approach’ *Bacillus subtilis* subsp. *subtilis* JJBS250 produced xylanase (15.16 U/ml) at pH 4.0, 35 °C and 200 rpm after 54 h. Using response surface methodology, medium components were further optimized to enhance xylanase production. Xylanase production ranged from 15.90 to 38.60 U/ml under different experimental conditions (Table 2). The results observed for xylanase were analyzed using the analysis of variance (ANOVA). The xylanase production (*Y*) was calculated from regression analysis as a function of these variables by the following equation:

$$\begin{aligned} \text{Xylanase production (U/ml):} \\ 26.43 - 0.41A + 3.46B + 1.58C - 1.79A^2 + 1.34B^2 \\ + 1.44AB - 0.25AC + 5.84BC \end{aligned}$$

Different criteria such as the coefficient of determination (adjusted R^2), correlation coefficient (predicted R^2), values of $P > F$, adequate precision and Model F value can be used for checking the quality of the model as given in Table S1. All these values justify the significance and variability in the model. The three-dimensional plots of interactions between the variables showed maximum xylanase production up to the concentration of variables raised to the optimal level

followed by decline afterwards (Fig. S5a–c). The experimental value for xylanase production was recorded as 38.60 U/ml, whereas predicted value was 37.63 U/ml. The predicted and experimental values are very close and hence, the model was successfully confirmed. There was a 2.54-fold improvement in xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS250 in cane molasses medium as a result of statistical optimization. Using statistical designs, there was a 3.8-fold increase in production of xylanase by *Aspergillus oryzae* LCI (Bhardwaj et al. 2017). More than 4.0-fold increase in cellulolytic and xylanolytic enzymes production was observed as a result of optimization in cane molasses medium by *S. thermophile* reported by Bala and Singh (2016). Khusro et al. (2016) observed more than 3.7-fold enhancement as a result of optimization in xylanase production by *Bacillus tequilensis* strain ARMATI. Kumar et al. (2014) reported a 4.5-fold increase in xylanase production in cane molasses using *Bacillus halodurans* TSEV1 after statistical optimization. Kumar et al. (2014) reported 1.46-fold improvement in the xylanase production by *Bacillus pumilus* VLK-1 using RSM. Coman and Bahrim (2011) reported that response surface methodology resulted in threefold increase in xylanase production by *Streptomyces* sp. P12–137 in SmF.

The process was validated by repeating experiment (no. 17, Table 2) under optimized conditions that led to the production of 38.25 U/ml, and thus, proving the validity of the process and the model.

Table 2 Experimental design and results of central composite design of response surface methodology

Run no.	Yeast extract (%)	Incubation period (h)	PEG 4000 (%)	Xylanase production (U/ml)
1	+1	+1	+1	38.60 ± 1.158
2	0	+α	+α	28.32 ± 0.849
3	+1	+1	−1	23.58 ± 0.471
4	−1	+1	−1	21.00 ± 0.525
5	0	+α	0	37.40 ± 1.122
6	−1	−1	+1	20.12 ± 0.603
7	0	0	0	26.44 ± 0.528
8	0	0	−α	24.32 ± 0.608
9	+1	−1	−1	24.40 ± 0.732
10	+α	0	0	20.52 ± 0.615
11	0	0	0	26.44 ± 0.528
12	0	0	0	26.44 ± 0.528
13	0	0	0	26.44 ± 0.528
14	0	0	0	26.44 ± 0.528
15	−1	−1	−1	27.40 ± 0.822
16	−α	0	0	22.16 ± 0.664
17	−1	+1	+1	36.84 ± 1.105
18	0	0	0	26.44 ± 0.528
19	+1	−1	+1	15.90 ± 0.318
20	0	−α	0	26.08 ± 0.652

Applications of xylanase in improvement of poultry feed nutrition

The efficacy of xylanase from *Bacillus subtilis* subsp. *subtilis* was evaluated in the improvement of poultry feed nutrition. High reducing sugars (7.750 mg/g feed) were released at 60 °C as compared to 37 °C (4.170 mg/g feed). The amount of reducing sugars increased with increase in enzyme doses but significant liberation was attained (7.870 mg/g substrate) with 10 IU/g poultry feed (Fig. 3a). After xylanase treatment, there was a continual increase in the release of reducing sugars and high amount of reducing sugars (95.540 mg/g substrate) was attained after 48 h (Fig. 3b). Kumari et al. (2015) reported enhanced liberation of nutritional components from poultry feed after phytase treatment. Mendes et al. (2013) observed improvement in the nutritional value of triticale-based diet (a hybrid of wheat and rye) using low doses of exogenous xylanase. Yegin (2017) reported maximum yields of reducing sugars of 89 mg/g, 212 mg/g and 357 mg/g from untreated, dilute sodium hydroxide and aqueous ammonia pretreated corn cobs, respectively, using crude xylanase from *Aureobasidium pullulans*. Zhang and Sang (2015) reported high liberation of reducing sugars from pretreated corn cobs using crude xylanase (2500 U/g sample) from

Fig. 3 Effect of different enzyme doses (a) and incubation time (b) on liberation of reducing sugar from poultry feed by the xylanase of *Bacillus subtilis* subsp. *subtilis* JJBS 250

Penicillium chrysogenum after 24 h. Damaso et al. (2004) observed enhanced hydrolysis of corn cobs using xylanase (3000 U) of *Thermomyces lanuginosus* IOC-4145 xylanase on enzymatic hydrolysis of corn cobs.

Optimization of xylanase production by *Bacillus subtilis* subsp. *subtilis* JJBS20 in cane molasses medium using ‘one variable at a time’ and statistical approaches found to be a beneficial tool for enhanced production. Statistical optimization resulted in enhanced xylanase production from 15.90 to 38.60 U/ml, which is 2.54-fold higher than the ‘one variable at a time approach’. In this present study, the xylanase produced was found to be effective in improving poultry feed nutrition. Xylanase addition (10 IU/g feed) enhanced liberation of reducing sugars (95.540 mg/g substrate) after 48 h at 60 °C.

Acknowledgements The authors acknowledge Maharshi Dayanand University, Rohtak for providing basic infrastructure facility for carrying out this research work. Mrs. Alokika acknowledges the financial

assistance as Junior Research fellowship (No. 09/382(0179)/2016-EMR-1) from Council of Scientific and Industrial Research (CSIR), New Delhi during the tenure of this research work.

Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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