Research paper

Sequential optimization of xylanase production by an indigenously isolated *Acinetobacter* species using solid state fermentation

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Abstract

*B*The present study aimed at sequential optimization of culture conditions for the enhanced production of xylanase from indigenously isolated *Acinetobacter sp*. by solid state fermentation. Among various agro-industrial residues utilized wheat bran was found to be the best. Various fermentation parameters for xylanase production were optimized using one factor at a time method. At the end of 72 hours, maximum enzyme activity (7.52 U/gds) was obtained using wheat bran with 75 % moisture content. Xylanase titres were further improved to 27.68U/gds using $L_9(3^4)$ orthogonal array method. Thus, approximately a four-fold increase in xylanase activity was achieved suggesting that newly isolated strain is an efficient xylanase producer.

Keywords: Xylanase, Wheat bran, Solid state fermentation, *Acinetobacter* sp.

Introduction

Xylan is an heterogeneous polysaccharide consisting of β-1,4-linked D-xylosyl residues along with a small fraction of arabinose, glucuronic acid and arabinoglucuronic acids linked to D-xylose back -bone (Wong *et al*., 1988). Enzymatic hydrolysis of xylan is catalyzed by different xylanolytic enzymes, most important are endo-1,4-β-xylanase and β-xylosidase, where the first attacks the main internal chain linkages and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides (Bakir *et al*., 2001).

These enzymes have great biotechnological functions in diverse fields of industrial applications such as delignification of pulp (Lama *et al*., 2004), as food additives in poultry, in wheat flour for improving dough handling and the quality of baked products, for extraction of coffee, plant oils and starch, in the improvement of nutritional properties of agricultural silage and grain feed, and in combination with pectinase and cellulase for clarification of fruit juices and recovery of fermentable sugars from hemicelluloses (Gupta and Kar 2008; Heck *et al*., 2006). However, the relatively high cost of enzyme production has hindered the industrial applications of enzymatic process (Battan *et al*., 2006). About 30-40% of the production cost of many industrial enzymes is accounted by the cost of growth substrate. Hence, abundantly available cost effective agricultural wastes by solid state fermentation are preferred. They not only provide alternative, cheap and renewable substrates but also help in reducing pollution problems (Revankar *et al*., 2007). The most commonly used substrate among them is wheat bran. It contains sufficient nutrients and is able to remain loose even in moist conditions thereby providing a large surface area (Archana and Satyanarayana, 1997). Wheat bran contains considerable amount of soluble sugar like glucose (42.5% dry weight), xylose (15.4% dry weight), arabinose (3.1% dry weight) and galactose (2.7% dry weight) required for the initiation of growth

and replication of microorganism (Lequart *et al.*, 1999). It contains hemicelluloses (45%) which may fulfill the role of inducers, and organic nitrogen sources (23%) that are essential for protein synthesis (Babu and Satyanarayana, 1996).

Other parameters that are critical in solid state fermentation include initial moisture content and inoculum size. At lowest and highest water content the decomposition rate of the total organic matter decreases and this in turn affects enzyme production (Pandey, 1994). The size of inoculum also plays a significant role in the production of metabolites under SSF. Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. But if the inoculum size increases beyond a certain limit the enzyme production decreases , may be because of depletion of nutrients which result in decrease in metabolic activity. Hence, a balance between biomass and nutrients would yield maximum enzyme yield (Sabu *et al.*, 2005). Thus microorganisms in solid state cultures grow under conditions closely resembling to their natural habitat, hence they are capable of producing enzymes and metabolites in higher yields than that in submerged fermentation (Asha and Prema, 2007; Pandey, 1994; Shah and Madamwar, 2005).

The next step is to make an enzyme production process commercially viable. Hence ,there is an utmost need to identify newer strains and further optimize its culture conditions and media components such as carbon source, nitrogen source, pH, temperature, inorganic salts and various cultivation conditions (Yang *et al.*, 2006). However, to optimize all the parameters and to establish the best possible conditions by interrelating all the parameters, numerous experiments have to be carried out which is not economical and practical. For a large number of variables, the conventional "one-factor-at-a-time" approach is laborious and time consuming. Application of statistical methods such as orthogonal array, reduces the number of experiments by developing specific design of experiment which also minimizes the error in determining the values for significant parameters (Lakshmi *et al.*, 2009, Maciel *et al.*, 2008). Orthogonal matrix method has successfully been applied to improve the culture media for the production of primary and secondary metabolites in fermentation process (Revankar *et al.*, 2006). It involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels). This approach facilitates identification of the influence of individual factors, establishing the relationship between variables and operational conditions and finally establish the performance at the optimum levels obtained with a few experimental sets. Hence, in the present work $L_9(3^4)$ orthogonal Matrix Method was used.

In this view main aim of the present work was exploration of

indigenously isolated *Acinetobacter sp.* for xylanase production and further improving xylanase titres by optimizing its fermentation medium using one factor at a time and orthogonal array method for solid state fermentation. Most studies till date have been carried out on xylanases isolated from different *Bacillus sp.* Experiments carried out on xylanase productionusing Acineto bacter has discussed in this paper.

Materials and Methods

Solid substrates

All agro-industrial residues were obtained locally from Navi Mumbai, India. All the substrates were used without any pretreatment.

Strain and Inoculum preparation

Indigenously isolated bacterial strain identified as a member of *Acinetobacter species* by 16sRNA sequencing was used in the present work. The organism was maintained through fortnightly transfer on basal medium agar slants having following composition: Xylan 10g/l; yeast extract 0.1g/l; $CaCl_2$ 0.2 g/l, K_2HPO_4 0.2 g/l; $MgSO_4$ 0.2 g/l, $MnCl_2$ 0.02 g/l, $(NH_4)_2SO_4$ 1g/l and agar 20g/l (Mandal *et al.*, 2008). After inoculation, slants were kept at 37°C for 48 hours. A loop full of the above culture was inoculated in 50 ml of sterile basal medium having following composition: 0.5% Xylan; 0.25% Peptone; 0.02% $MgSO_4$; 0.04% K_2HPO_4. After inoculation, the culture was incubated at 37 ±2°C with continuous agitation at 120 rpm for 24 hours. Culture with absorbance of one unit (at 660 nm) was used as inoculum in SSF.

Moistening medium

A salt solution containing $MgCl_2$ $6H_2O$ 6.6 g/l; K_2HPO_4 0.5 g/l; KH_2PO_4 0.5 g/l and $(NH_4)_2SO_4$ 2g/l with pH 6.7 was used as moistening medium for SSF (Gupta and Kar, 2009).

Preparation of SSF medium for inoculation

5g of dry substrate were taken in a 250ml Erlenmeyer flask. The contents of the flask were autoclaved at 121°C for 20 min. To the substrate, 5ml of inoculum, 2ml of moistening medium and appropriate amount of distilled water were added aseptically to adjust the initial moisture content to 75 %. The contents of flask were mixed thoroughly and incubated in a controlled humidity atmosphere at 37°C with substrate initial moisture content of 75 % for the desired period of time.

Optimization of different parameters by one factor at a time method

To select the best substrate for xylanase production different agro-industrial wastes viz; wheat bran, wheat husk, sugarcane bagasse, rice husk, coconut coir, corn cobs, saw dust, wheat straw were used. Five grams of the substrate were separately added to 250 ml Erlenmeyer flask to which 5ml of inoculum, 2ml of moistening medium and adequate quantity of sterile distilled water were added aseptically to adjust the initial moisture content to 75%.

To optimize the incubation time for maximum xylanase production, 5g of optimized substrate was added to 250 ml Erlenmeyer flask to which 5ml of inoculum, 2ml of moistening medium and adequate quantity of sterile distilled water were added aseptically to adjust the initial moisture content to 75%. The contents of flask were mixed thoroughly and incubated in a controlled humidity atmosphere at 37°C. After 24hours, 48hours, 72 hours and 96 hours respectively, Enzyme was extracted from different flasks and assayed for xylanase activity.

The effect of inoculum size for xylanase production was tested by varying optimized substrate to inoculum ratio ($^W/_v$) in the range of 1:0.1, 1:0.25, 1:0.5, 1:1, 1:1.5 and 1:2. Varying inoculums sizes as mentioned above were incorporated in Erlenmeyer flasks (250 ml) containing 5g of sterilized wheat bran along with 2 ml of the basal moistening medium. The moisture content was adjusted to 75% using appropriate amount of sterile distilled water. The contents of the flasks were mixed thoroughly and incubated at 37°C for 72 hours. The enzyme was then, extracted and assayed for xylanase activity.

To optimize the moisture content ,5g of wheat bran was added to Erlenmeyer flasks (250 ml) and autoclaved at 121°C for 20 minutes. To each flask, optimized volume of inoculum was added along with 2 ml of the basal moistening medium under aseptic conditions. The moisture content in different flasks was varied from 65% to 85% using sterile distilled water. The contents of the flask were mixed thoroughly and incubated at 37°C for 72 hours. The enzyme was then extracted and assayed for xylanase activity.

Optimization by Orthogonal Matrix Method

The design for $L_9(3^4)$ orthogonal array was developed and analyzed by Minitab 13.3 software. Table 1 documents the design of $L_9(3^4)$ orthogonal array and the concentration of different components used in the present study.

Xylanase Assay

To the fermented substrates, 50ml of 0.2 M Phosphate buffer (pH 7.0) was incorporated and mixed thoroughly by keeping the flasks on a rotary shaker at 150 rpm for 10min. Crude enzyme was extracted by direct filtration through a wet muslin cloth.

Filtrate was centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was collected and xylanase activity was determined by measuring total reducing sugars released from 1% birch wood xylan using Millers method (Miller, 1959). 0.3 ml of appropriately diluted enzyme was added to 1.6ml of 1% birch wood xylan, prepared in 0.2 M Phosphate buffer (pH 7.0) as the substrate, mixed thoroughly and incubated at 50° C for 30 minutes. The reaction was stopped by addition of 1ml dinitrosalicylic acid solution (DNS). One unit of xylanase activity was defined as the amount of enzyme that catalyses the release of 1 ìmol of reducing sugar equivalent to xylose per min under the specified assay conditions. Xylanase production was expressed as units (U) per gram of dry substrate. All the experiments, were carried out independently in duplicates and the results presented are the mean of the two values.

Results and Discussions

Optimization of different parameters by one factor at a time method

Figure 1 documents the effect of different substrates for xylanase activity. Various lignocellulosic substrates were tested for xylanase production by solid state fermentation, low xylanase activity was observed using coconut coir, sawdust and wheat straw as substrates. This may be due to the presence of polyphenols which may have inhibited growth of organism and enzyme production. Maximum xylanase activity of 6.25 U/gds was obtained with wheat bran as substrate. This may be due to the fact that wheat bran is rich in growth factors, vitamins and proteins which might have served as A source of carbon and nitrogen for the microorganisms (Lequart, 1999, Revankar *et al*., 2007). Further, it has low lignin and silica contents (Battan *et al*., 2006). Moreover, wheat bran contains sufficient nutrients and is able to remain free even in high moist condition providing large surface area (Archana and Satyanarayana, 1997).These
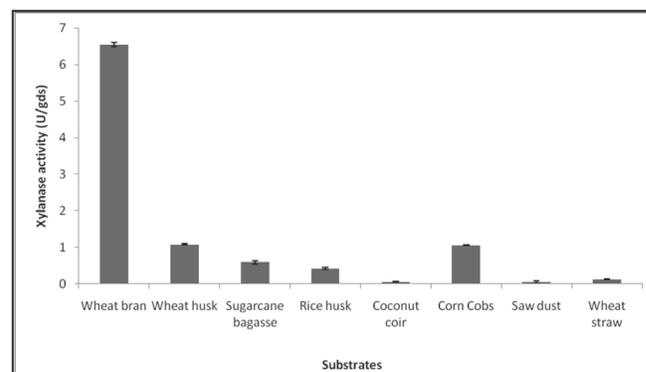

Figure1. Effect of different substrates for xylanase production by *Acinetobacter sp*.

results are in agreement with that reported in literature (Asha and Prema, 2007; Darah, 2006; Dhillon *et al.*, 2000; Gawande and Kamat ,1999). Hence, wheat bran was selected and used as substrate for further studies.

After selection of the substrate, the first step was to optimize the incubation period for xylanase production. In the present work, xylanase production was studied for about 96 hours (Figure 2). It was observed that with the increase in incubation time, enzyme production steadily increased from 0.31 U/gds after 24 h of incubation to a maximum of 6.83 U/gds at the end of 72 hours. However, with further increase in incubation time to 96 hours, a decline in xylanase activity was seen. The reduction in xylanase yield after optimum period may be probably due to proteolysis or depletion of nutrients available to the microorganism. These results are similar to those reported for bacterial isolates grown on wheat bran (Archana and Satyanarayana, 1997, Bakri *et al.*, 2008, Gawande, and Kamat, 1999). Hence, for further studies fermentation was carried out up to 72 hours.
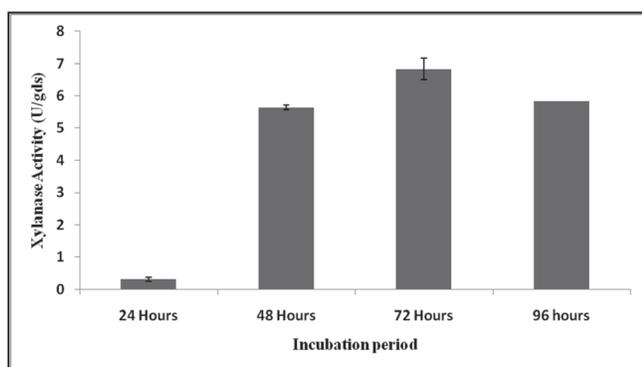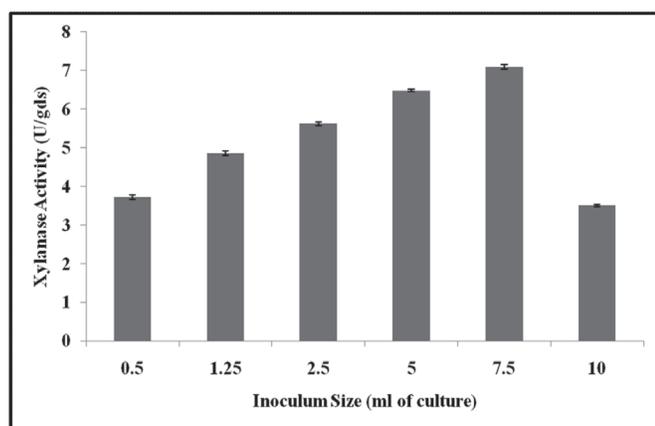
The next parameter that plays a significant role in the production of metabolites under SSF is inoculum size. Increased level of inoculum typically improves growth-related activities, but after a certain point, they serve to restrict gaseous exchange, reduce heat removal and increase the demand for nutrients from substrate (Simoes *et al.*, 2009)**.** Hence, optimization of inoculum size is important. The effect of inoculum for xylanase production was tested by varying wheat bran to inoculum ratio ($^{w}/_{v}$) in the range of 1:0.1, 1:0.25, 1:0.5, 1:1, 1:1.5, and 1:2, respectively. Figure 3 depicts the effect of varying inoculum on xylanase production. It was observed that with the increase in inoculum size from 1: 0.1 to 1:1.5 ($^{w}/_{v}$) increase in xylanase activity was achieved. This may be due to the enhanced utilization of solid substrate and thereby improving xylanase titres. Maximum enzyme production of 7.05 U/gds was obtained using ratio of 1:1.5. With further increase in inoculum to 1:2 $^{w}/v$, enzyme production was found to decline to 3.51 U/gds. This may be because of depletion of nutrients which resulted in decrease in metabolic activity, thereby decreasing enzyme activity (Simoes *et al.*, 2009).

Another critical factor for SSF is moisture content, as it is necessary for new cell synthesis, growth and enzyme production. Hence, the initial moisture content was optimized. Figure 4 represents the effect of varying moisture content on xylanase production. It can be seen that with increasing moisture content from 65 to 75% a considerable increase in xylanase activity was observed. Maximum xylanase production was obtained when the moisture content of the substrate was adjusted to 75% (7.14 U/gds). However, when the moisture content was increased beyond 75%, a substantial decrease in xylanase titres was observed. This may be the result of increase in water content at constant substrate volume that reduces the air content of the substrate (air occupied within the interparticle space), thereby, affecting xylanase activity (Asha
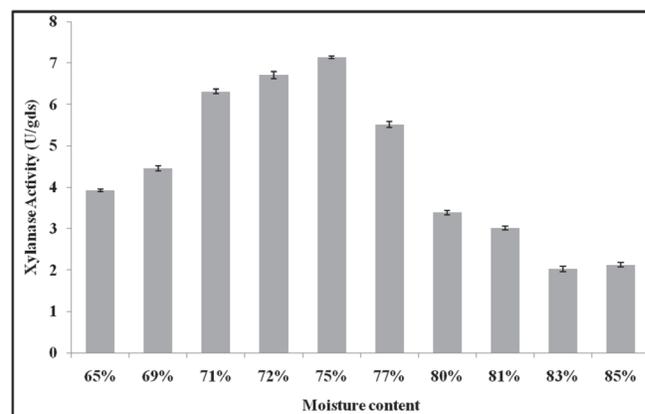

Figure 2: Effect of varying incubation period on xylanase production by *Acinetobacter sp.*

Figure 3: Effect of inoculum size on xylanase production by *Acinetobacter sp.*

Figure 4: Effect of moisture content on xylanse production by *Acinetobacter sp.*

Table 1: L (9)3⁴ Orthogonal Array design for xylanase production by *Acenitobacter sp.*

Run	Conc. of Xylose	Conc. of Ammonium Nitrate	Conc. of Moistening medium	pH of Moistening medium	Xylanase Activity (U/gds)
1	1%	1%	1X	5.7	11.5
2	1%	2%	2X	6.7	14.86
3	1%	3%	3X	7.7	12.5
4	2%	2%	2X	7.7	9.87
5	2%	2%	3X	5.7	20.03
6	2%	2%	1X	6.7	26.14
7	3%	1%	3X	6.7	16.11
8	3%	2%	1X	7.7	14.35
9	3%	3%	2X	5.7	18.9

and Prema 2007, Pandey 1994.). In addition, at the lower end the higher water contents beyond 70%, the decomposition rate of the total organic matter decreases and this in turn affects xylanase production (Asha and Prema 2007, Pandey 1994). Thus, 75% initial moisture content was selected for the further studies.

Media optimization by orthogonal matrix Method

Studies show that supplementation of agricultural residues with carbon and nitrogen source in media formulation enhances growth and subsequently, results in higher enzyme production (Darah, 2006). The orthogonal matrix method was used to evaluate the relative importance of various nutrients and optimize their concentrations for xylanase production by solid state fermentation.
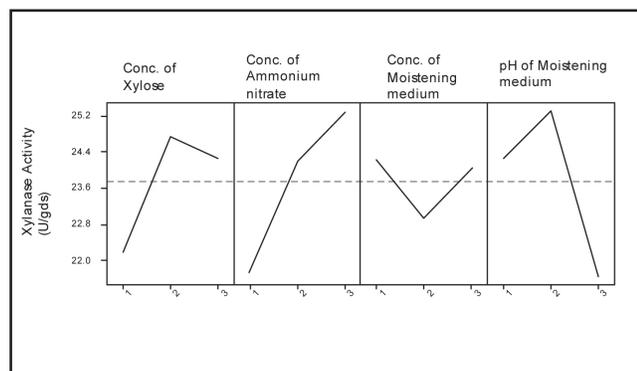
L₉(3⁴) orthogonal matrix was selected for *Acnetobacter sp.* with four variables at three different concentrations. Preliminary studies (results not reported) indicated that xylose as carbon source and ammonium nitrate as nitrogen source resulted in enhancement of xylanase activity. Hence, these variables were selected. The variables optimized in the present study involved A – Concentration of Xylose; B - Concentration of ammonium nitrate; C - Concentration of moistening medium and D - pH of moistening medium that may have a significant influence on the xylanase production. The experimental conditions were similar to that used for one factor at a time method. The next stage involved selecting the appropriate design structure for data analysis. The design for the L₉(3⁴) orthogonal array was developed and analyzed using "MINITAB 13.30" software. Table 1 shows L₉(3⁴) orthogonal array and the concentration of different media components that were used in the present study. The experimental results are included in the last column of the table.

Table 2 represents the response table for means (larger is better) obtained for L₉(3₄) orthogonal array. The last two rows in the table document delta values and the ranks for the system. Rank and delta values help to assess which factors have the greatest effect on the response characteristic effect. Higher the delta value more is the effect of that component on xylanase activity. According to the magnitude of order of delta, the order of effect of factors on xylanase activity was ammonium nitrate > pH of moistening agent > Xylose > concentration of moistening agent. The optimum levels of each factor obtained by statistical analysis are shown in Figure 7. This figure represents the main effect plot for the system. In the present study, it can be seen that for each of the four variables at three levels we find one level increases the mean compared to the other level. This difference is a main effect that is xylose at level 2, ammonium nitrate at level 3, concentration of Moistening agent at level 1 and pH of Moistening agent at level 2. These levels also represent the optimal concentrations of the individual components in the medium. Thus, the optimum levels

Table 2: Response table for Means

Level	Concentration of Xylose	Concentration of Ammonium Nitrate	Concentration of Moistening Medium	pH of moistening medium
1	12.95	12.49	17.33	16.81
2	18.68	16.41	14.54	19.03
3	16.45	19.18	16.21	12.24
Delta	5.72	6.68	2.78	6.79
Rank	3	2	4	1

Figure 5: Main effect plot for xylanase production by
Acinetobacter sp.

are as follows; xylose 2%, ammonium nitrate 3% and concentration of moistening agent 1 X and pH of moistening agent 6.7. Minitab software predicted a value of 28.34 U/gds for the above media composition for xylanase production using *Acinetobacter sp.* on experimental verification xylanase activity of 27.18U/gds was obtained that confirmed the findings. Thus, an optimum medium for xylanase production by *Acinetobacter sp.* was determined.

In order to assess potential of the new isolate *Acinetobacter sp.* as xylanase producer, xylanase activities obtained in this work were compared with those reported in literature. Depending on the strain used, different ranges of xylanase activities could be obtained using solid state fermentation. Using *Streptomyces sp.* Techapun *et al*, 2001 have reported xylanase activity of 3.5 IU and Widjajaja *et al.*, (2009) have reported 5U/ml using *Aspergillus niger sp.* where as Couri *et al.*, (2000) , Kheng *et al.*, (2005) could successfully obtain 30.62U/ml and 25.4 U/g respectively using different *Aspergillus sp.* We report in the present work 27.68U/gds of xylanase activity with the new strain *Acinetobacter sp.* This activity is higher and comparable with many of the reported strains Thus, it can be said that the new isolate, *Acinetobacter sp* is an efficient and excellent producer of xylanase and further experiments need to be conducted to explore as xylanase producer on commercial scale.

Conclusion

The solid state medium was optimized using one factor at a time and orthogonal array method. Wheat bran with 75% moisture content gave yield of 7.52U/gds at the end of 72 hours. The wheat bran medium supplemented xylose and ammonium nitrate was optimized using $L_9(3^4)$ orthogonal array method and a four fold increase in xylanase activity was achieved. Xylanase activities achieved using the new isolate are significantly higher than most of the reported strains. Thus, it can be said that the new isolate *Acinetobacter* species

efficiently produces xylanase with the conditions optimized in this study.

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