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ORIGINAL ARTICLE

Optimization of cellulase-free xylanase production by alkalophilic *Cellulosimicrobium* sp. CKMX1 in solid-state fermentation of apple pomace using central composite design and response surface methodology

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Abstract Microbial xylanases and associated enzymes degrade the xylans present in lignocellulose in nature. Xylanase production by *Cellulosimicrobium* sp. CKMX1, isolated from mushroom compost, produced a cellulase-free extracellular endo-1, 4-β-xylanase (EC 3.2.1.8) at 35 °C and pH 8.0. Apple pomace—an inexpensive and abundant source of carbon—supported maximal xylanase activity of 500.10 U/g dry bacterial pomace (DBP) under solid state fermentation. Culture conditions, e.g., type of medium, particle size of carbon source, incubation period, temperature, initial pH, and inoculum size, were optimized and xylanase activity was increased to 535.6 U/g DBP. CMCase, avicelase, FPase and β-glucosidase activities were not detected, highlighting the novelty of the xylanase enzyme produced by CKMX1. Further optimization of enzyme production was carried out using central composite design following response surface methodology with four independent variables (yeast extract, urea, Tween 20 and carboxymethyl cellulose), which resulted in very high levels of xylanase (861.90 U/g DBP). Preliminary identification of the bacterial isolate was made on the basis of morphological and biochemical characters and confirmed by partial 16Sr RNA gene sequencing, which identified CKMX1 as *Cellulosimicrobium* sp. CKMX1. A phylogenetic analysis based on the 16Sr RNA gene sequence placed the isolate within the genus *Cellulosimicrobium*, being related most closely to *Cellulosimicrobium cellulans* strain AMP-11 (97% similarity). The ability of this strain to produce cost-effective xylanase from apple pomace on a large scale will help in the waste management of apple pomace.

Key words Cellulase-free xylanase · *Cellulosimicrobium* sp. · Solid state fermentation · Apple pomace · Response surface methodology

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Introduction

Xylan is the major hemicellulosic constituent of hard and soft wood, and is the next most abundant renewable polysaccharide after cellulose. This complex heteropolysaccharide consists of a main chain of 1,4-β-D-xylose monomers and short chain branches consisting of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues. Xylanases and associated debranching enzymes produced by a variety of microorganisms including bacteria, yeast and filamentous fungi, bring about the hydrolysis of hemicelluloses (Maheshwari et al. 2000).

Xylanolytic enzymes are receiving increasing attention because of their potential applications in improving the digestibility of animal feed (Wong et al. 1988), pulp bleaching (Rifaat et al. 2005) and bioconversion of lignocelluloses into feedstocks and fuels (Kim et al. 2000). The xylan-degrading system includes endo-1,4-xylanases (1,4-β-xylan

xylanohydrolase; EC 3.2.1.8), which release long and short xylo-oligosaccharides, and other xylanases that attack only longer chains, and β-D-xylosidase (1,4-β-xylan xylohydrolase; EC 3.2.1.37), which remove D-xylose residues from short xylo-oligosaccharides (Biely 1985; Saha 2003).

Cellulase-free xylanases are important in the paper and pulp industry as alternatives to the use of toxic chlorinated compounds (Viikari 1994; Srinivasan and Rele 1999). For the last two decades the bleaching of pulp has become an issue of great concern, primarily because of the environmental hazards caused by the release of the adsorbable organic halogens and due to increasing public awareness thereof (Beg et al. 2001).

The commercial application of xylanase in various industrial processes has had limited success due to various factors. These include inaccessibility of substrate to xylanase enzymes because of physical limitations, the limited hydrolysis of xylans due to their branched nature, thermal instability, narrow pH range, end product inhibition and cost of enzyme production. The last two problems can be overcome to some extent by the use of cheap substrates and by employing the process of solid state fermentation (SSF). *Cellulosimicrobium* sp. CKMX1 grows well on apple pomace in solid-state culture conditions and produces a high level of xylanase. The optimal culture medium for this strain for SSF has not yet been developed and designing such a medium would improve significantly the yield and quality of xylanase.

Culture medium optimization by the traditional 'one-factor-at-a-time' technique requires a considerable amount of work and time. An alternate strategy is a statistical approach, e.g., factorial experimental design and response surface methodology (RSM), involving a minimum number of experiments for a large number factors, by which improvement in enzyme production has been demonstrated successfully (Ghanem et al. 2000; Bocchini et al. 2002). Although several reports are available on the production of xylanase in SSF from fungi and actinomycetes, in bacteria there are only few reports showing low enzyme yield (Archana and Satyanarayana 1997; Heck et al. 2005; Battan et al. 2006; Sindhu et al. 2006). Although very limited information is available in respect of xylanase production in SSF of apple pomace, previous research has described the beneficial effect of apple pomace as a good and cheap substrate for production of bacterial xylanases (Sharma 1998). Therefore, keeping in view the importance of this substrate, an attempt has been made to use apple pomace in combination with additional sources of carbon and nitrogen as substrate for xylanase production by *Cellulosimicrobium* sp. CKMX1 in SSF. Further optimization of process parameters to obtain high levels of cellulase-free xylanase was carried out using central composite design (CCD) following RSM.

Materials and methods

Culture and growth medium

A bacterial culture isolated originally from mushroom compost was obtained from the Culture Collection Centre of Microbiology Section, Department of Basic Sciences (Dr. Y.S. Parmar University of Horticulture and Forestry). The bacterial culture was grown and maintained in basal salt medium (BSM, pH 8.0) containing 0.5 % xylan with the following composition (g/L): Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0, 1 M MgSO4 (2 mL) and 1 M $CaCl_2$ (0.1 mL). The bacterial culture was maintained in 30% glycerol at −20 °C.

Apple pomace as substrate

Apple pomace was procured from the processing unit of the Horticultural Produce Marketing and Processing Corporation (HPMC; Parwanoo, Himachal Pradesh, India). The oven-dried material (60 °C for 48 h) was ground in an electric grinder and packed in air-tight containers for subsequent studies. The powder was screened through ISI meshes and fractions of mesh 10, 15, 20, 25, 30, 35 and 40 were collected.

Enzyme production and extraction

Solid state fermentation was carried out in Erlenmeyer flasks (250 mL) containing 10 g substrate (apple pomace) and 20 mL mineral salt solution (BSM) at pH 8.0 were autoclaved at 15 psi pressure for 20 min, cooled and inoculated with 2 mL bacterial suspension (OD 1.0 at 540 nm). After mixing, the flasks were incubated at 35 °C for 3 days. At the desired intervals, the flasks were taken out and the contents were extracted with 50 mL sterilized buffer (0.2 M, pH 8.0, Tris HCl). The flasks were kept in shaker for half an hour to ensure thorough mixing of apple pomace with the buffer. The flask contents were centrifuged at 8,000 rpm for 20 min at 4 °C. The culture supernatant was used as crude enzyme preparation (prior to centrifugation, samples were withdrawn for determining viable number of cells by the standard viable plate count technique).

Enzyme assay

Xylanase activity was assayed using 1% oat spelt xylan (Sigma, St, Louis, MO) in 0.2 M Tris-HCl buffer (pH 8.0) according to the calorimetric method of Miller (1959).The release of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNSA) method with a xylose standard curve. The reaction mixture contained 0.5 mL 1 % D-xylan in Tris-HCl buffer (0.2 M, pH 8.0) and 0.5 mL diluted enzyme. It was incubated at 50 °C for 5 min in a water bath with occasional shaking. After incubation, 3 mL DNSA

reagent was added into the test tubes, which also stopped the enzymatic reaction. The tubes were immersed in boiling water bath and removed after 15 min when color development was completed. Tubes were cooled to room temperature. The contents were transferred to a 25 mL volumetric flask and final volume made up with distilled water. Optical density was read at 540 nm in a Spectronic-20.

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugars per minute under given assay conditions. Xylanase activity is expressed as U/g dry bacterial pomace (DBP).

Response surface methodology

Using the 'one variable at a time' approach, the effect of media types, particle size of carbon source, temperature, pH, incubation temperature and inoculum size was studied. Based on these experiments, four independent variables were chosen for further optimization by RSM using CCD experiments (Table 1). Each variable was studied at three levels (−1, 0, +1). The experimental design included 21 flasks with three replicates at their central coded values (Chadha et al. 2004). The mathematical relationship of response (enzyme production) and variable A, B, C and D [yeast extract, urea, tween 20 and carboxymethyl cellulose (CMC)] was approximated by a quadratic model equation. The response value in each trial is the average of triplicate experiments.

Molecular taxonomic characterization

The bacterial isolate was grown in nutrient broth at 37 °C overnight. Bacterial cells were harvested by centrifugation at 5,000 *g* for 5 min and DNA was isolated from these bacterial cells by using a RealGenomics DNA Extraction Kit (Life Technologies, Delhi, India). The isolated DNA was finally suspended in 100 μl elution buffer and quantified on a 1% agarose gel. The total genomic DNA was kept at −20 °C before use (Sambrook and Russel 2001).

Species level identification of the strain was conducted by 16Sr RNA sequence comparison. PCR reactions were carried out in 20 μL reaction buffer containing ~50 ng template DNA, 20 pmol of each primer fC1 (5′-GCAAGTC-GAGCGGACAGATGGGAGC-3′) and reverse primer rC2 (5′-AACTCTCGTGGTGTGACGGGCGGTG-3′), 0.2 mM dNTPs and 1 U *Taq* polymerase (MP Biomedicals, Santa Ana, CA) in 1x PCR buffer. Reaction were cycled 35 times as 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min 30 s

Table 1 Actual and predicted values (unit/g dry substrate of apple pomace) of xylanase recorded in the response surface methodology (RSM) experimental setup. *CMC* Carboxymethyl cellulose

Sample no.	Yeast extract (g %)	Urea nitrogen (mg %)	Tween 20 (mL %)	CMC (g %)	Xylanase activity (actual)	Xylanase activity (predicted)
1	0.55	35.00	0.06	5.00	618.79	619.75
2	0.55	35.00	0.06	3.00	647.65	608.36
3	0.55	10.00	0.06	3.00	386.75	387.71
4	0.55	35.00	0.06	1.00	718.25	719.21
5	0.10	10.00	0.01	1.00	408.85	419.76
6	0.10	35.00	0.06	3.00	685.09	686.05
7	0.55	60.00	0.06	3.00	861.9	862.86
8	1.00	60.00	0.10	1.00	729.3	717.9
9	0.55	35.00	0.06	3.00	628.75	608.36
10	1.00	10.00	0.10	5.00	663	651.6
11	1.00	35.00	0.06	3.00	607.75	608.71
12	0.10	60.00	0.01	5.00	625.49	636.4
13	0.10	60.00	0.10	5.00	475.15	463.75
14	0.55	35.00	0.10	3.00	522.25	542.84
15	0.55	35.00	0.06	3.00	596.7	608.36
16	0.10	10.00	0.10	1.00	817.69	806.29
17	0.55	35.00	0.01	3.00	377.8	354.12
18	1.00	60.00	0.01	1.00	419.89	430.8
19	0.55	35.00	0.06	3.00	593.05	608.36
20	0.55	35.00	0.06	3.00	621.45	608.36
21	1.00	10.00	0.01	5.00	386.75	397.66

followed by final extension at 72 °C for 10 min. The PCR products were analyzed on a 1% agarose gel in 1x TAE buffer, run at 100 V for 1 h. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel using a gel extraction kit (RealGenomics Hi Yield ™ Gel/PCR DNA Extraction Kit); the eluted fragment was then sequenced using PCR primers.

Sequence analysis

The sequence was aligned with corresponding sequences of 16Sr RNA from the database using BLAST (Altschul et al. 1997). Multiple alignments were generated by the program MULTALIN (Corpet 1988). A phylogenetic tree was constructed with the help of ClustalW (Higgins et al. 1994). The tree was viewed with the help of TreeView (Page 1996).

Statistical analysis

The statistical software package Design-Expert 8.0.4 (StatEase, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. ANOVA was used to estimate statistical parameters.

Results

Evaluation of mineral salt medium for alkaline xylanase production

The composition of the medium plays an important role in enzyme production, therefore different media types were tested for xylanase production. *Cellulosimicrobium* sp. CKMX1 was grown on apple pomace supplemented with BSM, BSYEM (same as BSM, but with 0.5 % yeast extract added) and tap water as fermentation media. The organism grew best on apple pomace supplemented with BSYEM followed by BSM; minimum growth was observed upon fermentation in tap water (Fig. 1). Maximum xylanase activity (500.10 U/g DBP) was obtained when BSYEM was used as fermentation medium. CMCase, FPase, avicelase and β-glucosidase were not detected.

Effect of particle size on alkaline xylanase production

The effect of particle size on production of extracellular xylanase activity by *Cellulosimicrobium* sp. CKMX1 is shown in Fig. 2. Maximum xylanase (535.6 U/g DBP) production was obtained when the particle size used was minimum (40 mesh size). The enzyme activities at all particle sizes differed significantly. However, CMCase, FPase, avicelase and β-glucosidase were not detected.

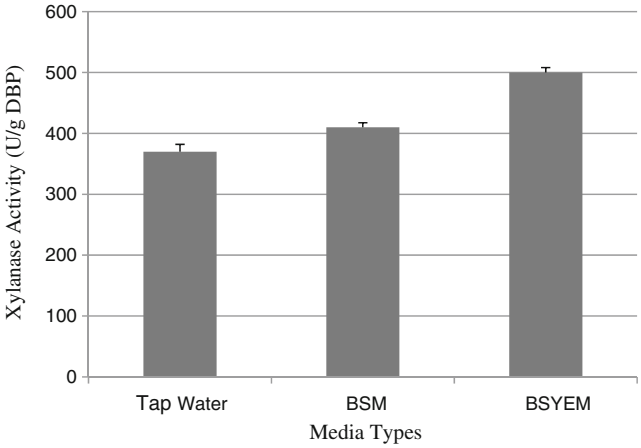

Fig. 1 Evaluation of mineral salt medium for production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in solid state fermentation (SSF). Each value represents the mean of five replicates. *Error bars* Standard deviation

Effect of incubation period on alkaline xylanase production

Maximum xylanase production (525 U/g DBP) was obtained at 72 h; further increase in incubation period decreased xylanase production (Fig. 3). The correlation coefficient (r=0.624) between different incubation temperatures, i.e., 0, 6, 12, 24, 48, 72 and 96 was found to be positively correlated and statistically significant at $P \leq 0.05$ for the production of xylanase. CMCase, FPase, avicelase and β-glucosidase were not detected at any time in the incubation period.

Effect of initial pH and temperature on alkaline xylanase production

Xylanase production was found to be highly dependent on pH and temperature. Maximum xylanase activity (535.10 U/g)

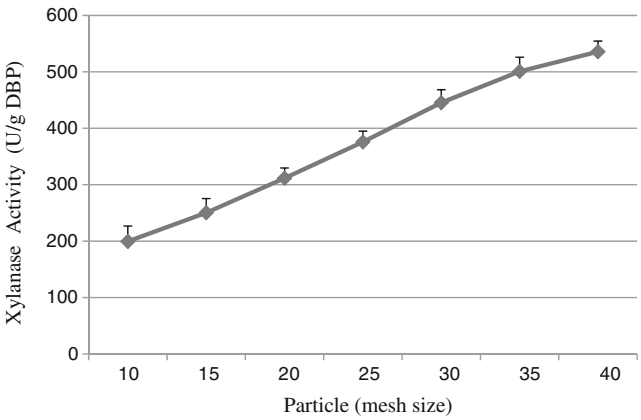

Fig. 2 Effect of particle size on production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in SSF. Each value represents the mean of five replicates. *Error bars* Standard deviation

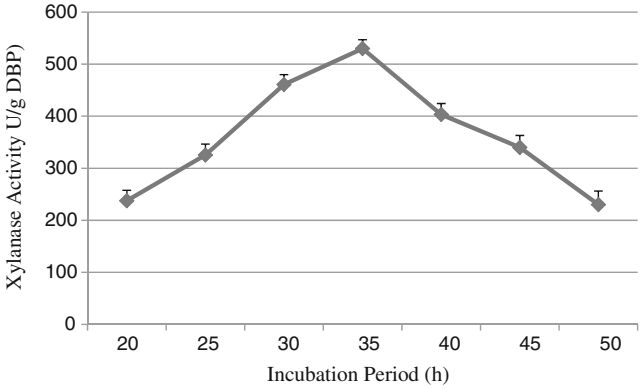

Fig. 3 Effect of incubation period on production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in SSF. Each value represents the mean of five replicates. *Error bars* Standard deviation

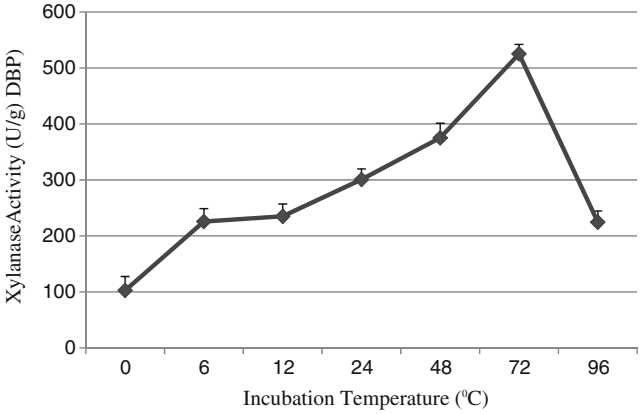

Fig. 5 Effect of incubation temperature on production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in SSF. Each value represents the mean of five replicates. *Error bars* Standard deviation

was observed at pH 8.0 (Fig. 4). This shows the alkalophilic nature of the present bacterial strain, which produces an enzyme active in the alkaline range. Incubation temperature is also a factor regulating enzyme synthesis. The effect of temperature on xylanase production by *Cellulosimicrobium* sp. CKMX1 was investigated by incubating the flasks over a wide range of temperature at 20, 25, 30, 35, 40, 45 and 50 °C using BSYEM at pH 8.0. As shown in Fig. 5, increasing the fermentation temperature from 20 °C to 50 °C affected significantly enzyme production, viable cell number, reducing sugar and final pH. The production of xylanase activity increased up to 35 °C, with any further increase in temperature resulting in a decrease in xylanase activity. The difference in xylanase production obtained at various temperatures was found to be statistically significant. The production of xylanases decreased drastically at temperature values lower or higher than the optimum temperature of 35 °C. CMCase, FPase, avicelase and β-glucosidase were not detected.

Effect of inoculum size on alkaline xylanase production

The effect of inoculum size (5 %, 10 % and 20 %) (v/w) on production of xylanase was observed in production medium (Fig. 6). Maximal xylanase yield (530.00 U/g DBP) was obtained with 10 % (v/w) inoculum, and the final cell number was also significantly higher. However, decreased xylanase activity was observed at lower (5 %) and higher inoculum (20 %) concentrations. The xylanase activity obtained at the 5 % inoculum level was 365.8 U/g DBP. CMCase, FPase, avicelase and β-glucosidase were not detected at any inoculum size used.

Optimization of enzyme production using RSM

The interaction of four independent variables, i.e., yeast extract, urea nitrogen, Tween 20 and CMC concentrations,

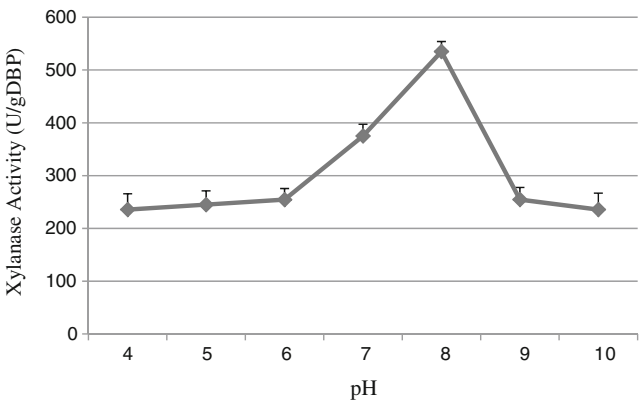

Fig. 4 Effect of initial pH on production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in SSF. Each value represents the mean of five replicates. *Error bars* Standard deviation

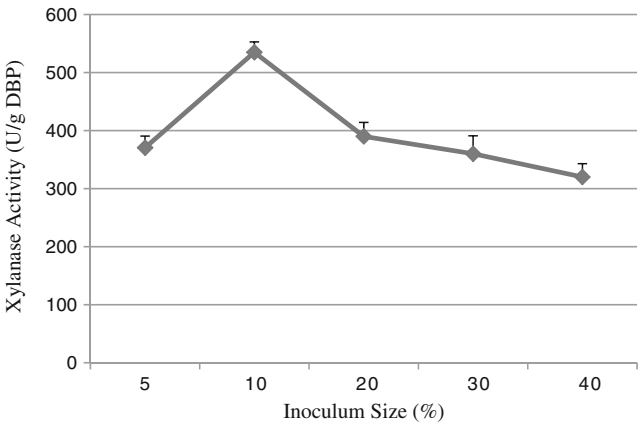

Fig. 6 Effect of inoculum size on production of alkaline xylanase by *Cellulosimicrobium* sp. CKMX1 during 72 h of incubation on apple pomace in SSF. Each value represents the mean of five replicates. *Error bars* Standard deviation

was studied for further optimization of enzyme production using CCD experiments. Regression analysis was performed to fit the response function to the experimental data. The results of RSM experiments for studying the effect of the four variables along with the mean actual and predicted response are presented in Table 1. The coefficient of determination (R^2) for xylanase production as a function of the independent variables was found to be 98 %, which showed that the model correlated well with measured data and was statistically significant at $P \leq 0.05$.

The regression equation obtained showed that the xylanase production may be best predicted by the model:

$$\text{Response (Xylanase activity)} =$$

$$613.08 - 38.67 * A + 237.58 * B + 98.86 * C49.73$$

$$* D21.56 * A * B + 40.90 * A * C + 240.88 * A * D$$

$$- 65.75 * B * C - 22.64 * B * D - 74.04 * C * D$$

$$+ 37.04 * A^2 + 14.94 * B^2 - 159.36 * C^2 + 59.14 * D^2$$

After eliminating the non-significant terms, the final response equation for xylanase production, is given as follows:

$$\text{Response (Xylanase activity)} =$$

$$613.08 - 38.67 + 237.58 * B + 98.86 * C49.73$$

$$* D21.56 + 40.90 * A * C + 240.88 * A * D - 65.75$$

$$* B * C - 22.64 - 74.04 * C * D + 37.04 + 14.94$$

$$- 159.36 * C^2 + 59.14 * D^2$$

where A is the level of yeast extract, B is urea nitrogen concentration, C is Tween 20 concentration and D is CMC concentration.

The corresponding analysis of variance (ANOVA) of the empirical model obtained along with the values of the coefficient of determination (R^2) and the adjusted determination coefficient (adj.R^2) are presented in Table 2. In each case, the lack-of-fit analysis gave non-significant *P*-values (>0.05) and *F*-values lower than the corresponding tabulated *F*-values, thus proving that the model obtained was highly significant. In addition, the high value of R^2 (0.98 or higher) indicated that the fitted models could explain at least 98 % of the total variation in the responses. These facts indicate that the quadratic models were appropriate to fit and describe satisfactorily the experimental data regarding xylanase production. Further, regression analysis showed that all the independent variables (yeast extract, urea nitrogen, Tween 20 and CMC concentrations) were significant ($P \leq 0.05$) for the production of xylanase (Table 2) in squared terms.

Table 2 Analysis of variance (ANOVA) for response surface quadratic model obtained for xylanase production

Source	Sum of squares	df	Mean square	*F* value[a]	*P* value Prob > F
Model	3.874E+005	14	27,670.06	40.79	<0.0001*
A yeast extract	2,990.74	1	2,990.74	4.41	0.0805**
B-Urea	1.129E+005	1	1.129E+005	166.41	<0.0001*
C-Tween 20	97,734.97	1	97,734.97	144.08	<0.0001*
D-CMC	4,946.15	1	4,946.15	7.29	0.0356*
AB	743.91	1	743.91	1.10	0.3353**
AC	13,379.21	1	13,379.21	19.72	0.0044*
AD	92,839.01	1	92,839.01	136.86	<0.0001*
BC	34,587.13	1	34,587.13	50.99	0.0004*
BD	820.11	1	820.11	1.21	0.3137**
CD	43,858.33	1	43,858.33	64.65	0.0002*
A^2	3,501.88	1	3,501.88	5.16	0.0635**
B^2	569.97	1	569.97	0.84	0.3947**
C^2	64,829.28	1	64,829.28	95.57	<0.0001*
D^2	8,927.83	1	8,927.83	13.16	0.0110*
Residual	4,070.10	6	678.35		
Lack of fit	1,988.48	2	994.24	1.91	0.2616
Pure error	2,081.63	4	520.41		
Corrected total	3.915E+005	20			
R^2value	0.989				
Adj. R^2value	0.965				
CV	4.41				

*Significant at $P \leq 0.05$, ** non-significant

[a] The Model F-value of 40.79 implies the model is significant. There is only a 0.01 % chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, D, AC, AD, BC, CD, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. A negative "Pred R-Squared" implies that the overall mean is a better predictor of your response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 23.202 indicates an adequate signal. This model can be used to navigate the design space

To investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum xylanase production by *Cellulosimicrobium* sp. CKMX1, the contour plot and three-dimensional response surfaces were plotted on the basis of the model equation. The effects of varying the concentration of yeast extract and one of the other variables are shown in Figs. 7 and 8, which demonstrates that the response surfaces for three combinations were similar to each other.

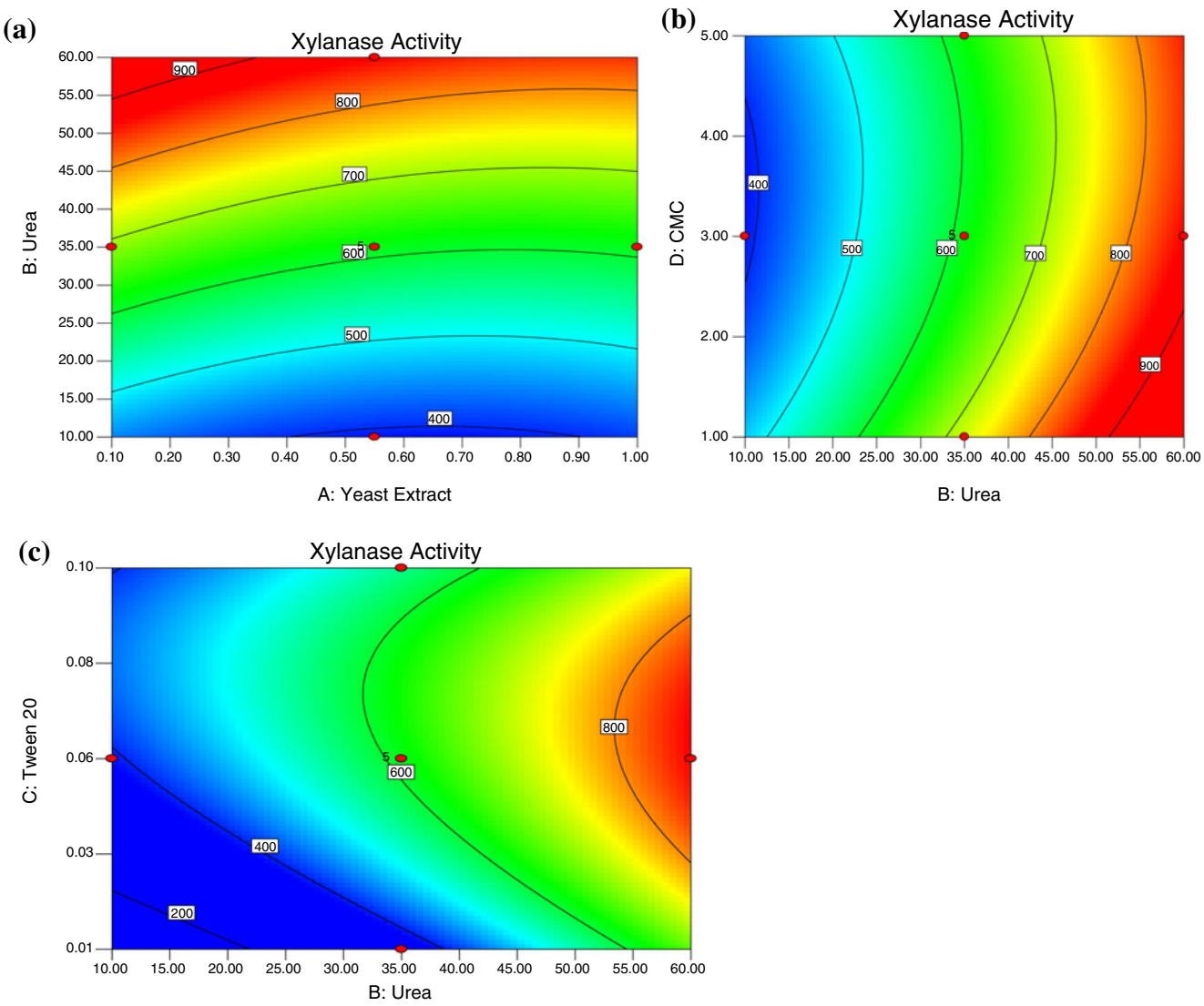

Fig. 7 **a**–**c** Contour plot showing the effect of the central composite design (CCD) experiment for alkali-stable xylanase production by *Cellulosimicrobium* sp. CKMX1. The interactions between yeast extract and urea nitrogen (**a**), Urea nitrogen and CMC (**b**) and urea nitrogen and Tween 20 (**c**) are shown. The values in the box indicate xylanase activity

Xylanase production in the original medium with the four components at their central levels was 535.1 U/g DBP. Design Expert predicted the maximum xylanase yield to be 862.86 U/g DBP in optimized medium composed of (g/L): 0.55% (w/v) yeast extract, 35 % mg (w/v) urea nitrogen, 0.06 % (v/w) Tween 20 and 3 % (w/v) CMC, which is very close to the actual level of xylanase produced in the optimized medium, which was 861.9 U/g DBP. Xylanase yield in the initial medium before optimization was only 535.1 U/g DBP, which is 1.6-fold less than the optimized level. There was a gradual increase in enzyme production from 408.85 U/g DBP to 861.9 U/g DBP when the yeast extract level was increased from 0.1 % to 0.55 % (Table 1). When the concentration of the yeast extract was increased further (0.55 % to 1 %) in the fermentation medium, keeping all other variables constant, bacterial growth and xylanase production were noted. CMCase, FPase, avicelase and β-glucosidase were not found in any of the media before or after optimization, suggesting that the optimized media components do not favor cellulase production. The kinetics of xylanase production in SSF using initial and optimized media showed maximum production after 72 h of incubation.

Phenotypic and metabolic characteristics of *Cellulosimicrobium* sp. CKMX1

The strain CKMX1 was characterized initially according to morphological, physiological and biochemical characteristics (Table 3). The isolated colonies on basal salt yeast extract medium after 48 h of incubation were cream-colored, circular with a smooth surface, convex elevation,

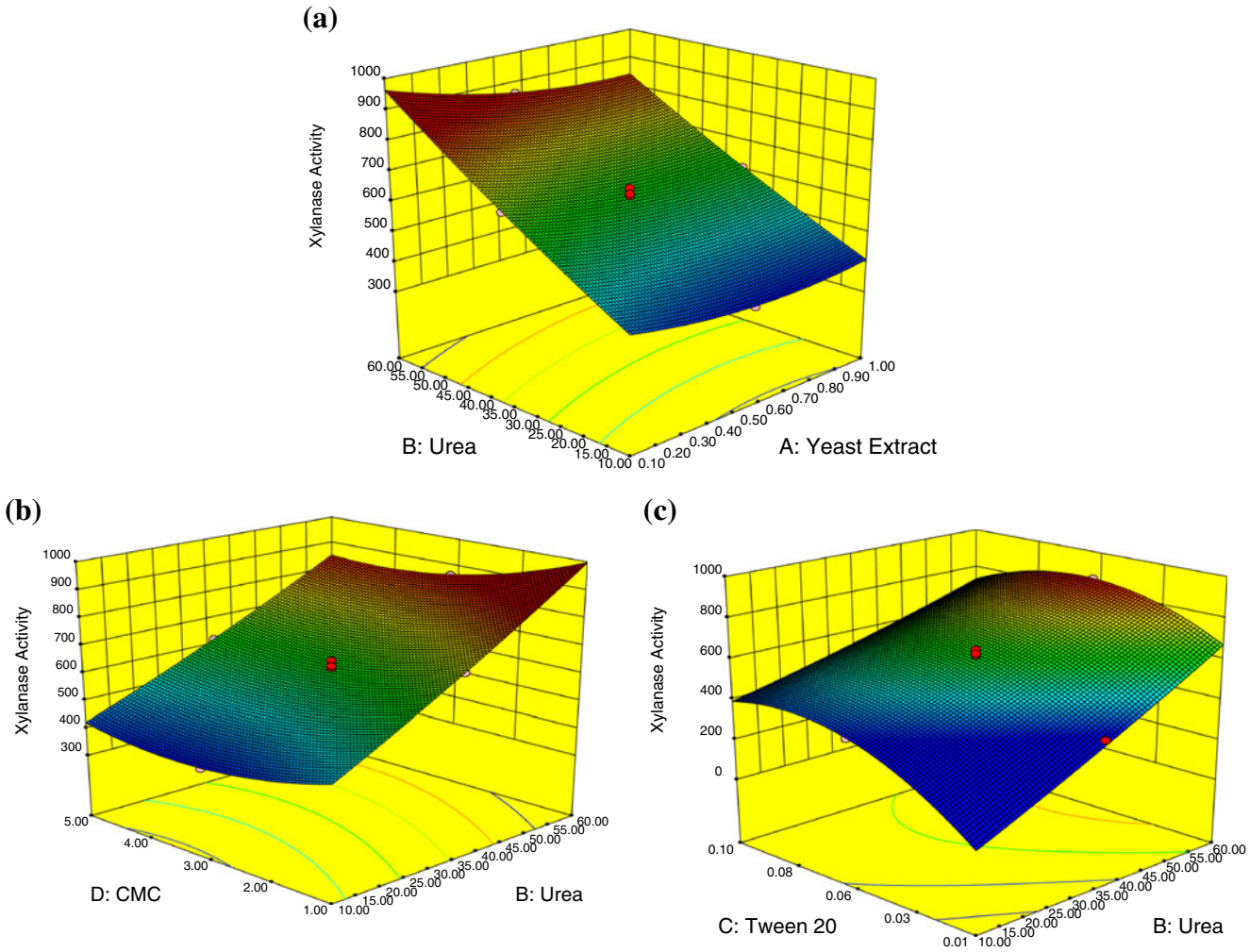

Fig. 8 Three dimensional (3D) response surface plot of the CCD experiment for alkali-stable xylanase production by *Cellulosimicrobium* sp. CKMX1. The interactions between yeast extract and urea nitrogen (**a**), Urea nitrogen and CMC (**b**) and urea nitrogen and Tween 20 (**c**) are shown

0.1–0.5 mm in diameter and entire edged. The morphological characteristics of the strain were as follows: substrate hyphae were present, the cells were Gram positive, non-spore forming and non-motile. Young cultures were composed of straight or curved rods. The rods occurred singly or in chains.

The physiological and biochemical characteristics of the isolated strain CKMX1 are given in Table 3. The isolate is an aerobic, catalase producing strain. Nitrate was reduced to nitrite. Starch was hydrolyzed through production of enzyme amylase. The isolate tested positive for gelatine liquefaction. The strain was positive for cellulolytic and esculin hydrolysis and could utilize a wide array of carbohydrates (assessed using a KB009 Hicarbohydrate™ Kit), including xylose, maltose, fructose, dextrose, galactose, trehalose, sucrose, L-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, cellobiose, xylitol, D-arabinose, as sole carbon source.

Phylogenetic analysis of *Cellulosimicrobium* sp. CKMX1 according to 16Sr RNA sequence

Universal primers were used successfully to amplify 16Sr RNA from bacterial isolate CKMX1, yielding an amplicon of the expected size, i.e., ~1,136 bp. The sequence of 16Sr RNA from CKMX1 was then analyzed using BLASTn analysis (http://www.ncbi.nlm.nih.gov/blast) and was found to have 97 % homology with several *Cellulosimicrobium cellulans* strains reported from different parts of the world. The 16Sr RNA sequence of CKMX1 was also compared with the corresponding sequences of eight different *Cellulosimicrobium* sp. (Table 4) reported from different parts of the world. Sequence analysis revealed that CKMX1 belongs to *Cellulosimicrobium* sp. CKMX1 as it showed maximum homology (97 %) with *Cellulosimicrobium cellulans* strain AMP-11 (accession no. HM104377).

Table 3 Differential phenotypic and metabolic characteristics of the *Cellulosimicrobium* sp. CKMX1

Characteristics	*Cellulosimicrobium* sp. CKMX1
Colony morphology	Circular/pale yellow/convex
Gram character	Gram +ve
Pleomorphic	+
Sporulatiing	−
Motile	−
Substrate hyphae	+
Gelatin liquefaction	+
Starch hydrolysis	+
Catalase	+
Cellulolytic	+
Nitrate reduction	+
Utilization of:	
Lactose	−
Xylose	+
Maltose	+
Fructose	+
Dextrose	+
Galactose	+
Raffinose	−
Trehalose	+
Melibiose	−
Sucrose	+
L-Arabinose	+
Mannose	+
Inulin	+
Sodium gluconate	+
Glycerol	+
Salicin	+
Dulcitol	−
Inositol	−
Sorbitol	−
Mannitol	−
Arabitol	−
Erythritol	−
α-Methyl-D-glucoside	−
Rhamnose	−
Cellobiose	+
Melezitose	−
α-Methyl-D-mannoside	−
Xylitol	+
ONPG	−
Esculin hydrolysis	+
D-Arabinose	+
Citrate utilization	−
Malonate utilization	−

To trace out the evolutionary patterns of the test isolate and to determine the relationship with other selected sequences at NCBI, a phylogenetic tree was also constructed using the neighbor-joining (J) method of mathematical averages (UPGMA) among 16Sr RNA sequence of CKMX1 and the corresponding sequence of eight different *Cellulosimicrobium* sp. Strain CKMX1 was united with quite high statistical support by the bootstrap estimates for 1,000 replications. The resulting phylogenetic tree (Fig. 9) also verified CKMX1 as *Cellulosimicrobium* sp. as the strain CKMX1 clustered closely with *Cellulosimicrobium cellulans* with high boot strap value (80 %). The 16Sr RNA sequence of the strain has been deposited in the GenBank database under accession number JN135476.

Based on above morphological, biochemical and molecular characterization, the strain CKMX1 was identified as *Cellulosimicrobium* sp. CKMX1

Discussion

The optimization of process parameters for SSF production of xylanolytic enzyme using apple pomace as substrate demonstrated clearly the impact of such parameters on the yield of enzyme as well as their independent nature in influencing the ability of *Cellulosimicrobium* sp. CKMX1 to synthesize the enzyme.

As also found in the present study, basal salt yeast extract medium (BSYEM) was reported earlier to enhance maximal xylanase (Sharma 1998) production on apple pomace. A reduction in enzyme yield in BSM without yeast extract might be due to the reduction in growth of the organism, as indicated by the presence of relatively fewer viable cells.

Particle size (specific surface area) is a critical factor in SSF. Apple pomace particles of small mesh size favored maximum enzyme production compared to larger particles (Fig. 2). This indicates that, SSF with substrate of small particle size provides sufficient surface area for adequate adsorption and penetration, resulting in adequate sugar diffusion, more bacterial growth and higher enzyme production. On the other hand, with larger particles, the reduced surface area/volume ratio provides a smaller surface for bacterial growth and might have inhibited absorption/penetration of bacterial cells on the substrate particles. These results are in agreement with those of Gupta and Madamwar (1994), who studied the effect of particle size on production of cellulolytic enzymes by *Trichoderma viride* CCMI 9414. A similar trend was reported for glucoamylase with wheat bran (Pandey 1990), and cellulose production with coir pith of small particle size (Muniswaran and Charyulu 1994). With smaller particles, the surface area for growth is greater but the interparticle porosity is less, and vice versa for larger particle size. These two opposing factors, i.e., decrease in surface area and increase in porosity, probably interact to determine the values corresponding to optimum growth and enzyme production.

Table 4 Percent homology of 16Sr RNA gene sequence of the CKMX1 isolate with other nucleotide sequences present in the database using BLASTn analysis

Seq A	Isolate	Length (bp)	Closest match	Accession no.	Length (bp)	Percent similarity
1	CKMX1	1,136	*Cellulosimicrobium cellulans* strain AMP-11 16S	HM104377	1433	97.0
1	CKMX1	1,136	*Cellulosimicrobium* sp. C10	GU183386	1384	97.0
1	CKMX1	1,136	*Cellulosimicrobium* sp. BD-29	GU085227	1427	97.0
1	CKMX1	1,136	*Cellulosimicrobium cellulans* strain TPBF10	EU249578	1282	97.0
1	CKMX1	1,136	*Cellulosimicrobium cartae* MSD 20106	X79456	1489	97.0
1	CKMX1	1,136	*Cellulosimicrobium funkei* strain 1P04AC	EU977646	1425	96.0
1	CKMX1	1,136	*Cellulosimicrobium funkei* strain W4083	AY523789	1444	96.0
1	CKMX1	1,136	Actinobacterium TB8	HQ241824	1378	96.0

Difference in the timing of appearance of xylanase activity (Fig. 3) suggests that the initial degradation of apple pomace is rapid. The increase in the production of xylanolytic activity with increase in incubation period up to 72 h may be due to increased growth of the organism. This is supported by the direct relationship between enzyme activity and final cell density. This observation is in agreement with results obtained by Archana and Satyanarayana (1997) and Wainø and Ingvorsen (2003) while working with *Bacillus licheniformis* and *Halorhabdus utahensis*.

Xylanase production has been found to be highly dependent on pH and temperature. The maximum activity of xylanase (535.1 U/g DBP), viable cell number (199.7 CFU×10^5) and reducing sugar (2,145 μg mL^{-1} g^{-1})

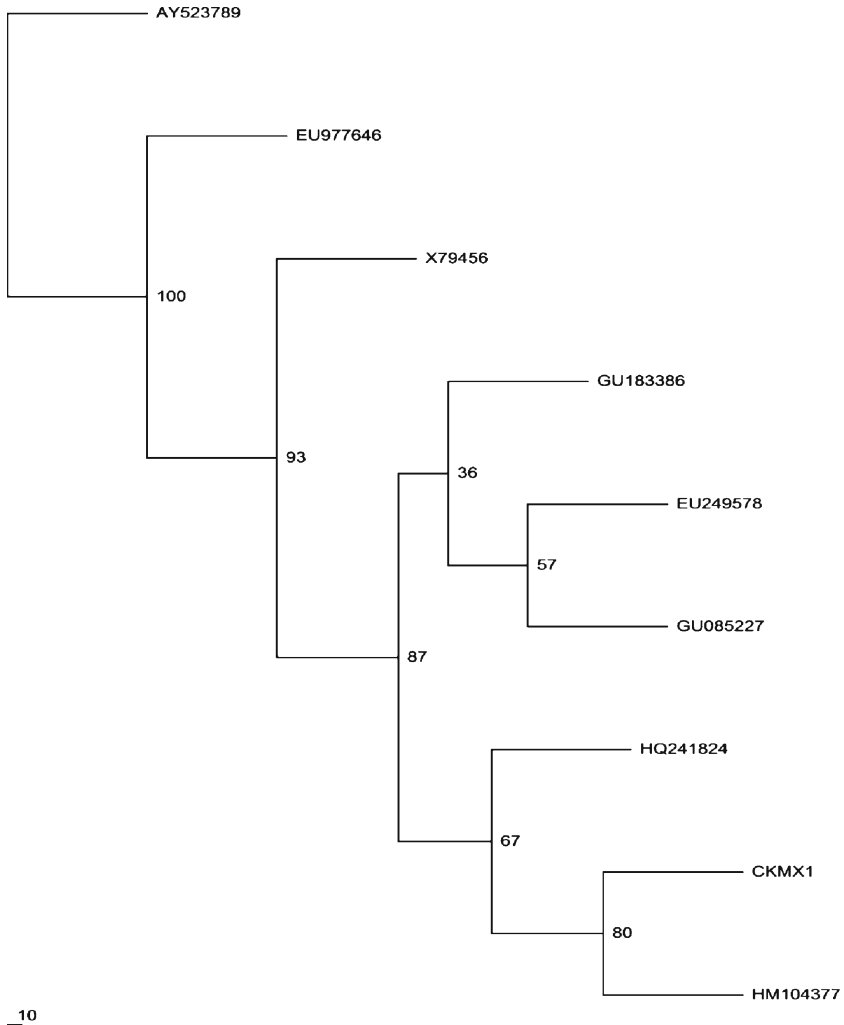

Fig. 9 Neighbor-joining tree based on 16Sr RNA gene sequences showing the phylogenetic relationship of isolate CKMX1 to related strains. Numbers at nodes indicate the level of bootstrap support based on data for 1,000 replicates; inferred values greater than 50 % only are presented. *Scale bar* Ten substitutions per nucleotide position

were obtained when *Cellulosimicrobium* sp. CKMX1 was grown at pH 8.0. Bansod et al. 1993 studied the effect of pH on xylanase and cellulase production using wheat bran and micro-crystalline cellulose by *Cephalosporium*, *Bacillus* sp. and *Aeromonas*. It was found that maximum xylanase production by *Cephalosporium* was obtained at pH 8.0–9.5; pH 8.0 supported maximum cellulase production by *Bacillus* sp. whereas *Chromohalobacter* sp. TPSV101 produced maximum xylanase activity at pH 9.0 (Prakash et al. 2009). Optimum growth and production of xylanase activity by *Cellulosimicrobium* sp. CKMX1 occurred at 35 °C (Fig. 5). An identical optimum temperature for xylanase production in SSF was reported by Beg et al. (2000) and Battan et al. (2006).

A 10 % inoculum (based on initial weight of apple pomace) supported the highest production of xylanase by *Cellulosimicrobium* sp. CKMX1. Similarly, 10 % inoculum was reported earlier by Sindhu et al. 2006 to produce maximal xylanase by *Bacillus megaterium* in SSF. In contrast other bacterial isolates (*B. licheniformis* A99 and *B. pumilus* ASH 7411) also produced highest xylanase when used at a 15 % inoculum level (Archana and Satyanarayana 1997; Battan et al. 2006). Inoculum size controls the initial lag phase of xylanase-producing microorganisms. A smaller inoculum size extends the lag phase whereas larger inoculum size increased the moisture content to a significant extent. Observations of the effect of inoculum size on maximal enzyme production in the present study agree well with these previously published observations.

Nowadays, there is growing acceptance of the use of statistical experimental designs in biotechnology to optimize culture medium components and conditions. Many studies have reported satisfactory optimization of xylanase production from microbial sources using a statistical approach (Wang et al. 2008). RSM and CCD was employed to optimize a fermentation medium for the production of xylanase by *Cellulosimicrobium* sp. CKMX1 at pH 8.0. The optimized medium resulted in a 1.6-fold increase in xylanase production. The application of statistical design for screening and optimization of culture conditions for the production of xylanolytic enzymes allows quick identification of the important factors, and the interactions between them. The RSM applied to the optimization of xylanase production in this investigation suggested the importance of a variety of factors at different levels. A high degree of similarity was observed between the predicted and experimental values, which reflected the accuracy and applicability of RSM to optimize the process for enzyme production. There have been reports on optimization of culture media using statistical approaches for a few bacterial xylanases processes but not for cellulase-free, alkali-stable xylanases in SSF of apple pomace. The statistical optimization approach is efficient and has been applied successfully to SSFs that have overcome the limitations of classical empirical methods (Yu et al. 1997). The results of CCD indicate the significance of yeast extract, urea nitrogen, Tween 20 and CMC on production of xylanase by *Cellulosimicrobium* sp. CKMX1. Despite some interactions, maximum interactions of different variables in the present investigation were found to be significant.

This is the first report on the isolation of *Cellulosimicrobium* sp. CKMX1 from mushroom compost. In this study, we characterized a strain of *Cellulosimicrobium* sp. capable of producing xylanase. According to the morphological and biochemical properties, and 16Sr RNA gene sequencing, the isolate was identified as a strain of *Cellulosimicrobium* sp. CKMX1. *Cellulosimicrobium* sp. CKMX1 an unusual bacterial strain in that it produces cellulase-free, alkali stable xylanase. This xylanase has been shown to solubilise pulp actively under alkaline conditions, which is one of the desirable criteria for industrial applications. In this study, the using ‘one variable at a time approach’, the effect of media types, particle size of carbon source, temperature, pH, incubation temperature and inoculum size was studied. Based on these observations, RSM was applied for the optimization of xylanase biosynthesis by SSF on *Cellulosimicrobium* sp. CKMX1. The existence of interactions between the independent variables and the response was established. The xylanase activity obtained with the optimized nutrient medium was 861.90 U/g DBP, which is 1.6-fold higher than the activity obtained with the basic medium. Above all, it takes only 72 h to achieve maximum xylanase production. Thus, with reduced batch time and cheap substrate, it can be concluded that utilization of apple pomace for SSF could lead to large-scale production of xylanases and also contribute to safe and economic waste management of apple pomace in the environment, where this waste is continuously accumulating and causing serious pollution problems.

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