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Optimization and Scale Up of Cellulase-Free Xylanase Production in Solid State Fermentation on Wheat Bran by *Cellulosimicrobium* sp. MTCC 10645

Rajashri D. Kamble[1,*] and Anandrao R. Jadhav[2]

[1]*Department of Biotechnology, Tatyasaheb Kore Institute of Engineering and Technology,Warananagar, Pin 416113. Tal. Panhala, Dist. Kolhapur.*

[2]*Department of Microbiology, Smt. K.R.P. Kanya Mahavidyalaya, Islampur, Pin 415 414, Tal: Walwa Dist: Sangli, M.S., India*

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Abstract

The production of cellulase-free xylanase was investigated with a locally isolated and identified strain, *Cellulosimicrobium* sp. MTCC 10645 in solid state fermentation. Different fermentation conditions were standardised for the growth and xylanase activity, the optimum being 72 h growth at pH 7.0, cultivation temperature 40°C and substrate to moisture ratio of 1:1.8 (w/v). Among different lignocellulosic substrates wheat bran was found to be best substrate for xylanase production. The inoculum size of 10% resulted in maximum production of xylanase. The enzyme production was stimulated by the addition of yeast extract and casein. Addition of glucose, xylose and carboxy methyl cellulose repressed the production of xylanase. Under optimized conditions, xylanase production in solid state fermentation was 4,962 $\pm$ 45.08 U/gds.

Keywords: Xylanase; solid-state fermentation; lignocellulosic substrates; wheat bran

1. Introduction

Xylanases (E.C.3.2.1.8) are the key enzymes, which play an important role in the breakdown of xylan. Wheat bran is a rich source of xylan (40%) and xylose (29%). Therefore, it is an attractive substrate for production of xylanase enzyme (Polizeli *et al.,* 2005). Xylan, a major component of hemicellulose, is a heterogeneous polysaccharide consisting of β-1,4 linked to D-xylosyl residues on the backbone, but also containing arabinose, glucuronic acid and arabino glucuronic acid linked to D-xylose backbone (Archana and Satanarayan, 1997). Enzymatic hydrolysis of xylan is catalysed by a group of xylanolytic enzymes such as endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase, and esterase (Techapun *et al.,* 2003). Among the xylanolytic enzymes, endo-1,4-β-xylanase (E.C. 3.2.1.8) and β-xylosidase are most important, first enzyme attacks the main internal chain linkages and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides (Thiago and Kellaway, 1982).

A variety of microorganisms including bacteria (Archana and Satanarayan, 1997), fungi (Polizeli *et al.,* 2005), actinomycetes and yeasts (Flores *et al*., 1997) have been reported to produce xylanase under solid state fermentation (SSF). Solid state fermentation is the growth of micro-organism on moist substrates in the absence of free flowing water. SSF offers distinct advantages over submerged fermentation (SmF) including economy of space, simplicity of media, no complex machinery, greater product yields, and reduced energy demand (Raimbault and Alazard, 1980). Fungi and actinomycetes as compared to bacteria have been reported widely for xylanase production in SSF due to the enhanced enzyme yield (Gessesse and Memo, 1999; Heck *et al*., 2005; Sindhu *et al.,* 2006).

Wheat bran was used for xylanase production in SmF by the *Bacillus licheniformis* isolate under different conditions. However, xylanase yield was lower compared to the yield under SSF, which is being reported here. Although wheat bran is a rich source of xylan and xylose, and is abundantly available in India. The objective of the present work was to optimize various fermentation parameters for xylanase production by a locally isolated bacterial strain, *Cellulosimicrobium* sp. MTCC 10645 on wheat bran under SSF and production enhancement by scaling up the solid state system.

2. Materials and Methods

2.1. Materials

Oat spelt xylan (Himedia Laboratories Pvt. Ltd., India) was used for enzyme assay. Wheat bran, gram bran, rice husk, rice bran, wood dust and apple pomace were collected locally and used after drying, grinding and

* Corresponding author. e-mail: rajashri.kamble@gmail.com.

sieving (500 mm. particle size). Other than autoclaving, no other pretreatment of these lignocellulosic substrates were necessary. All other reagents were of analytical grade.

2.1.1. Microbial strain and its growth conditions

The bacterial strain used in the present study was isolated from compost soil, which is potentially a good source of xylanase producing micro-organisms (Khandeparkar and Bhosle, 2006). The strain was identified on the basis of morphological, physiological, biochemical characteristics as well as 16S rRNA sequencing by Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial strain was maintained and stored in the laboratory for further study at 4°C on nutrient agar medium supplemented with 0.1% (w/v) oat spelt xylan. Xylanolysis of the bacterial strain was tested on xylan-red agar medium containing oat spelt xylan covalently linked with cibacron brilliant red 3-BA dye (Ten *et al.*, 2003).

2.1.2. Inoculum prepararion and production of xylanase in SSF

Inoculum was prepared by transferring one loopful of bacterial cells from 48h old culture into 2 ml of fermentation medium and incubated at 40°C for 48 h. The medium containing bacterial cells (O.D. 1.0) was used to inoculate 20 ml of fermentation medium. Erlenmeyer flasks (250 ml) containing 10.0 g of wheat bran moistened with 18.0 ml of the basal salt solution (BSS: substrate to moisture ratio 1:1.8) were inoculated with 10% (w/v) inoculum (48 h old) and incubated at 40°C. The composition of the basal salt solution was (g/l) NaCl, 30.00; KCl, 0.75; $MgSO_4$, 7.00; NH_4Cl, 0.5; K_2HPO_4, 2.5; KH_2PO_4 0.5; trace metal solution, 1.00 ml; Distilled water, 1.0. The pH of the medium was adjusted to 7.0. Composition of the trace metal solution was (g/l) H_3BO_3, 2.85; $MnCl_2.7\ H_2O$, 1.80; $FeSO_4.7H_2O$, 2.49; Na Tartarate, 1.77; $CuCl_2$, 0.03; $ZnCl_2$, 0.02; $COCl_2$, 0.04; $Na_2MoO_4.2H_2O$, 0.02; Distilled water, 1.0.

2.1.3. Enzyme recovery

After every 12 h of incubation period, the flasks were removed and contents mixed with 30.0 ml of 50 mM phosphate buffer (pH 7.0). The enzyme was extracted by squeezing fermented bran through a wet muslin cloth and vortexed. The sample was centrifuged at 5000 x g for 10 min at 4°C. Supernatant was filtered through Whatman No. 1 filter paper and the clear filtrate was used as crude xylanase preparation. Prior to centrifugation, the samples were withdrawn for determining viable number of cells by standard viable plate count technique (Babu and Satyanarayana, 1995).

2.1.4. Enzyme assay

Xylanase activity was determined by mixing 0.9 ml of 1% (w/v) birch wood xylan (prepared in 50 mM Na-citrate buffer, pH 5.3) with 0.1 ml of suitably diluted enzyme and the mixture was incubated at 50°C for 5 min (Bailey *et al.*, 1992). The reaction was stopped by the addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) and the contents was boiled for 5 min. After cooling, the colour development was read at 540 nm. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute per ml under the assay conditions. Xylanase production was expressed as units (U) per gram dry substrate.

2.1.5. Statistical analysis

All the experiments were carried out in triplicates. The analyses were done in duplicates. The mean values are shown in the figures and tables Data were expressed as mean ± standard deviation for all experiments and statistical significance was calculated according to student two-tailed t test. Values corresponding to $p<0.001$ were considered statistically significant.

2.2. Effect of various lignocellulosic substrates on enzyme production

The bacterial strain was inoculated in 250 ml Erlenmeyer flasks each containing 10.0 g of various lignocellulosic substrates such as wheat bran, gram bran, rice husk, rice bran, wood dust and apple pomace and moistened with 18.0 ml mineral salt solution. The flasks were incubated at 40°C for 72 h. The enzyme was then extracted and assayed for activity as described above.

2.3. Effect of incubation period and temperature

Effect of incubation period on xylanase production was determined by assays of the enzyme after 24 h, 48 h, 72 h and 96 h at 50°C. Effect of temperature on xylanase production was studied by incubating the strain at 30°C, 35°C, 40°C, 45°C and 50°C.

2.4. Effect of moisture content

The influence of initial moisture level on the enzyme production was evaluated by varying the ratio of wheat bran to basal salt solution (1:1.5, 1:1.8, 1:2.0, 1:2.5 and 1:3.0 w/v). The activity of xylanase was determined after an incubation of 72h at 40°C.

2.5. Size of inoculum

Wheat bran (10 g) moistened with 18 ml basal salt solution were autoclaved and inoculated at a level of 5, 10, 15 and 20% (v/w of wheat bran) using 18 h old bacterial culture broth. The enzyme was extracted from each set following an incubation of 72 h at 40°C.

2.6. 2 Effect of sugars, non-sugars and inorganic-organic nitrogen sources

Moistened wheat bran was supplemented with xylose, glucose and carboxy methyl cellulose at 0.5% (w/v) concentration and their effect on xylanase production after incubation at 40°C for 72 h was observed. The effect of different inorganic and organic nitrogen sources (0.25% w/v) such as yeast extract, peptone, ammonium chloride, casein and potassium nitrate were also tested.

2.7. Scale up of xylanase production in solid state

The bacterial strain was cultivated in an aluminum tray ($20\times8\times5\ cm^3$) containing 80 g of wheat bran moistened with BSS (ratio of 1:1.8), and other conditions as optimized in the 250 ml Erlenmeyer flasks as mentioned earlier. The trays were covered with aluminum foil and sterilized at 121°C for 20 min and cooled and inoculated with 10% of 48 h old inoculum. The trays were incubated at 40°C for 96 h. Samples were withdrawn after 12 h of incubation and xylanase was assayed as described earlier.

3. Results and Discussion

3.1. Microbial strain and its growth conditions

The bacterial strain was deposited as *Cellulosimicrobium* sp. with MTCC designation no. 10645. It was Gram positive and rod shaped, moderate thermophile, growing optimally at 40°C. It was observed that the strain can grow well in the range of 25°C- 45°C. The strain was an alkalophile being capable of growing at pH values up to 11.0. Morphological, physiological and biochemical properties of the strain are shown in Table 1.

Table 1. Morphological, physiological and biochemical properties of isolated strain

Tests	Characteristics	Tests	Characteristics
Colony morphology		Growth on NaCl (%)	
Configuration	Circular	2.0	+
Margin	Entire	4.0	+
Elevation	Raised	6.0	-
Surface	Moist	10.0	-
Colony color	Lemon-Yellow	12.0	-
Opacity	Opaque	Biochemical tests	
Gram's reaction	Positive	Methyl red	-
Cell shape	Short rods	Voges Proskauer	-
Size (µm)	0.5-1.0	Casein hydyolysis	+
spores	-	Citrate	-
Motility	Motile	Indole	-
Anaerobic growth	Facultative	Gelatin hydrolysis	+
Morphological tests		Starch hydrolysis	+
Growth at temperatures		Esculin hydrolysis	+
4°C	+	Catalase test	+
15 °C	+	Oxidase	-
25 °C	+	Growth on MCA	-
30 °C	+	Tween-40	-
37 °C	+	Tween-60	-
40 °C	++	Tween-80	-
55 °C	-	Urease	-
Growth at pH		Arginine	-
5.0	-	Nitrate	+
6.0	+	Acid production from	
7.0	++	Sorbitol	-
8.0	+	Salicin	+
9.0	+	Adonitol	-
10.0	+	Inositol	-
11.0	+	Trehalose	+
12.0	-	Mannitol	-
		Raffinose	+
		Fructose	+
		Mellibiose	+

The phylogenetic analysis on the basis of 16S rRNA sequence is given in Figure 1. The nucleotide length of the rRNA is 1408 bp and NCBI (National Centre for biotechnology Information) accession number is FR729925.1. The strain is phylogenetically very much similar (99%) to *Cellulomonas* sp. MN60.3. Its xylanolytic ability was observed by a detecting prominent zone of clearance on xylan-red medium (Figure 2). On the basis of above observations, the bacterial strain was identified as *Cellulosimicrobium* sp. GSV4 (MTCC 10645). Similarly, Kim *et. al*. also isolated *Cellulosimicrobium* sp. strain HY-13 from the gut of *Eisenia fetida*, which is prominent xylanase producer (Kim *et al*., 2009).

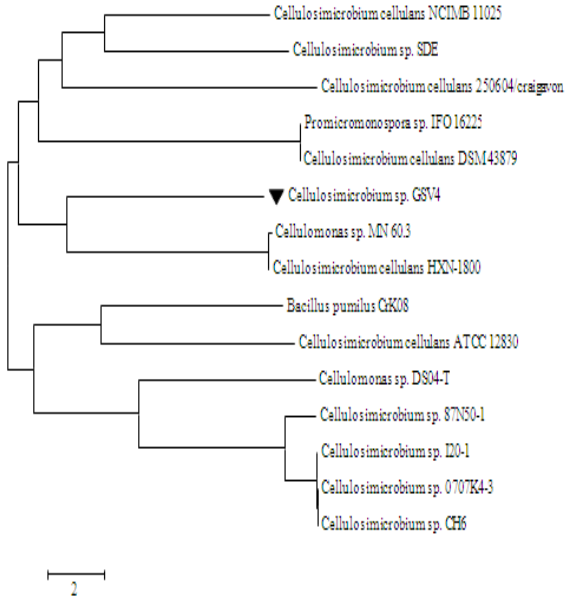

Figure 1. The phylogenetic tree of isolated strain MTCC 10645

Figure 2. Zone of clearance on xylan red agar medium by isolated strain MTCC 10645

3.2. Enzyme production in SSF using various lignocellulosic substrates

Among the lignocellulosic substrates tested, wheat bran gave maximum yield of xylanase (1,091 ± 35.01 U/gds) as compared to other substrates viz. gram bran, rice husk, rice bran, wood dust and apple pomace after 72 h of incubation at 40°C as given in Table 2. Enzyme production was negligible on wood dust and rice bran whereas gram bran supported a very low yield of xylanase. Wheat bran was the best substrate for xylanase production due to its nutritional content and a large surface area (Babu and Satyanarayana, 1995). The wheat bran contained xylan and protein, which were served as substrate as well as carbon and nitrogen source for microorganisms, respectively (Thiago and Kellaway, 1982). Heck *et al*. also reported xylanase production by *Bacillus coagulans* BL69 using

wheat bran in solid state cultivation (Heck *et al.,* 2005). Similar results on the production of xylanase in SSF using wheat bran by *Bacillus licheniformis* A99 (Archana and Satyanarayan, 1997), *Bacillus* sp. (Gessesse and Mamo, 1999), *Bacillus pumilus* ASH (Battan *et al.,* 2006), *Bacillus megaterium* (Sindhu *et al.,* 2006), *Bacillus* sp. (Gupta and Kar, 2008) were in agreement with the present results.

Table 2. Production of xylanase by *Cellulosimicrobium* sp. MTCC 10645 isolate under SSF on various agro-residues at 72 h of incubation; Temperature 40°C; pH 7.0

Agro-residues (w/v)	Xylanase activity (U/gds)
Wheat bran	1,091 ± 29.01
Gram bran	282.3 ± 6.31
Rice husk	620 ± 19.19
Rice bran	165 ± 5.78
Wood dust	91.8 ± 0.50
Apple pomace	501.9 ± 20.91

3.3. Effect of incubation period and temperature on xylanase production

Xylanase production by the bacterial isolate under SSF showed that a low level of xylanase activity was detected in earlier stages of incubation and enzyme activity steadily increased to a maximum level of 1,100 ± 28.06 U/gds by 72h of incubation (Figure 3). A decrease in enzyme activity upto 801.1 ± 25.09 U/gds was observed with a further increase in incubation period. Similar findings have been reported with *Bacillus lichenifoermis* where enzyme production reached a maximum level after 72 h in wheat bran medium (Archana and Satyanarayan, 1997). Feniksova *et al*. also reported optimum incubation period of 72 h for maximum xylanase production by *Bacillus subtilis* in solid state as well as surface culture conditions (Feniksova *et al.,* 1960). The reduction in xylanase yield after optimum period was probably due to the depletion of nutrients available to the microorganism or due to proteolysis (Flores *et al.,* 1997). The duration needed for incubation might depend on the growth rate of microorganism and its enzyme production pattern (Battan *et al*., 2007).

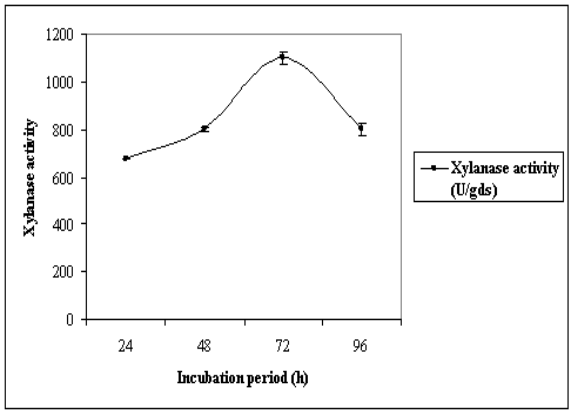

Figure 3. Effect of incubation period on xylanase production by *Cellulosimicrobium* sp. MTCC 10645 on wheat bran as substrate in SSF Temperature 40ºC; pH 7.0.

The results of the influence of temperature on xylanase production are shown in Figure 4, showing that the optimum temperature for xylanase production was 40°C (1215.01 ± 35.05 U/gds), which was also the optimum growth temperature of the bacterial strain. Incubation at temperatures below and above 40°C greatly reduced the enzyme activity. Similarly, maximum xylanase production by *Bacillus* sp. NTU-06 at incubation temperature 40°C was reported by Wang et al. (Wang *et al.,* 2010). Gupta and Kar also reported that *Bacillus* sp. was able to produce maximum amount of xylanase when grown at 40°C (Gupta and Kar, 2008). Most of the researchers reported several *Bacillus* isolates giving maximum xylanase production at incubation temperature in the range of 30 °C-40 °C (Sindhu *et al.,* 2006; Heck *et al.,* 2005; Gessesse and Mamo, 1999).

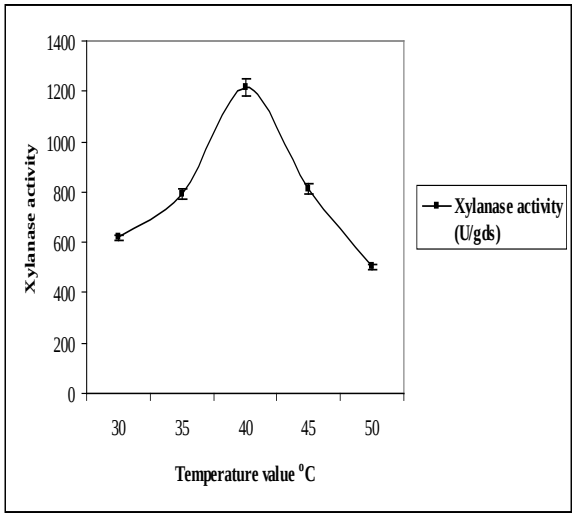

Figure 4. Effect of temperature on xylanase production by *Cellulosimicrobium* sp. MTCC 10645 on wheat bran as substrate in SSF Temperature 40°C; pH 7.0

3.4. Effect of moisture content on xylanase production

As indicated in Figure 5, xylanase production was maximum (1001.02 ± 18.10 U/gds) when wheat bran and moisture content ratio was 1: 1.8. Similarly, when *Arthrobacter* sp. MTCC 5214 grown in medium having wheat bran and moisture content ratio 1: 1.8 gave enhanced xylanase production (Khandeparkar and Bhosle, 2006). The moisture content in SSF is an important factor that determines success of the process. Moisture content higher than optimum decreases porosity of substrate, causes alternation in particle size, gummy texture and lowers oxygen transfer (Feniksova *et al.,* 1960; Raimbault and Alazard, 1980). A lower moisture level leads into a reduction in solubility of nutrients from the solid substrate (Ikasri and Mitchell, 1994).

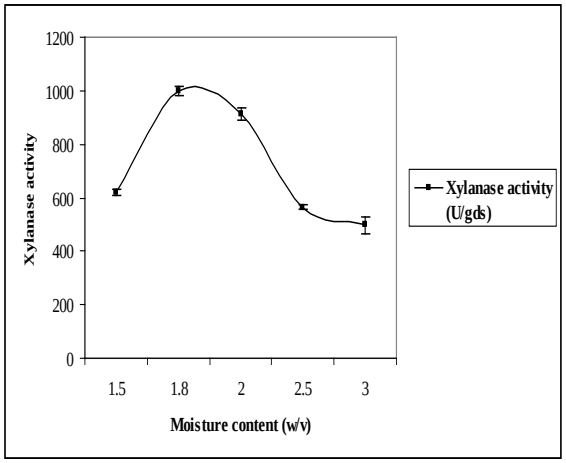

Figure 5. Effect of moisture content on xylanase production by *Cellulosimicrobium* sp. MTCC 10645 on wheat bran as substrate in SSF; Temperature 40°C; pH 7.0

3.5. Effect of inoculum size on xylanase production

A 10% inoculum (based on the initial weight of wheat bran) supported the highest level of xylanase by *Cellulosimicrobium* sp. MTCC 10645. A further increase in inoculum size resulted in a decline in the enzyme titre as shown in Figure 6. An inoculum size of 10% was found adequate for good growth of the culture and consequently enzyme production. Similarly, *Bacillus megaterium* gave maximum xylanase production when fermentation medium was inoculated with 10% of inoculum (Sindhu *et al.*, 2006). Gupta and Kar also reported the 10% inoculum was optimum inoculum level for *Bacillus* isolate to give enhanced xylanase production (Gupta and Kar, 2008).

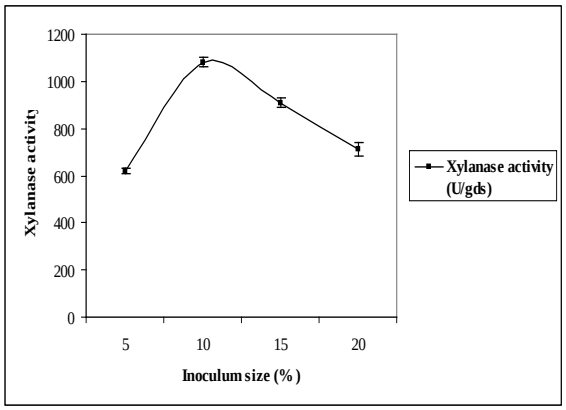

Figure 6. Effect of inoculum size on xylanase production by *Cellulosimicrobium sp.* MTCC 10645 on wheat bran as substrate in SSF Temperature 40°C; pH 7.0

3.6. Effect of sugars, non-sugars and inorganic-organic nitrogen sources on xylanase production

Wheat bran was supplemented with additives to enhance xylanase titre in fermentation medium. Glucose, xylose and carboxy methyl cellulose caused reduction in xylanase activity as shown in Table 3. The observations by Battan et al. stated that xylanase activity was not observed until the glucose was depleted from culture medium during the growth of *Bacillus pumilus* ASH in medium containing both xylan and glucose (Battan *et al.,* 2007). Xylose, glucose, lactose and cellobiose reduced xylanase production in *Bacillus licheniformis* A99 (Archana and Satyanarayan, 1997). Xylanase production in *Arthrobacter* sp. MTCC 5214 was found reduced when medium is supplemented with xylose and glucose (Khandeparkar and Bhosle, 2006). In the present study, xylanase production was observed improved when wheat bran is supplemented with yeast extract and casein, whereas peptone caused only a marginal increase in enzyme activity. On the other hand, the activity of xylanase was lower in the presence of additives such as ammonium chloride and potassium nitrate as reported in Table 4. Sindhu *et al.* also reported yeast extract, peptone and casein supported improvement in xylanase production by *Bacillus megaterium* in solid state fermentation, while ammonium chloride and potassium nitrate markedly reduced xylanase production (Sindhu *et al.*, 2006).

Table 3. Effect of sugars and non-sugars on xylanase production by *Cellulosimicrobium sp.* MTCC 10645 on wheat bran as substrate in SSF Temperature 40°C; pH 7.0

0.5 % (w/v)	**Xylanase activity (U/gds)**
Control	1281.1 ± 34.43
Xylose	1081.02 ± 21.0
Glucose	619.10 ± 10.13
Carboxy methyl cellulose	910.0 ± 21.33

The control contained only moistened wheat bran

Table 4. Effect of Inorganic and organic nitrogen sources on xylanase production by *Cellulosimicrobium sp.* MTCC 10645 on wheat bran as substrate in SSF Temperature 40°C; pH 7.0

0.25 % (w/v)	**Xylanase activity (U gds^{-1})**
Control	1200.0 ± 25.56
Yeast extract	1389.1 ± 12.90
Peptone	1209.0 ± 12.13
Ammonium chloride	912.0 ± 9.81
Casein	1410.01 ± 36.76
Potassium nitrate	54.60 ± 7.08

The control contained only moistened wheat bran

3.7. Scale up of xylanase production in solid state

Xylanase production was enhanced by scaling up the solid state system by using enamel trays containing 80 g wheat bran. When SSF was performed in trays for xylanase production using bulk quantities of wheat bran (80 g), xylanase production was higher if compared with Erlenmeyer flasks. Cultivation in large enamel trays yielded 4,962 ± 45.08 U/gds in static state when compared to the value obtained in 250 ml flasks (965 ± 19.80 U/gds). The improvement of xylanase production in trays is more as compared to flasks may be due to efficient aeration, more surface area resulted in rapid heat transfer and better mass. A slight decrease (12.14%) in enzyme production by

scaling up has been reported in *Bacillus megaterium* (Sindhu *et al.,* 2006), while in *Bacillus licheniformis (*Archana and Satyanarayan, 1997), scaling up stimulate xylanase production. It may be possible to further improve the enzyme yield with higher quantities of the substrate thus making wheat bran a potential solid substrate for xylanase production.

Due to the abundance and renewability of wheat bran, it can be good substrate and solid state support for xylanase production. The present study results showed optimization of process parameters and scaling up to obtain higher production of xylanase. The prospects seem good for developing an enzymatic production process using such cheap agro-residues.

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