Applied Biological Research 15 (2): 137-144; (2013)

OPTIMIZATION OF CULTURAL CONDITIONS FOR CELLULASE-FREE XYLANASE PRODUCTION BY MUTANT STRAIN OF ALKALOPHILIC *Cellulosimicrobium* sp. CKMX1 IN SUBMERGED FERMENTATION

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(Received 10 August, 2013; accepted 16 November, 2013)

ABSTRACT

This paper reports optimization of cultural conditions for xylanase production by mutant strains of *Cellulosimicrobium* sp. CKMX1 in submerged fermentation. Alkalophilic *Cellulosimicrobium* sp. CKMX1, capable of producing xylanase, was isolated from mushroom compost. Xylanase production by this strain was enhanced by ethyl methanesulfonate (5-70 mg mL^{-1}) mutation and by optimizing the process parameters in submerged fermentation. Mutant strain E_5 with hyper xylanase production was obtained after treating wild strain with ethyl methanesulfonate on the basis of xylanase activity index and xylanase activity in liquid medium. Cultural conditions were optimized for parent and mutant strain and incubation period of 72 h, incubation temperature of 35 ± 1^0 C, pH of 8.0; 1% inoculum size: and xylan as carbon source yielded high xylanase. Mutant E_5 was 22.99% more efficient than parent strain. Results indicated the potential of alkalophilic *Cellulosimicrobium* sp. CKMX1 and its hyper xylanase producing mutant strain E_5 to be promising for pulp and paper industry.

Key words: Alkalophilic bacterium, *Cellulosimicrobium*, mushroom compost, mutation, xylanase

INTRODUCTION

Biodegradation of xylan, a component of plant cell wall, is a complex process that requires the combined action of several enzymes. Among these xylanase (1,4-ß-D-xylan xylanohydrolase; EC 3.2.1.8) which cleaves internal linkages on ß-1,4-xylose backbone, plays a key role in biodegradation (Roy and Rowshanul, 2009). Xylanases are wide-spread among fungi (Polizeli *et al.*, 2005), actinomycetes (Ninawe and Kuhad, 2006), yeasts (Petrescu *et al.*, 2000) and bacteria (Sindhu *et al.*, 2006). Several microorganisms produce multiple xylanases, implying a strategy for effective hydrolysis of ß-1,4-xylan. Currently, the biotechnological relevance of xylanases has expanded distinctly in paper manufacturing, animal feed processing and in the production of bioethanol (Ouyang *et al.*, 2011). Xylanases are used in pulp and paper industry to minimize the use of chlorine chemicals and bring about improved pulp brightness. Xylanases are produced either by solid state or submerged fermentation, but submerged fermentation is recognized for its superior enzyme production where crude fermented product may directly be used as an source. Traditional mutation and selection techniques have been exploited for improvement of bacterial cultures and to enhance xylanase production level in microbes. Quantitative enhancement is the foremost approach which involves strain improvement mostly by physical and chemical mutations (Ellaiah *et al.*, 2002). Chemicals such as ethyl methanesulfonate, N-methyl-N′-nitro-N-nitrosoguanidine (Haq *et al.*, 2004; Xu *et al.*, 2005) and physical agents such as UV have been commonly used. The production of xylanase is dependent on the cultural conditions of batch fermentation such as time course, incubation temperature, initial pH,

carbon source, nitrogen source, inoculum size, etc. Incubation time, temperature and initial pH play important role in the production and stability of xylanase in batch fermentation (Kansoh and Gammal, 2001). Among the several strains isolated from mushroom compost, one strain which showed maximum zone of clearance was characterized on the basis of the nucleotide sequence of the 16S rRNA gene analysis and the strain was found to be *Cellulosimicrobium* sp CKMX1. This study reports isolation, mutation and optimization of cultural conditions of xylanase producing strain *Cellulosimicrobium* sp. CKMX1 obtained from mushroom compost.

MATERIALS AND METHODS

Microbial strain isolation and screening

The xylan degrading bacterium *Cellulosimicrobium* sp. was isolated by enrichment and adaptation technique. For this, bulked compost samples (50 g each) from different developmental stages were incubated at 35±1°C in 150 mL basal salt medium (BSM, pH 7.0) containing 10 g wood dust for 10 days on a rotary shaker. An aliquot (5 mL) of this enrichment culture was reinoculated into fresh medium containing CP-60 and wood dust, respectively, for 5 days at 35±1°C on a rotary shaker. An aliquot of this medium was again inoculated into 50 ml fresh medium containing 16-20 pieces (1 cm^2) of Whatman filter paper No.1 for 5 days. Finally, xylanolytic bacterium was isolated on BSM containing 0.5% xylan. BSM with 0.5% xylan composed of 6.0 g Na_2HPO_4 , 3.0 g KH_2PO_4 , 0.5 g NaCl and 1.0 g NH_4Cl per litre water to which separately sterilized solution of 1 M $MgSO_4$ (2 mL) and 1 M $CaCl_2$ (0.1 mL) were added. The bacterial strain was grown at 35±1°C and maintained in both liquid and solid media containing 0.5% xylan and stored at 4°C. All the chemicals used were analytical grade reagents purchased either from BDH or E. Merck while xylan (oat spelt) was obtained from Sigma-Aldrich, USA.

For mutagenesis, 1 mL of 24 h old culture was centrifuged at 1000 rpm for 2 min. and pellet dissolved in tris-HCl buffer (1 mL, pH 7.0). Ethyl methanesulfonate (EMS) solution (5-70 mg mL^{-1}) was added to the sample in different Eppendorf tubes and a concentration showing 1% survival after 20 min. was selected. This concentration was used for carrying out mutation at 5, 25, 50 and 70 min. time interval so as to optimize the time required for 3 log kill. Accordingly the survival curve was prepared. Survivors with variable morphology and size of clear zone with respect to wild strain were screened on the basis of xylanase activity index (Singh *et al.*, 1995) and most efficient one selected on the basis of its high enzyme production capacity in liquid medium.

$$\text{Xylanase activity index} = \frac{\text{Average diameter of clear zone} - \text{Diameter of colony}}{\text{Diameter of colony}}$$

Optimization of xylanase production conditions

The most efficient strain was cultivated in 20 mL production medium with variable inoculum size (v/v) (1-15%) at different pH (6.0-10.0) and temperatures (30-50°C) for 72 h under standard shaking conditions to assay the enzyme produced. Effect of substrates on enzyme production was studied by substituting xylan (0.5%) in production medium with various substrates (w/v) viz., sucrose, glucose, apple pomace, xylose and carboxymethyl cellulose (CMC) to assess their suitability to promote xylanase production. Xylanase production was studied in an incubator shaker maintained at 115 rpm upto 5 days using 24 h old culture (OD: 1.0 at 540 nm). Xylanase activity was later assayed to optimize production conditions. Basal salt yeast extract medium (BSYEM) [20 mL] supplemented with 0.5% yeast extract containing all the ingredient of BSM including 0.5% xylan was used as medium for xylanase production. The flasks containing BSYEM were inoculated with bacterial suspension (OD: 1.0 at 540 nm) and incubated under shaken (120 rpm) conditions for 72 h at 35°C. At the end of incubation period, cell density was determined by quantitative plating. The sample was centrifuged at 12000 rpm for 15 min. at 4°C, the culture supernatant filter sterilized and used as crude enzyme preparation.

Xylanase assay

Xylanase activity was determined as per Dubey and Johri (1987) using 0.5 mL D-xylan (1%) in tris-HCl buffer (0.2 M, pH 8.0) and 0.5 mL of appropriately diluted enzyme. Mixture was incubated at 55±1°C for 5 min. The total reducing sugars produced were determined by boiling with 3 mL dinitro-salicylic acid reagent (DNSA) for 15 min. using xylose as standard (Miller, 1959). The contents were transferred to 250 mL volumetric flask, final volume made up with distilled water and optical density measured at 540 nm in a Spectronic-20. One unit of xylanase activity was defined as the amount of enzyme that catalyzes the release of 1 µM reducing sugar as xylose equivalent per minute under the specified assay conditions.

***Assay of CMCase, FPase, avicelase and β*-glucosidase activities**

In addition to xylanase production the bacterial strains were assayed for carboxymethy cellulase [CMCase] and filter paperase [FPase] enzyme activities as per the method of Reese and Mandels (1963). The optical density measured at 540 nm in a Spectronic-20. One unit of CMCase activity was defined as the amount of enzyme which produced 4 mg of reducing sugars from CMC as substrate in 1 h at 55°C whereas 1 unit of FPase activity was defined as the amount of enzyme that produced one µmol glucose mL^{-1} min^{-1} under the specific assay conditions. Further, avicelase and *β*-glucosidase activities were also assayed as per the method of Berghem and Petterson (1973). One unit of avicelase activity was defined as amount of enzyme required to liberate reducing sugar equivalent to 5µg glucose under defined assay conditions whereas 1 unit of *β*-glucosidase activity was defined as the amount of enzyme which liberated 5µg ρ-nitrophenol under defined assay conditions.

All the experiments were conducted in triplicate along with equal number of controls. The data obtained were subjected to analysis of variance technique using completely randomized design (Gomez and Gomez, 1984).

RESULTS

A total of 86 xylanolytic bacterial isolates were isolated from the enriched culture by direct plating of serially diluted samples on BSM containing xylan as sole carbon source. The most predominant bacterial isolate capable of using xylan and capable of forming clear zone around colony (xylanase activity index: 2.66 at 72 h of incubation) was selected for further characterization, mutation and optimization processes. The alkalophilic bacterium, isolated from mushroom compost, was identified as *Cellulosimicrobium* sp. strain CKMX1 under the GenBank accession No. JN135476.

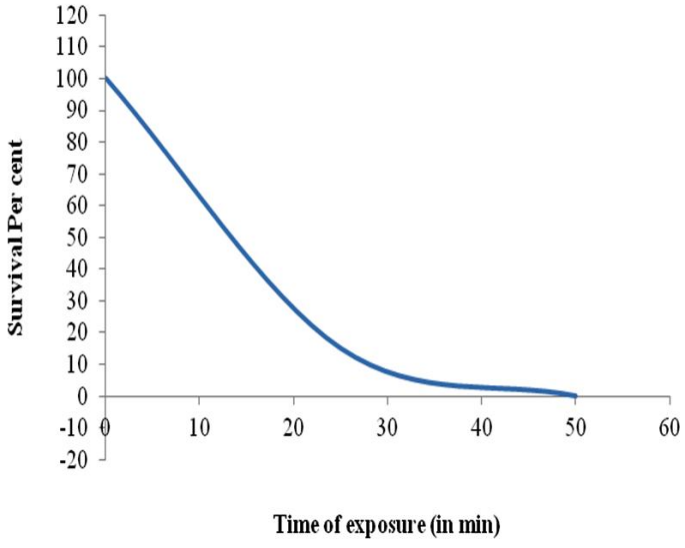

Fig. 1: Survival curve of *Cellulosimicrobium* sp. CKMX1 after exposure with ethyl methanesulfonate (70 mg mL^{-1}) at different time interval

Screening and selection of mutants by mutagenesis

Mutagenesis studies revealed that EMS treatment @ 70 mg mL^{-1} resulted in 0.33% survival after 30 min. incubation (Fig. 1). Increase in EMS concentration (from 5 to 70 mg mL^{-1}) declined %survivors while the mutant frequency increased. The highest concentration was further evaluated at different time exposures (0 to 50 min.). At 50 min. EMS exposure (70 mg mL^{-1}) 99.99% kill was recorded. The treatment of

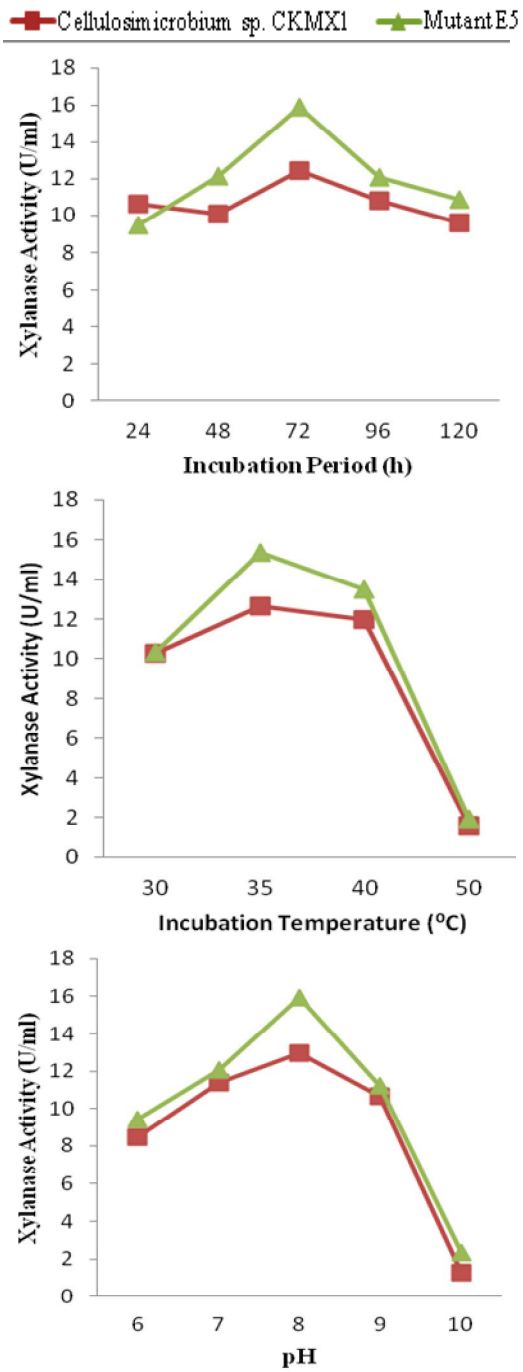

Fig. 2: Effect of incubation time, temperature and pH on xylanase production by *Cellulosimicrobium* sp. CKMX1 and its mutant strain E_5 in BSM under submerged fermentation conditions

Cellulosimicrobium sp. CKMX1 with selected concentration and 50 min. exposure time resulted in 11 putative mutants on the basis of clear zone formation. These putative mutants were allowed to grow in liquid medium for enzyme production. Mutagenesis using EMS mutagen exhibited significant increase in enzyme activity. Of all the putative mutants tested, mutant E_5 with xylanase activity index of 4.12 was selected. The strain showed 22.99% increase in xylanase activity (14.87 U mL^{-1}) in liquid medium as compared to wild strain (12.09 U mL^{-1})

Effect of incubation period, temperature and pH on xylanase production

Xylanase production by *Cellulosimicrobium* sp. CKMX1 and its mutant strain E_5 under submerged fermentation showed that a low xylanase activity in earlier stages of incubation but enzyme activity steadily reached a maximum of 12.45 U mL^{-1} in parent strain and 15.87 U mL^{-1} in mutant strain E_5 at 72 h of incubation (Fig. 2). Further increase in incubation period decreased xylanase activity. However, CMCase, FPase, avicelase and β-glucosidase were not detected at any time in incubation period.

Microbes are known to produce high enzyme titre at their optimum growth temperature. *Cellulosimicrobium* sp. CKMX1 gave maximum xylanase (12.66 U mL^{-1}) at 35°C. Similarly, mutant strain E_5 produced 15.36 U xylanase mL^{-1}. Above or below 35°C, the production of xylanase was gradually reduced, both in parent and its mutant strains. CMCase, FPase, avicelase and β-glucosidase were not detected at any incubation temperature.

The pH of medium is one of the regulatory parameters during fermentation. Maximum enzyme production of 15.92 U mL^{-1} was achieved by mutant E_5 at pH 8.0. At this pH parent strain showed the enzyme activity of 12.96 U mL^{-1}. The xylanase production progressively decreased when pH of medium was increased or decreased from the optimum value. This showed the alkalophilic nature of parent and mutant strains which produced the enzymes active in alkaline range, except for mutant M_{11}. CMCase, FPase, avicelase and β-glucosidase were not detected.

Effect of inoculum size and substrate on xylanase production

When 1% inoculum (v/v) of 24 h old culture was added to the production medium xylanase production was highest (14.94U mL^{-1}) in mutated strain E_5 as compared to12.13U mL^{-1} in parent strain (Fig. 3). The enzyme titre declined with increase in inoculum size beyond 1%. CMCase, FPase, avicelase and β-glucosidase activities were not detected at any inoculum size used.

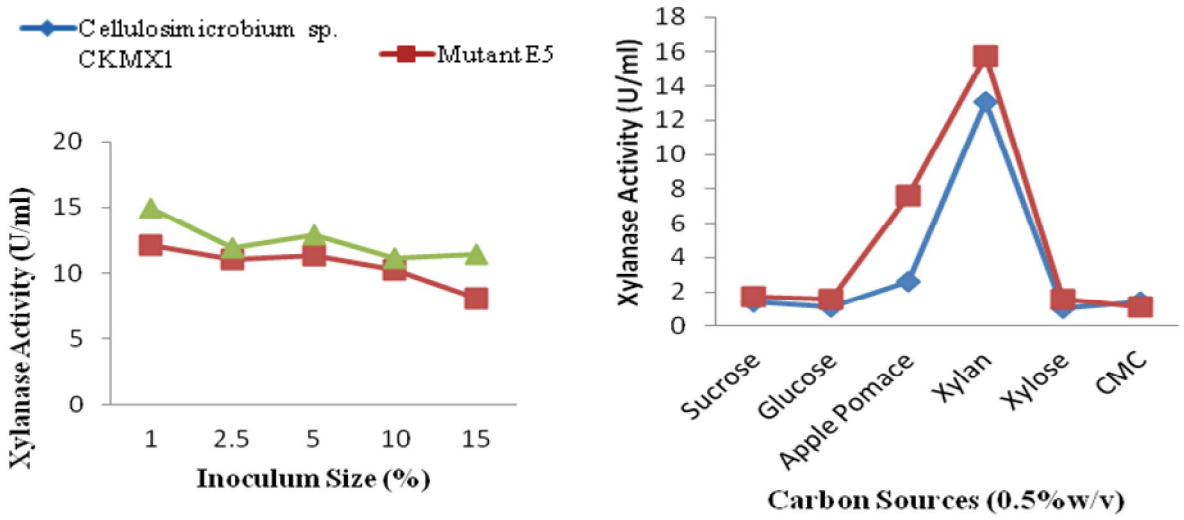

Fig. 3: Effect of inoculum size (%) and carbon source (w/v%) on xylanase production by *Cellulosimicrobium* sp. CKMX1 and its mutated strain E_5 in basal salt medium under submerged fermentation

Of the substrates tested for xylanase production in Smf, the substrates possessing some lignocellulosic material in growth medium exhibited higher xylanolytic activity but less than that produced in growth medium containing 0.5% xylan (Fig. 3). Among the different substrates tested, xylan supported highest xylanase production followed by apple pomace for parent strain. However, in case of mutant strain E_5 maximum production was observed in xylan as carbon source followed by apple pomace and xylose.

DISCUSSION

Recently the interest in xylanases has markedly increased due to its potential application in pulping and bleaching processes using cellulose-free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment. In this respect, the isolation of more efficient xylanase producing microbes is an essential step. Although xylanase production from several microorganisms has been reported (Roy and Rowshanul, 2009; Polizeli *et al*., 2005; Ninawe and Kuhad, 2006; Sindhu *et al*., 2006), in the present study one hyper xylanase producing isolate *Cellulosimicrobium* sp. CKMX1 (Accession No. JN135476) was obtained from mushroom compost [Walia *et al*., 2012]. Kamble and Jadhav (2012) and Kim *et al*. (2009) also reported production of xylanase from *Cellulosimicrobium* sp.

To enhance xylanase production level in microbes, different strategies have been developed. The traditional mutagenesis approach enhances xylanase activity is successfully employed in fungal (Singh *et al*., 1995; Kuhad *et al*., 1998; Bakri *et al*., 2008) and bacterial (Subramaniyan *et al*., 2001) strains. Mutagens induce mutation through lesion or modification in base sequence of DNA that remained un-repaired. An attempt was made to develop *Cellulosimicrobium* sp. CKMX1 mutant strains by mutagenesis and select efficient strain for xylanase production. The parent strain was treated with EMS. The EMS treated mutant strain E_5 exhibited 22.99% improvement in xylanase enzyme activity in liquid medium as compared to wild strain. This is in agreement with Haq *et al*. (2004; 2009) who screened 28 putative mutants of parental strain *Bacillus licheniformis* GUB30^{UCM} for xylanase production in liquid medium and found mutant *B. licheniformis* strain EMS-200^{40} highly efficient in α-amylase production. Hamad *et al*. (2001), Kotchoni and Shonukan (2002) and Shafique *et al*. (2009) also developed efficient mutants of *Bacillus* sp. by EMS treatment. The chemical mutagens cause permanent changes in DNA sequence, bring about transitions from G:C→A:T and have preferential effect on DNA replication.

Fermentation profile of an organism is affected by nutritional and physiological factors such as incubation time, pH, inoculum size, temperature and carbon and nitrogen sources. The optimization of medium composition and cultural conditions were carried out based on stepwise modifications of governing parameters for xylanase production. Under optimized submerged fermentation conditions i.e. at incubation temperature of 35±1°C; incubation period of 72 h; pH of 8.0; 1% inoculums size, and xylan as carbon source, *Cellulosimicrobium* sp. CKMX1 mutant strain E_5 produced high xylanase as compared to unoptimized conditions. Further, cellulase activity was not detected in cell free supernatant thereby indicating that xylanase was cellulose-free. Time course optimization is a significant step in fermentation experiments as it actually determines the incubation period for any microbial culture. So, the rate of xylanase production by parent and mutant strains were studied. The maximum xylanase production was observed after 72 h of incubation. The increase in production of xylanolytic activity upto 72 h may be due to the increased growth of organism which is supported by the direct relationship of enzyme activity and final cell density. The results are in agreement with Archana and Satyanarayana (1997) and Waino and Ingvorsum (2003) who worked with *Bacillus licheniformis* and *Halorhabdus atahensis*. Xylanase production has been found to be highly dependent on pH and temperature. Optimum growth and production of xylanase activity by *Cellulosimicrobium* sp. CKMX1 occurred at 35±1°C. Similar optimum temperature for xylanase production in Smf has been reported for *Bacillus* sp. (Battan *et al*., 2007; Sanghi *et al*., 2009; Nagar *et al*., 2010). The effect of varying pH on xylanase production for parent and mutant strains maximum xylanase production at pH 8.0 suggesting that organisms are alkalophilic in nature. Kohli *et al.* (2001) and Nagar *et al.* (2010) reported pH 6 as optimum pH for xylanase production. However, Dhillon and Khanna (2000), Battan *et al.* (2007) and Sanghi *et al*. (2009) reported neutral pH range for production by *Bacillus* sp. and Prakash *et al.* (2009) reported pH 9.0 for enzyme production by *Chromohalobacter* sp. TPSU101. An inoculum level of 1% supported highest xylanase production by parent and mutant strain. Several researchers have reported the use of 1.0 to 5.0% v/v inoculum for hyper production of xylanase (Kar *et al*., 2006; Battan *et al*., 2007; Nagar *et al*., 2010). Differential enzyme yield with respect to inoculum size may be attributed to the fact that increase in inoculum size upto optimum level causes faster progressive decline in the number of free substrate molecules until an equilibrium is achieved resulting in maximum xylanase production. Increase in inoculum size above optimum level results in competition for substrate disturbing stable substrate-inoculum relationship necessary for enzyme production (Bhatt *et al*., 1994). Carbon source, an essential constituent of microbial fermentation medium, affects the overall cellular growth and metabolism. When xylan in the production medium was replaced with other synthetic mono-, di-, oligo- and poly-saccharide, xylan supported highest xylanase production for both wild (13.1 mL^{-1}) and mutant E_5 (15.75 U mL^{-1}). It is, therefore, apparent that presence of xylan induces xylanase biosynthesis and releases mono, di and largely oligosaccharide on enzymatic degradation. Similar observations have been reported by Dwivedi *et al*. (2009) and Oliveira *et al.* (2006).

Conclusion: The mutant strain E_5 of *Cellulosimicrobium* sp. CKMX1 was 22.99% more effective in producing alkaline xylanase as compare to the wild strain. Therefore, the characteristics of parent strain and mutant E_5 including their alkalophilic nature makes them suitable for paper and pulp industry.

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