Optimization of Process Parameters for Cellulase Production by *Bacillus licheniformis* MTCC 429 using RSM and Molecular Characterization of Cellulase Gene

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Abstract

World is threaten to energy crisis which has advances research in bioenergy and specifically development of biofuels to replace petroleum products have increased the use of microbial enzyme like cellulases and xylanases as well as amylases for generation of reducing sugars for their conversion into bioethanol. Extensive research has been carried out in this view but alkaline cellulase production and molecular characterization is not studied in detail so far, this study will aid to achieve it. Optimization of fermentation parameters for production of cellulase was evaluated with the help of Response Surface Methodology (RSM) a statistical design, initial pH (9), moisture ratio (1:1) and incubation time (72 h) (run no.4) were found to be ideal parameters for optimum production of cellulase, substrate Jatropha seed cake without any pre-treatment was found to be an ideal source for cellulase production by *Bacillus licheniformis* under solid state fermentation. Cellulase gene of size 786 bp was isolated later using PCR techniques, confirmed with sequence analysis and ligated to pRSET A vector for the transformation to *E. coli* DH5α. Positive clones were identified and sequenced to justify the cloning. Sequence of *Bacillus licheniformis* endo-β-1,4-glucanase (Cel12A) gene showed 100% similarity with endoglucanase gene sequence from *Bacillus licheniformis* ATCC 14580 genome, shows successful cloning of Cel12A gene into pRSET A vector.

Keywords: Cellulase; Cloning; Isolation; Production

Introduction

Among various bacterial genera reported for production of various industrially important enzymes so far, *Bacillus* genus had widely been explored for production of various enzymes. The genus *Bacillus* established by Cohn in 1872, has undergone taxonomic changes. In the 2nd edition of the taxonomic outline of Bergey's manual of Systematic Bacteriology [1,2] phylogenetic classification schemes, accomplished mainly by the analysis of 16S rDNA sequence similarities were included in the family of *Bacillaceae*. The genus *Bacillus* is made up by 94 species. *Bacillus* species are historically clustered into six large groups based on numerous physiological, biochemical and morphological characters. The groups contain various *Bacillus* spp. which are described below:

Group I includes *B. polymyxa* as a reference organism, comprises facultative anaerobic species that ferment a variety of sugars and have reasonably fastidious growth requirements in the form of vitamins and amino acids. Numerous extracellular enzymes such as amylases, glucanases including cellulases, pectinases and pullulanases are secreted by members of this group.

Group II consists of *B. subtilis* and its relatives, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus*. Some species such as *B. anthracis*, *B. cereus*, *B. licheniformis* and *B. thuringiensis*, secrete numerous extracellular enzymes including many commercially important amylases, β-glucanases and proteases.

Group III species are perhaps taxonomically the least defined and are rather physiologically heterogeneous. This group is based on *Brevibacillus brevis* which is a strict aerobe that does not produce appreciable acid from sugars and differentiates into an oval endospore that distends the sporangium. Other species in this group might include *B. badius* and "*B. freudenreichii*".

Group IV is a phylogenetically homogeneous group of species including *B. sphaericus*, the psychrophiles *B. nsolitus*, *B. psychrophilus* and some other species.

Group V is composed of the thermophilic bacilli, represented by *B. stearothermophilus*, as a separate group. This includes a physiologically and morphologically heterogeneous collection of species with various metabolic pathways.

Group VI, the acidophilic thermophiles have recently been allocated to the new genus *Alicyclobacillus* in which thermophily appears to have independently evolved in many lineages. Recent researches, based on a "pan-genomic" approach, support the division of *Bacillus* into further new genera and revealed unexpected groupings suggesting that the final picture of *Bacillus* taxonomy is still far from drawn [3,4]. *Bacillus* species are an important source of insecticides, antibiotics, vitamins and enzymes [5].

Bacillus species have been major industrial workhorse industrial microorganisms with roles in microbiology, which have been used thousand years back, since the production of natto by solid-state fermentation of soyabeans using *Bacillus subtilis*. Natto was first practiced in Japan [6]. The role of *Bacillus* had continually expanded in the past century. Molecular biology techniques have contributed a lot for the development of strains and production strategies. *Bacillus* species are attractive industrial organisms for a variety of reasons, (1) High growth rates leading to short fermentation cycle times. (2) Capacity to

secrete proteins into the extracellular medium and (3) GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *B. subtilis* and *Bacillus licheniformis* [4]. At present there is enough information about the biochemistry, physiology, and genetics of *B. subtilis, B. licheniformis* and other species, which facilitates further development and greater exploitation of these organisms in industrial processes.

Moreover, *Bacillus thuringiensis* a member of genus *Bacillus* produces an endotoxin which has given a new era in the development of the BT plant varieties having defense mechanism against insect pests. One of the most popular examples is BT-cotton. This approach has helped farmers to get higher yields and more resistance towards the pests.

Bacillus subtilis and related *Bacillus* strains continue to be the dominant enzyme-producing microorganisms in industries. These organisms are an important source of industrial extracellular enzymes, including proteases and amylases. The world market for industrial enzymes is estimated to be 1.6 billion US$, split between food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%) [7]. Most detergent proteases currently used in the market are serine proteases of *Bacillus* spp. [8]. It is estimated that enzymes of *Bacillus* spp. make up about 50% of the total enzyme market. Many researchers have studied various *Bacillus* spp. for production of extracellular enzymes like amylases, xylanases, proteases and cellulases using submerged or solid state fermentation technology [9-15].

Response surface methodology is applied to optimize and model the number of variable which leads to the optimization of process parameters with combination of experimental design by interpolation of first or second polynomial equation in systemic order [16]. It is a useful package which is being used worldwide successfully, integrates statistical approach to deduce the effect of multiple variables on the response with its preinformation about relationships amongst them [17,18]. As it is now being considered as the best statistical package to optimize the process parameters it was applied in the present study to obtain the better yield of the cellulase enzyme.

Recent advances in bioenergy research and specifically development of biofuels to replace petroleum products have increased the use of microbial enzyme like cellulases and xylanases as well as amylases for generation of reducing sugars for their conversion into bioethanol. *B. licheniformis* in biofuel research has been used in first generation biofuel technology. A thermostable α-amylase from *B. licheniformis* has been used for the liquefaction of starch containing substrates like wheat flour and corn meal [19,20]. The hydrolysates obtained thus will further be converted into ethanol using yeasts like *Saccharomyces cerevisiae*. Second generation biofuel technology focuses on degradation of cellulosic plant biomass into reducing sugars for fermentation into bioethanol instead of hydrolysis of food materials like starch for fermentation into ethanol [21-23]. Hence second generation biofuels require better understanding of enzymes which can efficiently degrade plant cellulosic and hemicellulosic biomass into fermentable sugars.

There have, however, been several reports of hydrolytic enzymes contributing to hydrolysis of plant cell walls from various strains of *B. licheniformis* [24-38].

The study described in this chapter suggests the use of *Bacillus licheniformis* MTCC 429 for production of an endoglucanase under solid state fermentation using Jatropha deoiled seed cake as a substrate.

In addition amplification of the gene encoding alkaline cellulase from *Bacillus licheniformis,* its cloning and sequence analysis has also been carried out.

Materials and Methods

Inoculum preparation and inoculation

For the development of inoculum, culture was transferred from stock to 100 ml nutrient broth and the inoculated flasks were incubated overnight at 37°C and 120 rpm. Cells were harvested from the broth and their absorbance (A) was checked at 660 nm. Accordingly, cells with inoculum size of $A660=0.5$ (10% inoculum (volume per mass) per 5 g of substrate were harvested, washed and resuspended in sterile distilled water [10]. Inoculum thus prepared was used for solid state fermentation.

Solid state fermentation

Cellulase production was studied in flasks containing 5 g of Jatropha deoiled seed cake as a substrate. The basal medium used for cellulase production was (g/L), yeast extract (5), peptone (5), potassium di-hydrogen phosphate (1), sodium chloride (5) and magnesium sulphate (0.2) [39]. The basal medium was sterilized at 121°C for 15 min at 15 lbs pressure, allowed to cool and inoculated with 10% (v/w) inoculum [13]. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and incubated at 37°C.

Optimizaton of fermentation parameters by response surface methodology (RSM) using Box-Behnken Design (BBD)

Bacterial culture: The bacterial culture *Bacillus licheniformis* MTCC 429 obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India, was subjected for cellulase production by solid state fermentation.

Substrate: Jatropha deoiled seed cake was obtained from biodiesel plant, Department of Bioenergy research, Anand Agriculture University, Anand, Gujarat.

Cellulase production by solid state fermentation: RSM using BBD was applied for optimization of cellulase production which involves full factorial search by observing simultaneous, systematic and efficient variation of important components on the fermentation process. Jatropha deoiled seed cake was selected as substrate for the optimization of cellulase production by response surface methodology. Three important parameters namely initial pH of the medium (X_1), moisture ratio (X_2) and incubation time (X_3) were selected as independent variables and the enzyme activity (cellulase U/g) was the dependent response variable. Each of these independent variables was studied at three different levels as per BBD in three variables with a total of 15 experimental runs. Cellulase activity (U/g) corresponding to the combined effects of three variables was studied in their specified ranges as shown in Table 1.

The temperature was kept constant at 37°C throughout the experiment. All the flasks were analyzed for cellulase activity at specific

Process Variables	Range and Level		
	-1	0	+1
Initial pH (X_1)	7.0	8.0	9.0
Moisture ratio (X_2)	1:0.5	1:1	1:1.5
Incubation time (X_3)	24 h	48 h	72 h

Table 1: Experimental range and coded levels of process variables for cellulase production.

No.	Initial pH X_1	Moisture ratio X_2	Incubation time X_3
1	1	1	0
2	-1	-1	0
3	0	0	0
4	1	0	1
5	0	-1	-1
6	-1	0	-1
7	0	0	0
8	0	1	1
9	-1	0	1
10	-1	1	0
11	0	1	-1
12	0	0	0
13	0	-1	1
14	1	0	-1
15	1	-1	0

Table 2: Full factorial Box-Behnken Design for production of cellulase from *Bacillus licheniformis*.

time intervals as planned in BBD. The plan of BBD in the coded levels of the three independent variables is shown in Table 2.

For statistical calculations the levels of independent variables were coded as

$$x_i = (X_i - X_0) / \delta X_i \quad (5.1)$$

Where, X_i is the actual value of variable; X_0 is the midpoint of the range of   ;

δX_i is the step change in X_i and x_i is the coded value for X_i , i = 1,2,3.

Response surface methodology allows the modeling of a second order equation that describes the process. Cellulase production data was analyzed and response surface model given by Equation (5.2) was fitted with multiple regressions through the least squares method.

Enzyme activity values were fitted by the second order polynomial equation

$$Y = \beta 0 + \sum \beta i\, xi + \sum \beta ii\, xi^2 + \sum \sum \beta ij\, xi\, xj \quad (5.2)$$

Where, Y is the predicted response variable,

β0 is the intercept,

β*i*, β*ii* and β*ij* are linear, quadratic and interaction coefficients respectively,

xi and *xj* are the coded forms of the independent variables.

Interpretation and data analysis

The results of the experimental design were analysed and interpreted using the MINITAB version 16 (PA, USA) statistical software. Prediction of optimum fermentation parameters and shape of the curves generated by the model was also done by the same software.

Enzyme extraction

The crude enzyme from each flask was extracted by adding 20 ml of 0.05 M glycine-NaOH buffer (pH 9.0) and then the entire content was filtered through a wet muslin cloth by squeezing. The extract thus obtained was centrifuged at 8,000 × g for 15 min. The volume of supernatant was measured and it was used to determine enzyme activity and concentration of soluble proteins.

Enzyme assay

Endo-β-1,4-glucanase assay: The enzyme extract was diluted with 0.05 M glycine-NaOH buffer (pH 9.0) (0.5 ml) mixed with 2% CMC (0.5 ml) and incubated in a water bath at 50°C for 30 min. The reaction was terminated using DNS reagent followed by boiling and reducing sugar contents were estimated [40]. One unit of enzyme was defined as one micromole of glucose liberated per min under standard assay conditions.

Endoglucanase gene cloning

Genomic DNA isolation: Bacterial culture was inoculated into 100 ml of brain heart infusion broth and was allowed to grow overnight on a rotary shaker at 37°C. Next day, cells were harvested by centrifugation at 6,000 g for 15 min at 4°C and resuspended in 5 ml of SET buffer (SET buffer: 75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 8.0).

To carry out cell lysis, lysozyme was added to a concentration of 1 mg/ml and incubated at 37°C for 0.5-1.0 h. 1/10th volume of 10% SDS along with 0.5 mg/ml of proteinase K were added and the mixture was incubated at 55°C with occasional inversions for 2 h. 1/3rd volume of 5 M NaCl and equal volume of chloroform were added to the tubes and were mixed by inversion for 0.5 h at room temperature. After mixing the tubes were centrifuged at 6,000 × g for 15 min. The aqueous phase was transferred to a new tube using a blunt ended tip. Finally DNA was precipitated by adding equal volume of isopropanol. Precipitated DNA was gently transferred into micro centrifuge tubes and centrifuged. The pellet obtained was washed with 70% ethanol at 10,000 × g for 10 min. Supernatant was discarded and ethanol was evaporated the pellet obtained was dissolved in appropriate volume of TE buffer [12].

Primer design

A single set of primer was designed manually, complimentary to the *Bacillus licheniformis* Cel12A gene sequence available in UNIPROTKB nucleotide database. This includes Forward primer 5'-GGATCCATGAAAAACAACCATTTGCTA-3') containing BamHI recognition site (underlined) and reverse primer (5'-CTCGAGTCAGCGGACCGTTACGTCCCA-3') with XhoI recognition site.

Amplification of endoglucanase gene

The gene for cellulase was amplified using primer pair generated on the basis of the sequence of cellulase gene of the same genus. Primers were obtained from Integrated DNA Technologies (http://eu.idtdna.com/). The Cel12A gene was amplified through Polymerase Chain Reaction (PCR) having total reaction volume of 20 μl, which contained 10 ng of template genomic DNA, 10 Ƥmol of each primer, 2.5 mmol of each dNTP, 2 μl of 10X Taq buffer, and 2U of Taq DNA polymerase. The PCR procedure comprised 30 cycles of 1 min at 94°C, 30 sec at 58°C, and 1 min at 72°C. A final extension at 72°C for 5 min was performed after 30 cycles. The amplified product was checked on 1% agarose gel stained with ethidium bromide and observed on an UV transilluminator.

Elution of amplified gene

Elution of amplified gene product was carried out with the help of gel extraction kit (Merck, India). The procedures were followed according to the manufacturer's protocol provided along with the Gel extraction kit.

Restriction digestion of Cel12A gene and pRSETA vector

Digestion of the insert and the expression vector were carried out

by taking 1 µg of pRSETA vector and 300 ng of insert. 5 units of both the enzymes were added to the mixture with, 2 µl BSA (Bovine serum albumin) and 10 µl buffer. Total volume of the mixture was made up to 100 µl with nuclease free water and incubated at 37°C overnight, followed by incubation at 65°C for 10 min for the deactivation of the enzymes. Confirmation of the digestion process was carried out by separating the mixtures on 1% agarose gel. Again insert and vector were eluted and purified from agarose gel.

Cloning

The purified PCR fragment and pRSETA vector were dissolved in appropriate amount of TE buffer and the DNA concentration was determined by absorbance at 260 nm. Into a 0.5 ml micro centrifuge tube, a ligation reaction of 20 µl was kept having 3:1 ratio of insert: vector along with 10 × ligation buffer 2 µl and T4 DNA ligase enzyme 1 µl. The reaction was incubated at 16°C overnight.

Transformation

Preparation of competent cells of *E. coli* DH5α: For preparation of competent cells, a well isolated, single colony of DH5α was picked from the Luria Bertani agar plate and inoculated into 5 ml of LB broth and incubated at 37°C (in a shaker) overnight. 1 ml of the overnight culture was further inoculated into 100 ml of LB broth and the flask was incubated at 37°C in a shaker till the O.D $A600$ reached 0.600. The cultured flask was chilled on ice for 10-20 min and it was aseptically transferred into sterile centrifuge tubes and spun at 4,000 × g for 8 min at 4°C. The supernatant was discarded and to the cell pellet, approximately 15 ml of cold 0.1 M $CaCl_2$ solution was added aseptically. The pellet was suspended using pre-chilled pipette tips by aspiration.

The tubes were transferred to ice for 30 min followed by centrifugation at 4,000 × g for 8 min at 4°C. The aqueous phase was discarded and pellet was resuspended gently in 0.6 ml of cold 0.1 M $CaCl_2$ solution. These competent cells of *E. coli* DH5α were used for transformation [41].

Transformation of Cel12A gene in *E. coli* DH5α

14 µl of ligated sample was mixed with 100 µl of competent cells and the tube was kept on ice for 30 min. Heat shock treatment was given to the sample tube for 2 min at 42°C, followed by incubation on ice for 5 min. To the sample tube, 900 µl LB was added and incubated at 37°C for 1h. The sample was centrifuged at 6,000 × g for 5 min, the supernatant was discarded (around 900 µl) and the pellet was resuspended in 100 µl of Luria broth. Aliquots of the sample were spread on Luria agar plates containing 100 µg/ml of ampicillin. The transformants were selected by their ability to grow on ampicillin containing LB plates [41].

Plasmid DNA isolation

Plasmids were isolated using alkali lysis method. The transformants from the ampicillin plate were picked up and inoculated into 10 ml of Luria broth and were incubated under shaking condition at 37°C. 1.5 ml of cell suspension was transferred into the micro centrifuge tube and centrifuged at 10,000 × g for 1 min at 4°C. Supernatant was completely removed and pellet was resuspended in 100 µl of solution-I (glucose 0.9%, 0.025 M Tris, 0.01 M EDTA). Then 200 µl of solution-II (0.2 N NaOH, 1% SDS) was added and tubes were invert mixed slowly once and then 150 µl of solution-III (3 M potassium acetate, 11.5% glacial acetic acid) was added and tubes were invert mixed once more. Cell debris and proteins were removed by centrifugation at 19.000 g at 4°C. 0.8^{th} volume of isopropanol was added to the solution and tubes were incubated at room temperature for 2 h. The tubes were centrifuged at

20,000 × g for 25 min at 4°C, pellet washed with chilled 70% ethanol and air dried. Finally plasmids were resuspended in 35 µl of MQ water. 5 µl of plasmid were checked on 1% agarose gel.

Confirmation of clones through gene specific PCR

Confirmation of plasmids containing Cel12A gene was carried out with the gene specific PCR. The gene specific primers were used to amplify gene of interest using plasmids isolated from *E. coli* DH5α cells as templates. PCR conditions were similar as described for amplification of Cel12A from genomic DNA of *Bacillus licheniformis.* The amplified product was checked on 1% agarose gel stained with ethidium bromide and observed on an UV transilluminator.

Sequencing

The confirmed recombinant plasmids were processed for sequencing. The plasmid was sequenced by automated DNA analyzer 3730 using ABI PRISM® BigDye™ Terminator Cycle Sequencing 3.1 (Applied Biosystems, Foster City, CA). The sequencing was carried out with the help of universal primers of T7 promoter.

Results and Discussion

Optimization of fermentation parameters by response surface methodology using box behnken design

Optimization of any process or process conditions can be effectively carried out with the involvement of Response Surface Methodology. Response Surface Methodology has been effectively employed for optimization of fermentation parameters for solid state fermentation by many research groups. This method has been successfully applied in the optimization of fermentation medium components, conditions for enzymatic hydrolysis and fermentation processes [37,42-45]. It allows the calculation of maximum enzyme production based on few sets of experiments in which all the factors are varied within selected range and also to study interactive effects of various process parameters [46]. The results of 15 run BBD in three variables, initial pH of the medium, substrate to moisture ratio and incubation time for the optimization of cellulase production are shown in Table 3.

ANOVA (analysis of variance) was employed for the determination of significant effects of variables for cellulase production. Cellulase

S.No	Process variables			
	Initial pH X_1	Moisture ratio X_2	Incubation time X_3	Endo-β-1,4-glucanase activity (U/g)
1	1	1	0	1.267
2	-1	-1	0	0.437
3	0	0	0	1.496
4	1	0	1	2.111
5	0	-1	-1	0.837
6	-1	0	-1	0.378
7	0	0	0	1.326
8	0	1	1	1.948
9	-1	0	1	1.844
10	-1	1	0	0.156
11	0	1	-1	0.459
12	0	0	0	1.126
13	0	-1	1	1.882
14	1	0	-1	0.556
15	1	-1	0	0.378

Table 3: Full factorial Box-Behnken Design for production of cellulase from *Bacillus licheniformis* and its activity.

production varied markedly with the conditions tested in the range of 0.156-2.11 U/g. It was observed from various experimental runs that cellulase production was quite high with higher pH and incubation time. Moreover, it was observed that the moisture ratio at 1:1 gave maximum cellulase activity which decreased with increased moisture ratio. The lowest level of endo-β-1,4-glucanase was observed with Run no 10, having low point value of initial pH, pH 7.0 (coded value -1), highest point value of moisture ratio, 1:1.5 (coded value +1) and medium value of incubation time 48 h (coded value 0). Having all the conditions not in favor for production of enzyme, strain *Bacillus licheniformis* showed very less production of endo-β-1,4-glucanase (0.156 U/g). With increasing initial pH of the medium (coded value 1), increase in enzyme production was observed. Maximum endo-β-1,4-glucanase activity was supported at pH 9.0 (coded value 1) (Run no. 4). Along with increased pH, incubation time also supported production of enzyme at its medium value (coded value 1). The experimental results suggest that the variables selected for the fermentation process had strong effect on cellulase production.

The coefficient for the linear effect of incubation time was highly significant while initial pH was statistically significant with 10% error. However, in square terms all three variables were found to exert significant influence on endo-β-1,4-glucanase production ($p<0.05$). In addition, the interactive effect between initial pH and moisture ratio was significant at ($p<0.05$) and had a positive influence on endo-β-1,4-glucanase production (Table 4).

On the basis of these experimental values, statistical testing was carried out using the Fisher's 'F'-test and students 'T'-test. Analysis of variance for cellulase production shows that fitted second order response surface model is highly significant with F test = 13.41 (P = 0.005) as shown in Table 5.

The fitted second order response surface model as specified by

Term	Endo-β-1,4-glucanase	
	Coefficient	***P***
Constant	1.31603	0.000
X_1	0.18705	0.064
X_2	0.03705	0.659
X_3	0.69443	0.000
$X_1 X_1$	-0.40800	0.017
$X_2 X_2$	-0.34875	0.030
$X_3 X_3$	0.31420	0.043
$X_1 X_2$	0.29258	0.047
$X_1 X_3$	0.02223	0.850
$X_2 X_3$	0.11107	0.366

Table 4: Estimated regression coefficient and corresponding *p* value for endo-β-1,4-glucanase activity.

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean of squares (MS)	F	P
Regression	9	6.03745	0.67083	13.41	0.005
Linear	3	4.14869	1.38290	27.64	0.002
Square	3	1.49503	0.49834	9.96	0.015
Interaction	3	0.39373	0.13124	2.62	0.163
Residual Error	5	0.25020	0.05004		
Lack-of-Fit	3	0.18146	0.06049	1.76	0.382
Pure Error	2	0.06874	0.03437		
Total	14	6.28766			

Table 5: ANOVA of endo-β-1,4-glucanase activity: Effect of initial pH, moisture ratio, and incubation time.
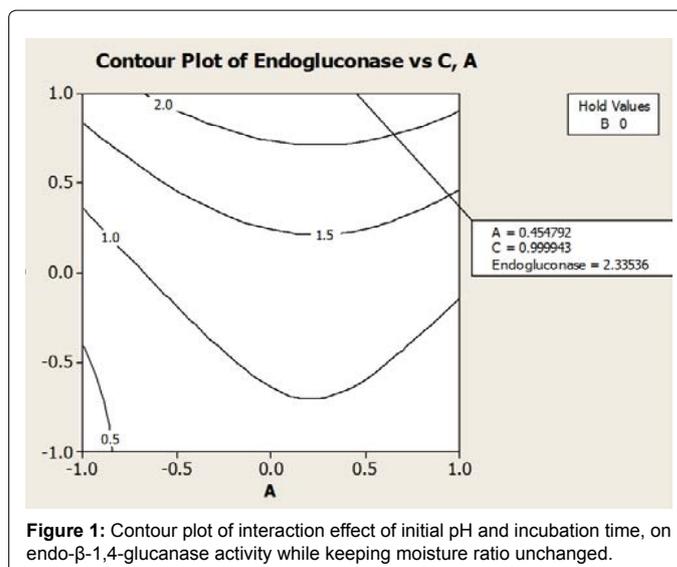

Figure 1: Contour plot of interaction effect of initial pH and incubation time, on endo-β-1,4-glucanase activity while keeping moisture ratio unchanged.

Equation (5.3) for cellulase activity in coded process variables is:

$$\eta \text{ (Endo-β-1,4-glucosidase)} = (1.3160) + (0.1870\,(X_1)) + (0.0370\,(X_2)) + (0.6944\,(X_3)) + (-0.4080\,(X_1{}^2)) + (-0.3487\,(X_2{}^2)) + (0.3142\,(X_3{}^2)) + (0.2925\,(X_1 X_2)) + (0.0223\,(X_1 X_3)) + (0.1107\,(X_2 X_3)) \qquad (5.3)$$

The coefficient of determination R^2 for the above predicted Equation (5.3) was 96%. Therefore, this equation can be used for predicting the response at any combination of three predicted variables in and around their experimental range.

The enzyme activities (η) at specified combination of the three variables can be predicted by substituting the corresponding coded values in the equation.

The contour plot based on Equation 5.3 was prepared using MINITAB 16 software. The contour plot (Figure 1) shows the behavioral change with respect to simultaneous change in two variables. Proper choice of fermentation parameters is desirable for maximum enzyme production and contour plots based on well fitted model provides these choices [46]. Contour plot was prepared for three pairs of variables which were having significant interaction effects in maximizing cellulase production at specific hold values. The behavior of cellulase activity (U/g) with respect to change in initial pH (A) and incubation time (C) and moisture ratio at hold values is shown in Figure 1. From the contour plot, it is evident that, the increase in initial pH and incubation time had positive effect in yielding higher cellulase activity.

All the parameters affected enzyme production in terms of squares. One of the parameters tested is the initial pH of the medium, which is an important parameter considered in any production optimization process because of the fact that any enzyme produced by microorganisms can be stable at a particular pH value. *Bacillus* is generally able to grow over a wide range of pH values. The enzymes produced by this genus are also stable over a wide range of pH. The pH values below and above the optimum value required for growth as well as enzyme production lead to slower growth rate along with low levels of enzyme secretion. Moreover, the extracellular enzyme produced will also have conditions which lead to the inactivation of the enzyme. *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12) and it required an alkaline pH (8-10) for growth and enzyme secretion [47]. Optimization of fermentation conditions like initial pH value of the medium is hence an important parameter.

The change of moisture content and incubation time was significant. Studies related to interaction of moisture ratio and incubation time are not reported so far, but at the same time selection of appropriate time for enzyme extraction was found to be an important parameter in various studies. The time of fermentation played an important role in the study. Maximum enzyme production was obtained after 72 h of fermentation. *Bacillus sp.* has been reported to produce maximum enzyme after 72 h fermentation using various substrates by many researchers [11,13,25,48-50]. Results obtained in the present study are in complete agreement with the reports on cellulase production by *Bacillus* spp.

The moisture content of the substrate is one of the critical factors influencing the outcome of SSF, and is governed by the water-holding-capacity of the substrate, the type of end-product and the requirement of the microorganism. At low moisture content, metabolic and enzymatic activities are reduced. Higher moisture levels decrease the porosity of substrate leading to gummy structure, which limits oxygen transfer and reduces growth [25,51-53].

Reductions in enzyme yield with high moisture content [54] and with low moisture content [55] have also been reported.

Application of RSM with BBD predicted that maximum cellulase production must occur at decoded values of process parameters as initial pH (X_1=+0.454), moisture ratio (X_2=0) and incubation time (X_3=+0.99). The model predicted cellulase production 2.33536 U/g at optimum parameters.

The optimization of cellulase production under solid state fermentation showed an interesting study on utilization of Jatropha deoiled seed cake as a substrate using *Bacillus licheniformis* as the producing microorganism. Though it gives insights into use of the substrate and abilities of microorganisms to utilize such a toxic substrate without any pretreatment, lower yield of the endo-β-1,4-glucanase suggests the use of some other ways to increase the production of the enzyme. The potential of the alkaline cellulases secreted by *Bacillus*

spp. are very well known along with many reports by various research groups. Endoglucanase from *Bacillus licheniformis* was produced at pH 9.0. Hence, studying this enzyme further will be an interesting research topic. More over literature survey and sequence data present in NCBI showed a few studies on gene amplification, cloning and expression of endoglucanase from *Bacillus licheniformis*. It was also found that predicted protein sequence of endoglucanase from *Bacillus licheniformis* do not contain cellulose binding module. The lower activities observed in the present study might also be due to lack of CBM in the cellulase.

Considering all the above facts, it was decided to study this enzyme with available molecular biology techniques for gene amplification, cloning and over expression followed by purification of recombinant protein.

Molecular characterization

Isolation of genomic DNA: Figure 2 depicts good quality genomic DNA from *Bacillus licheniformis*. DNA obtained had a purity ratio of 1.6. (*A*260 nm: *A*280 nm). Genomic DNA thus obtained was used for the endoglucanase gene amplification by PCR using universal primers.

Amplification of cell2A: The presence of a gene for cellulase in *Bacillus* spp. has been reported by many researchers [56-60]. The endoglucanases found among various strains of *Bacillus* spp. are of different gene sizes with different properties. Alkaline cellulases

from *Bacillus licheniformis* have been reported by Liu et al. [31]. Characterization of Cel12A from the library generated by Liu et al. [31] suggests a predicted open reading frame of 261 amino acids. The genes for endoglucanase enzyme reported by researchers have different sizes according to the class of the enzyme like Cel9A, Cel12A, Cel5A. The gene encoding Cel12A was found in *Bacillus licheniformis* having alkaline pH optima and good thermal stabilities. Hence the gene sequence available in NCBI GenBank database was searched for Cel12A of *Bacillus licheniformis* and primers were designed according to the full length sequence of the enzyme. This set of primers gave specific amplification, producing a sharp band on an agarose gel. The expected product size for this primer pair was 786 bp and nearly 800 base pairs of specific amplification obtained, as confirmed by agarose gel electrophoresis. The PCR product thus obtained was eluted from the gel using a gel extraction kit (Merck, India). This eluted product was further used for the cloning of the insert into vector (Figure 3).

Restriction digestion: Agarose gel electrophoresis after the restriction digestion of insert as well as vector pRSETA showed sharp bands at ~800 bp and 2.9 kb respectively. The figure shows bands of insert (Cel12A gene) and plasmid pRSETA on agarose gel along with
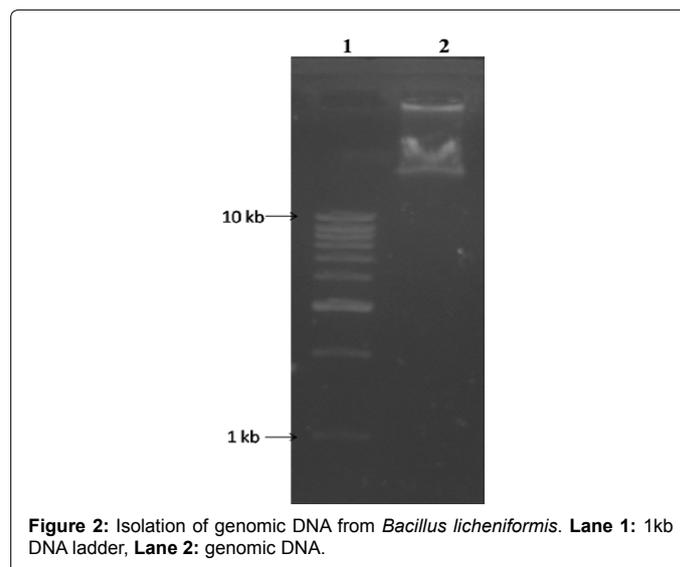

Figure 2: Isolation of genomic DNA from *Bacillus licheniformis*. **Lane 1:** 1kb DNA ladder, **Lane 2:** genomic DNA.
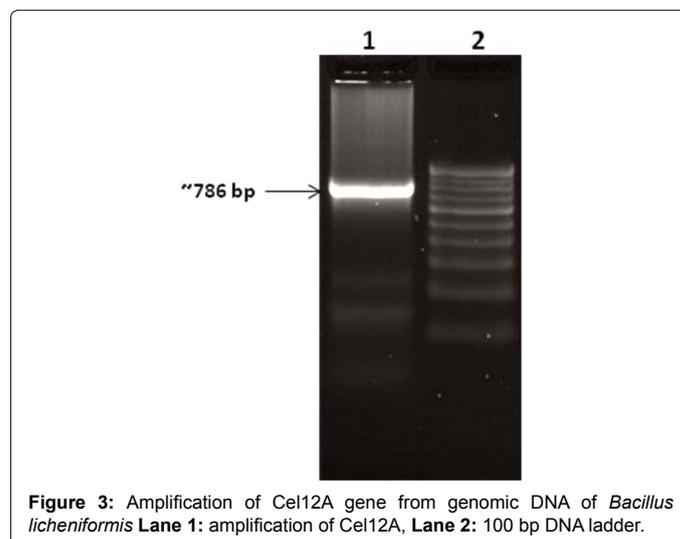

Figure 3: Amplification of Cel12A gene from genomic DNA of *Bacillus licheniformis* **Lane 1:** amplification of Cel12A, **Lane 2:** 100 bp DNA ladder.

the appropriate markers (Figure 4). Results obtained in figure shows linearization of plasmids; hence it confirms that plasmid as well as insert was successfully digested by *BamHI* and *XhoI* restriction enzymes.

Cloning of cellulase gene: Purified insert and vector (pRSETA) with sticky ends were ligated using T4 DNA ligase (NEB). Ligated

mixture was then transformed into competent cells of *E. coli* DH5α by heat shock method. The transformed cells were then plated on Luria Agar plate containing 50 μg/ ml ampicillin. The transformants were further used to isolate plasmid DNA by alkali lysis method and selected with application of gene specific primers (Cel12AFP and Cel12ARP) for the amplification of Cel12A gene and by restriction digestion and release of insert [61].

Sequencing and BLAST analysis: The plasmids showing amplification of Cel12A gene with gene specific PCR were sent for sequencing. A nucleotide sequence of the 1000 bp was obtained. Out of 1000 bp sequence a 786 bp open reading frame was found to be coding for Cel12A. The open reading frame encoding Cel12A was confirmed with BLAST analysis. The BLAST analysis showed its 100% identity towards glycosyl hydrolase family 12 enzymes of *Bacillus licheniformis*. Sequence similarity data is as shown in the Table 6. The sequence of the Cel12A gene from *Bacillus licheniformis* was submitted to NCBI under the Accession number: JQ846014.

BLAST analysis of deduced amino acid sequence: The deduced amino acid sequence encoding Cel12A was generated from the sequence data available in the form of nucleotides. The amino acid sequence was aligned with the amino acid sequences of other organisms using UniprotKB programme. The results obtained are as below.

Figure 4: Restriction digestion of Cel12A and pRSETA (a) **Lane 1:** undigested insert, **Lane 2:** undigested vector pRSETA (b) **Lane 1:** digested insert, **Lane 2:** digested vector, **Lane 3:** 1 kb DNA ladder.

Figure 5: Jalview representation of multiple sequence alignment of *B. licheniformis* (Cel12A) with other glycosyl hydrolase family β-glucanases BL: *Bacillus licheniformis* (Cel12A) deduced amino acid sequence. BLATCC: *Bacillus licheniformis* DSM13 (Cel12A). BS: *Bacillus sp*; PW: *Pectobacterium wasabiae;* PC: *Pectobacterium carotovorum* subsp. *Carotovorum*; PM: *Paenibacillus mucilaginosus*; EC: *Erwinia carotovora* subsp. *atroseptica* (strain SCRI 1043 / ATCC BAA-672).

Entry	Protein names	Organism	Length	Identity	Score
Q65FM6	Glycoside Hydrolase 12 (Uncharacterized protein)	*Bacillus licheniformis* (strain DSM 13)	261	100%	1435
Q7X4S4	Endo-beta-1,4-glucanase (EC 3.2.1.4)	*Bacillus licheniformis*	261	100%	1435
E5W7D6	Endo-beta-1,4-glucanase	*Bacillus* sp. BT1B_CT2	261	100%	1435
I0UJW3	Glycoside hydrolase family protein	*Bacillus licheniformis* WX-02	261	99%	1430
H1AD15	Endoglucanase	*Bacillus licheniformis*	261	94%	1382
C6DDT4	Cellulase (EC 3.2.1.4)	*Pectobacterium carotovorum* subsp. *carotovorum*	264	66%	970

Table 6: Sequence similarity of Cel12A with the sequences available in the database.

The Jalview representation of multiple sequence alignment shows similarity of sequences of various organisms' glycosyl hydrolase 12 with the deduced amino acid sequence of Cel12A (Figure 5). The similarity shown by the amino acid sequence confirms the existence of alkaline cellulase in *Bacillus licheniformis*. Moreover, it also states that the cloned gene sequence will encode an endoglucanase having properties like alkaline cellulases from *Bacillus sp.* Further expression of the cloned gene will provide insights about function of the expressed protein and its biochemical properties.

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