Original article

Response surface optimization of cellulase production from *Aneurinibacillus aneurinilyticus* BKT-9: An isolate of urban Himalayan freshwater

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ARTICLE INFO

ABSTRACT

Due to their vast industrial potential, cellulases have been regarded as the potential biocatalysts by both the academicians and the industrial research groups. In the present study, culturable bacterial strains of Himalayan Urban freshwater lake were investigated for cellulose degrading activities. Initially, a total of 140 bacterial strains were isolated and only 45 isolates were found to possess cellulose degrading property. On the basis of preliminary screening involving cellulase activity assay on CMC agar (with clear zone of hydrolysis) and biosafety assessment testing, only single isolate named as BKT-9 was selected for the cellulase production studies. Strain BKT-9 was characterized at the molecular level using rRNA gene sequencing and its sequence homology analysis revealed its identity as *Aneurinibacillus aneurinilyticus*. Further, various physico-chemical parameters and culture conditions were optimized using one factor approach to enhance cellulase production levels in the strain BKT-9. Subsequently, RSM based statistical optimization led to formulation of cellulase production medium, wherein the bacterial strain exhibited ~60 folds increase in enzyme activity as compared to un-optimized culture medium. Further studies are being suggested to scale up cellulase production in *A. aneurinilyticus* strain BKT-9 so that it can be utilized for biomass saccharification at an industrial level.

1. Introduction

Cellulose, the most abundant renewable bio-resource produced on earth, is found as a principal component in the plant cell wall and is a polymer of glucose linked by β-1, 4- glucosidic bonds (Jarvis, 2003; Saha et al., 2006; Sethi et al., 2013; Tomme et al., 1995; Zhang and Lynd, 2004). Globally, it is the most abundant natural and attractive raw material available for bioconversion into numerous products as well as required in many industries such as animal feed, biofuel, food, detergent, manure, paper, pharmaceutical, textile and waste management (Kasana et al., 2008; Shajahan et al., 2017). Enormous quantities of cellulosic resources are being utilized for various industrial applications throughout the globe but still there are huge quantities of partially hydrolyzed or unutilized resources accumulating in the biosphere and moreover, some are being poorly utilized because of the elevated process costs (Lee et al., 2008). In most of the microbial bioprocesses, the effective bioconversion of cellulose rich materials is confronted by its microcrystalline structure. Although, conventional methods including acid, alkali and heat treatments propose good outcome, yet they likewise deliver secondary contaminations. Therefore, application of microbial enzymes has been anticipated as an efficient alternative for enhancing bioavailability and further bioconversion of the

cellulosic bioresources (Premalatha et al., 2015). Microbial bioprocesses also offer pollution-free, economical and cost-effective alternatives to physico-chemical cellulase degradation (Ding et al., 2008). Cellulases catalyze the hydrolysis of cellulose into bioavailable sugars. Cellulases refers to a group of enzymes (glycosyl hydrolases) including endoglucanase, exoglucanase and β-glucosidase, exhibiting varied action on cellulose degradation and are required to act synergistically for complete enzymatic hydrolysis of cellulosic biomass (Bhat, 2000; Yang et al., 2014).

In cellulose saccharification, endogluconase acts randomly on glucose-polymer chains, cleaves internal β-1,4-glycosidic bonds to release small fibers consisting of free-reducing ends and non-reducing ends, followed by exoglucanase acting on free ends of the chains to release cellobiose that is finally acted upon by β-glucosidase to release glucose as the end product (Allardyce et al., 2010). Most of these cellulolytic enzymes are of microbial origin and illustrate considerable variations in their enzymatic activity and stability. This leads to the isolation and characterization of new cellulase producing microbial resources (Premalatha et al., 2015). Cellulase production has been studied in many aerobic, anaerobic, mesophillic or thermophilic bacterial strains belonging to genera *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteriodes, Acetovibrio, Streptomyces, Microbispora, Fibrobacter*, and *Paenibacillus* (Irfan et al., 2017; Liang et al., 2014; Saravanan et al., 2013; Sreena and Sebastian, 2018). Optimization of media components and physicochemical process variables plays a vital role in enhancing production of an indigenous and inexpensive cellulase (Shajahan et al. 2017). In the present study, Response Surface Methodology (RSM) was used to evaluate the interactions of independent physicochemical process variables for optimization of cellulase production in *A. aneurinilyticus* strain BKT-9. RSM is a statistical tool, in which several variables are tested simultaneously (Parajo et al., 1992; Srinubabu et al., 2007). The multivariate approach has benefits included decrease in the number of investigational runs, enhances statistical justification potentials and specifies whether parameters interact or not. Central composite design (CCD) is widely used statistical experimental design. Although rotatable feature is an anticipated property of a CCD, in the case of difficulty to extending the star points beyond the experimental region defined by the upper and lower limits of each factor (Lundstedt et al., 1998). This study also aimed at isolation and screening of cellulolytic bacteria from Dal Lake, an urban fresh water Himalayan Lake and optimization of the nutritional supplements and environmental parameters for improving cellulase production in this potential isolate.

2. Material and methods

2.1. Screening and selection of the cellulose degrading bacterial isolates

To isolates culturable bacteria from Dal lake waters, samples were drawn from different sites of the lake during winter and summer seasons and pooled respectively. The collected Lake water samples were serially diluted in sterile distilled water up to 10^{-4} and inoculated on the nutrient agar medium and incubated at 37 °C. Morphologically distinct colonies were isolated, purified and maintained at 4 °C on nutrient agar slants. Cellulolytic/CMCase activity of the bacterial isolates were screened on nutrient agar plates containing 0.5% carboxy methyl cellulose (CMC). The plates were incubated at 37 °C for 48 h and were flooded with 0.1% Congo red solution followed by 1 M NaCl. Presence of a clear zone around the growth indicated the hydrolysis of CMC (Teather and Wood, 1982).

2.2. Safety assessment and selection of the best bacterial isolate for enzyme production

As the bacterial strains were isolated from an environmental sample, it is imperative to perform some of the biosafety tests. Therefore, couple of tests were performed to preliminary investigate the safety of cultured bacterial strains as described below:

2.2.1. Blood agar test
The bacterial isolates were grown on nutrient agar plates containing 5% blood, incubated at 37 °C for 3 days and the plates were observed for hemolytic reaction. β-hemolysis leads to formation of a clear zone of hydrolysis of red blood cells, α-hemolysis leads to formation of a green zone and γ-hemolysis results in formation of no clear or green zone of hemolysis around the bacterial colonies (Gupta and Malik, 2007).

2.2.2. Gelatinase test
The bacterial isolates were grown on nutrient agar plates containing 0.4% gelatin using disc diffusion method. After incubation at 37 °C for 3 days, plates were flooded with saturated ammonium sulfate solution and observed for clear zones around the colonies indicates a positive reaction (Gupta and Malik, 2007).

2.2.3. DNase test
The bacterial isolates were grown on nutrient agar plates containing 0.2% DNA using disc diffusion method. After incubation at 37 °C for 48 h, plates were flooded with 1 N HCl and observed for clear, pinkish zone around the bacterial colonies (Gupta and Malik, 2007). Formation of clear, pinkish zone was considered positive for DNAse production.

2.2.4. Antibiotic susceptibility test
Susceptibility of the potential cellulase producing bacterial isolates to the different antibiotics was tested using a Disc Diffusion Assay on nutrient agar plates using antibiotic discs (Himedia Laboratories, Pvt. Ltd., Mumbai, India) consisting amikacin (25 mcg), amoxicillin (25 μg), cephalexin (30 μg), cefazolin (30 μg), co-trimoxazole (25 μg), gentamicin (10 μg), streptomycin (10 μg), amoxyclav (30 μg), tetracycline (30 μg), norfloxacin (10 μg), cefixime (5 μg) and roxithromycin (30 μg). Inhibition zone were observed around the individual antibiotic discs (Gupta and Malik, 2007). The strains showing antibiotic resistant character and haemolytic, gelatinase and DNase activity in the biosafety assessment tests were not taken for further studies.

2.3. Molecular identification of the selected bacterial strain

After the preliminary investigations on cellulase enzyme production and biosafety testing, the isolate with best enzyme producing potential and negative pathogenicity was selected for enzyme production and identified on the basis of Gram staining and molecular biology techniques (Kumar et al., 2018).

2.3.1. 16S rDNA characterization
DNA was isolated from the bacterial isolate BKT-9 at Xcelris Labs Ahmedabad, India. Quality was evaluated on 1.2% Agarose Gel; a single band of high-molecular weight DNA was observed. Isolated DNA was amplified using 16S rRNA Specific Primer (8Fand 1492R) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon of 1500 bp was observed. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1487 bp 16S rDNA was generated from forward

and reverse sequence data using Aligner software. The 16S rDNA sequence was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first 15 sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGA6 (Kumar et al., 2018).

2.4. Extracellular Cellulase/CMCase enzyme activity assay

Cellulase activity was measured following standardized procedure of Miller by estimating reducing sugar content (Miller, 1959). In brief, 0.5 mL of cell free supernatant was taken as crude enzyme to form a reaction mixture with 0.5 mL of 0.5% carboxymethyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and incubated at 37 °C in a shaking water bath for 30 min. The reaction was terminated by adding 3 mL of DNS reagent and the colour was developed by boiling the mixture for 5 min. The reaction mixture was cooled down and diluted in 20 mL of distilled water. Absorbance of samples was measured at 540 nm against a blank containing all the reagents except crude enzyme.

2.4.1. Intracellular enzyme activity assay

To check intracellular enzyme activity, 1 mL culture broth was taken and centrifuged to collect the cell pellet. The pellet was resuspended in 300 μl Bugbuster (Novagen) for inducing cell lysis at 37 °C for 1 h and after centrifugation supernatant was used as crude enzyme for performing enzyme activity assay as per standard protocol (Miller, 1959).

2.5. Selection of the culture medium and physicochemical process variables for optimizing bacterial growth and enzyme production

Cellulase production in any bacterial strain can be varied and affected by different physico-chemical parameters, which could considerably affect the enzyme production in different ratios. Therefore in the current study, cellulase enzyme production was optimized using growth media under differential conditions consisting salt concentration, incubation temperatures, pH of media, aerobic/anaerobic conditions, and incubation time to select optimal production media and growth factors (Berg et al., 1972; Ratnakomala et al., 2019; Sethi et al., 2013). The experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL medium. After sterilization by autoclaving, the flasks were cooled and inoculated with 1 mL freshly grown culture containing 10^6 CFU/mL.

2.5.1. Optimization of growth media

Flasks containing the different growth medium (selected on the basis of previous literature) namely Nutrient Broth (containing Peptone 10 g/L, Beef extract 10 g/L, Sodium chloride 5 g/L; pH 7.3 ± 0.1), Luria Broth (consisting Casein enzymic hydrolysate 10 g/L, Sodium chloride 10 g/L, Yeast extract 5 g/L; pH 7.5 ± 0.2), Berg's Minimal Salt Media (containing $NaNO_3$ 0.2%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02%, $MnSO_4 \cdot H_2O$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.002%, $CaCl_2 \cdot 2H_2O$ 0.002%, Cellulose 1%) and YT Medium (containing Casein enzymic hydrolysate 16 g/L, Yeast extract 10 g/L, Sodium chloride 5 g/L; pH 7.0 ± 0.2) were taken, inoculated and incubated at 37 °C temperature (Berg et al., 1972). At the end of incubation period, 1 mL sample was drawn, centrifuged at 5000 rpm for 5 min and pellet was washed and resuspended in saline. The OD was recorded at 600 nm to observe bacterial growth and cellulase activity from CFS was assayed as per standard procedure mentioned above.

2.5.2. Optimization of medium pH

Flasks containing 100 mL YT medium broth were taken and the pH of the broth was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 in different flasks using 1 N HCl and 1 N NaOH and sterilized at 150 psi for 20 min. The culture media were inoculated and incubated at particular temperature. At the end of incubation period, 1 mL sample was drawn, centrifuged at 5000 rpm for 5 min and pellet was washed and resuspended in saline. The OD was recorded at 600 nm to determine bacterial growth. Cellulase activity from CFS was assayed as per standard procedure (Miller, 1959).

2.5.3. Optimization of salt concentration

Flasks containing YT broth were supplemented with NaCl (*w/v*) at a concentration of 0.1–20% w/v and inoculated. At the end of incubation period, 1 mL sample was drawn, centrifuged at 5000 rpm for 5 min and pellet was washed and resuspended in saline to measure absorbance at 600 nm. Cellulase activity from CFS was assayed as per standard procedure of Miller (1959) as mentioned above.

2.5.4. Optimization of physical parameters for enhancing cellulase/CMCase production
2.5.4.1. Optimization of incubation temperature. Flasks containing selected YT culture medium were inoculated as described above and incubated at different temperatures such as 5 °C, 25 °C, 30 °C, 37 °C, 42 °C and 60 °C. At the end of the incubation period, 1 mL sample was drawn, centrifuged at 5000 rpm for 5 min and pellet was washed and resuspended in saline to record absorbance at 600 nm. Cellulase activity in the CFS was assayed using standard procedure as mentioned above.

2.5.4.2. Aerobic vs. anaerobic conditions. Flasks with YT broth were taken, inoculated and incubated under aerobic and stationary, aerobic and shaking and anaerobic conditions (covered with a layer of mineral oil). At the end of the incubation period, 1 mL sample was drawn, centrifuged at 5000 rpm for 5 min and pellet was washed and resuspended in saline to assess bacterial density by taking absorbance at 600 nm and estimate CMCase activity.

2.5.4.3. Shaking vs. stationary conditions. Culture broth in the selected media were incubated under shaking conditions (100 rpm, 150 rpm and 200 rpm) to examine their effect on biomass and enzyme production.

2.5.4.4. Incubation time period. The defined production YT medium (100 mL in 250 mL flasks) was inoculated with 1% inoculum (*v/v*) of 18 h old starter culture. The flasks were incubated for 52 h at 30 °C and 150 rpm. To study the growth pattern and cellulase production with respect to time, samples were drawn regularly at 4 h intervals and analyzed for biomass production as well as cellulase activity in the cell free supernatant using standard protocols as mentioned above. The optimal incubation period with maximum cellulose production was selected for further RSM based studies as a fixed factor. Evaluations of above parameters eventually lead to the formulation of production media and physico-chemical conditions for enhanced bacterial cell culturing as well as enzyme production. Further, these parameters were kept fixed during statistical media optimization through RSM.

2.6. Optimization of enzyme production at shake flask level by one factor approach

In order to further enhance bacterial growth and enzyme activity, medium components such as carbon, inorganic nitrogen, salt sources and substrate concentrations were changed according to one-factor analysis (Sethi et al., 2013; Shajahan et al., 2017). The

medium components producing higher cellulase enzyme activity were considered suitable for further RSM based studies (CMCase activity assayed as per Miller, 1959).

2.6.1. Selection of the best carbon sources

The effect of various carbon sources such as glucose, fructose, sucrose, mannitol and maltose at the concentration of 0.5% w/v was examined in the production medium (Premalatha et al., 2015; Sethi et al., 2013).

2.6.2. Selection of the best nitrogen sources

Various nitrogen sources such as urea, ammonium acetate, ammonium sulphate, ammonium nitrate and ammonium chloride at the concentration of 0.05% *w/v* were examined for evaluating their effect on enzyme production (Premalatha et al., 2015; Sethi et al., 2013).

2.6.3. Selection of the best salt sources

The effect of salt supplementation was examined by adding various salts such as calcium sulfate, magnesium chloride, magnesium sulphate, manganese sulphate and ferrous sulfate at a concentration of 0.1% *w/v* in the selected production medium (Pereira et al., 2017).

2.6.4. Selection of substrate concentration

To study the improvement in cellulase production, 0.1%, 0.5% and 1% *w/v* CMC was used as an enzyme inducer in the selected production medium (Liang et al., 2014; Islam and Roy 2018).

2.7. Optimization of enzyme production by response surface methodology (RSM)

Based on the above experiments, important process variables were selected for statistical medium optimization of cellulose production in the bacterial isolate BKT-9. Response surface methodology (RSM) is an effective statistical tool which follows a combination of mathematical and statistical techniques to study cumulative effect of the process variables on enzyme production (Gunst et al., 1996). Experimental design was made by using Design Expert software version 12.0. A central composite rotatable design (CCRD) was used to optimize cellulase production as a function of four variables including carbon source, inorganic nitrogen source, salt and substrate concentration and their interactions in

each experiment. The experimental plan generated a full-factorial screening design with a total of 21 experiments including 5 replicate center points. The influence of variables like glucose (5–14 gm/L, ammonium chloride (0.4–1 gm/L), $MnSO_4$ (0.3–0.7%) and CMC (0.55–1.5%) was studied on enzyme activity as described in Table 1.

2.8. Experimental validation of the statistically defined production process

On the basis of the results obtained during statistical analysis, the optimized medium was formulated, adjusted to pH 7 and inoculated with 1% inoculum and incubated at 30 °C, 150 rpm for 36 h and enzyme activity was estimated according to Miller (1959). The optimized medium for validation of model consist of fixed parameters (tryptone 1.6%, yeast extract 1%, NaCl 0.6%) and RSM optimized parameters (11.1g/L, ammonium chloride 0.77g/L, manganese sulphate 0.49%, CMC 0.5%). The predicted response surface model was validated in triplicates.

3. Results

### 3.1. Isolation, screening, selection and identification of the bacterial isolate

Among 140 bacterial strains isolated from the Dal Lake waters (66 from winter and 74 from summer samples respectively) only 45 isolates were found to possess cellulolytic activity on 0.5% CMC agar and showed the zone of hydrolysis ranging from 0.8 to 2.5 cm. Five isolates exhibiting hydrolysis greater than 2 cm zone diameter were tested for biosafety of the culture. Isolate BKT-9 did not generate hydrolysis zone whereas other four isolates showed zone of hydrolysis on blood agar and was scored positive in gelatinase and Dnase tests. Therefore, isolate BKT-9 was selected for further study. Furthermore, the antibiotic susceptibility test of BKT-9 showed susceptibility to all the 12 tested antibiotics, with maximum zone of inhibition for Amikacin 25 μg (5 cm) and minimum for Amoxyclav 30 μg (2 cm). Results were depicted in Fig. 1.

3.2. Identification of the selected bacterial strain

The selected bacterial isolate BKT-9 was found to be Gram-positive, rod shaped in structure while observation under

Table 1
CCD design matrix and response in terms of cellulase production (IU/L).

| Run	Glucose (g/L)	Ammonium chloride (g/L)	Manganese sulphate (% w/v)	CMC (% w/v)	Actual value (IU/L) Response	Predicted value (IU/L) Response
1	9.5	0.7	0.5	1.84	55.41	55.98
2	9.5	0.7	0.5	1	69.01	69.36
3	9.5	0.7	0.16	1	42.82	43.07
4	14	0.4	0.3	1.5	59.57	59.30
5	5	0.4	0.3	0.5	35.35	35.08
6	14	0.4	0.7	1.5	64.07	63.54
7	14	1	0.3	0.5	73.69	73.42
8	9.5	0.7	0.5	1	69.30	69.36
9	9.5	1.2	0.5	1	67.68	68.24
10	9.5	0.7	0.5	1	69.47	69.36
11	9.5	0.19	0.5	1	68.75	69.31
12	9.5	0.7	0.5	1	69.76	69.36
13	9.5	0.7	0.5	0.16	72.18	72.75
14	5	0.4	0.7	0.5	57.09	56.56
15	1.93	0.7	0.5	1	35.00	35.57
16	5	1	0.3	1.5	31.45	31.19
17	9.5	0.7	0.83	1	61.38	62.26
18	14	1	0.7	0.5	73.92	73.38
19	17.06	0.7	0.5	1	68.44	69.01
20	5	1	0.7	1.5	51.67	51.14
21	9.5	0.7	0.5	1	70.58	69.36
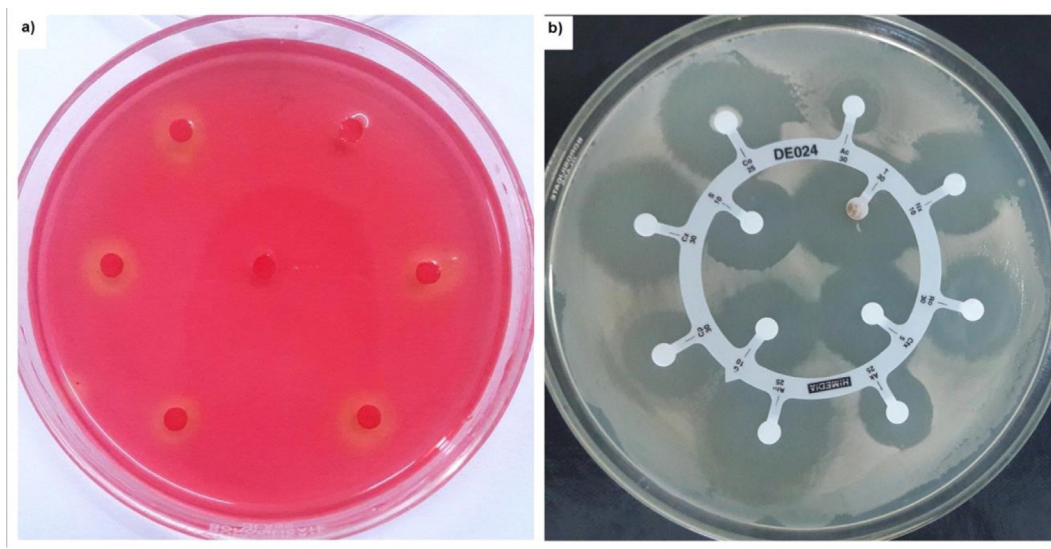
Fig. 1. (a) Zone of hydrolysis produced on CMC agar while screening of isolated bacterial strains for cellulase production (b) Antibiotic susceptibility of *Aneurinibacillus aneurinilyticus* BKT-9.

microscope (100× oil immersion objective) as shown in Fig. 2a. Genotypic characterization based on nucleotide homology, a phylogenetic analysis of 16S rDNA sequence revealed that the isolate BKT-9 has more than 98% similarity with *Aneurinibacillus aneurinilyticus* ATCC 12856 and the phylogenetic tree is given as Fig. 2b. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1496 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

(Kumar et al., 2018). The sequence has been submitted to GenBank under accession no. MT131245.

3.3. Selection of culture medium and physico-chemical process variables for optimizing bacterial growth and enzyme production

Among different media, the selected isolate BKT-9 showed maximum growth in YT medium. The isolate exhibited growth at a broad range of temperature 25–60 °C and pH 7–10 with optimal growth at 30 °C and pH 7. The isolate was observed to show increased growth on supplementation of YT medium with 0.1% NaCl under aerobic conditions at 150 rpm. Composition of the formulated YT medium consists of tryptone 1.6%, yeast extract 1% and NaCl 0.6%. Furthermore, the isolate was not able to grow under anaerobic conditions where oxygen supply was cut off by putting a thin layer of mineral oil over top of the inoculated medium. These parameters were taken into consideration for further formulation of cellulase production medium and to study their cumulative effect on enzyme activity is depicted in Fig. 3.
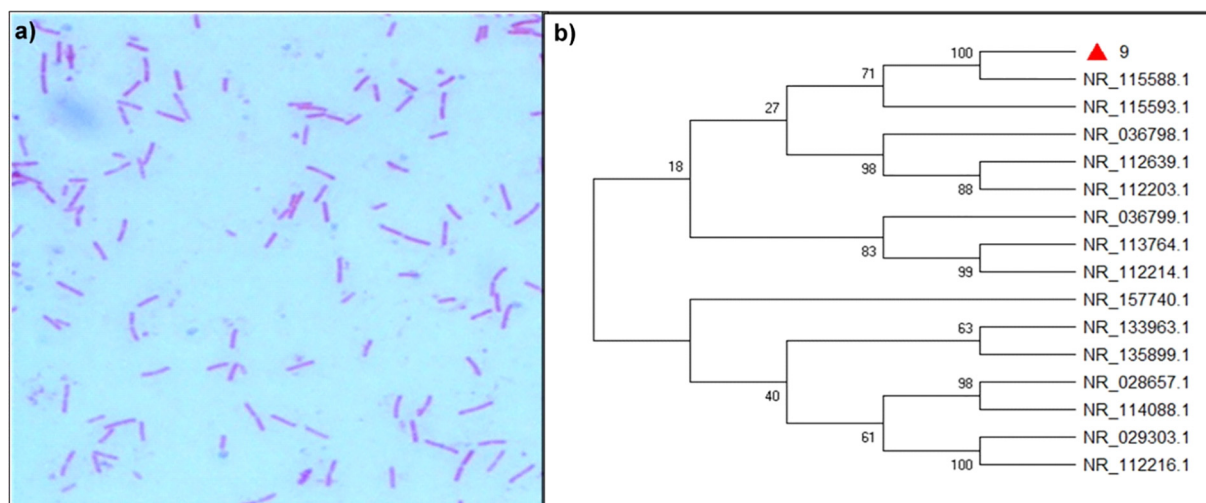
Fig. 2. (a) Microscopic examination of BKT-9 isolate under 100X oil immersion objective and (b) Phylogenetic tree constructed using the Neighbor-Joining method based on the 16S rDNA sequence of *Aneurinibacillus aneurinilyticus* BKT-9.

Maximum biomass in terms of A600nm was observed at 36 h of incubation as evident from Fig. 3f. Maximum cellulase production was also achieved at 36 h with an enzyme activity of 1.41 IU/L at this stage. The strain also showed an intracellular cellulase activity of 0.3 IU/L but was significantly less than extracellular enzyme activity therefore not considered in further experiments.

3.4. Optimization of enzyme production at shake flask level by one factor approach

The medium variables have a significant role in attaining maximum growth and enzyme production. Optimization makes it easier to evaluate the effect of several medium variables for achieving optimum conditions of a particular response (Ratnakomala et al., 2019). Therefore variables such as different carbon, inorganic nitrogen and salt sources and substrate concen-

tration (CMC) were optimized under shake flask conditions by varying one factor at a time.

3.5. Carbon source

All the tested carbon sources were found to supplement bacterial growth and enzyme production. However, glycerol showed maximum growth but did not enhance enzyme activity. Medium containing glucose showed maximum enzyme activity of 17.1 IU/L that is 12 folds higher as compared to control.

3.5.1. Inorganic nitrogen source

Among the nitrogen sources used ammonium nitrate was found to decrease the enzyme activity and other four sources were found to supplement both growth and enzyme activity. The maximum activity of 1.7 IU/L was recorded in medium supplemented with
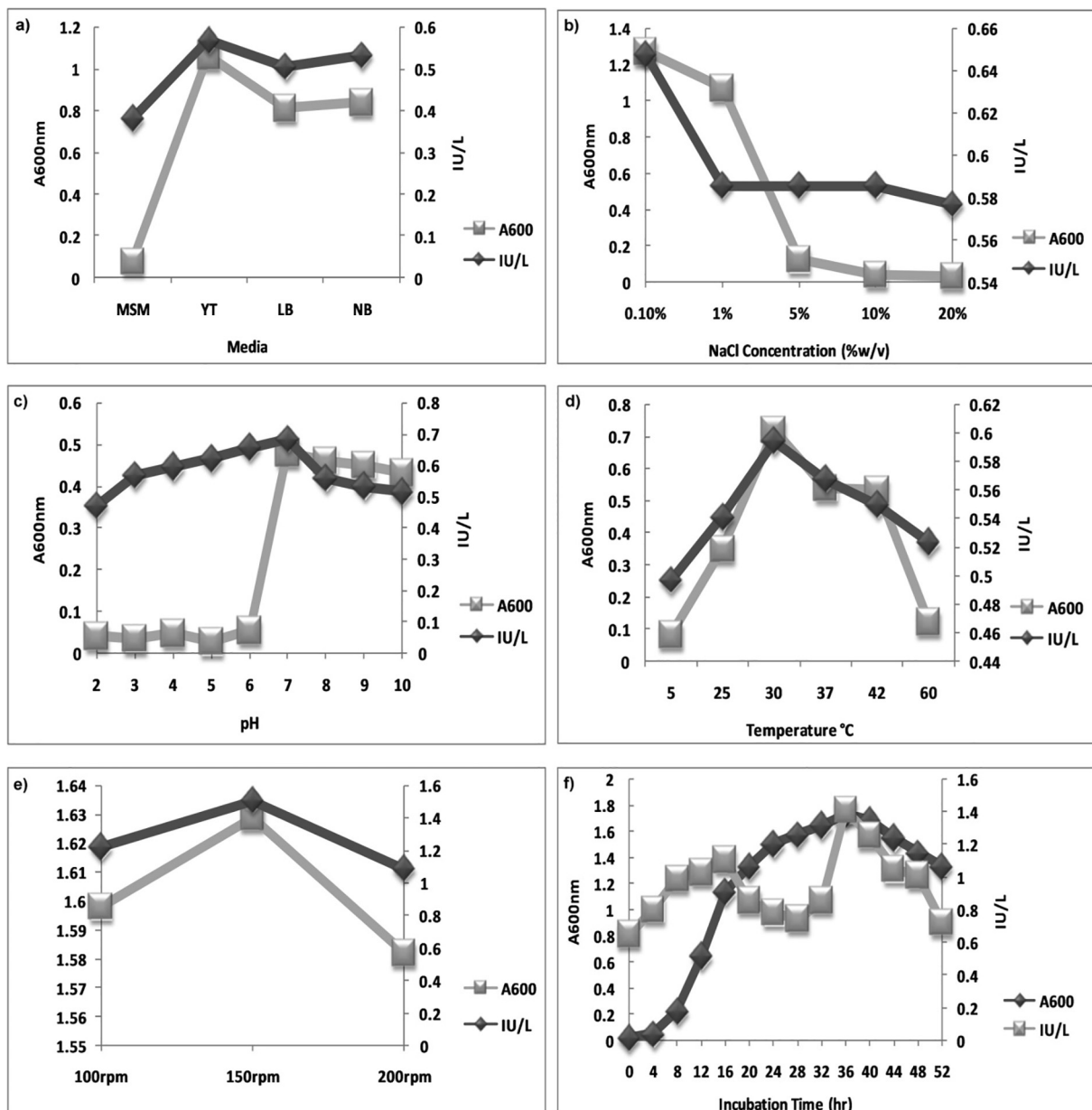

Fig. 3. Bacterial growth (A600nm) vs. enzyme production (IU/L) under different conditions: (a) Medium; (b) NaCl concentration; (c) pH; (d) Temperature; (e) Shaking and (f) Incubation period.

ammonium chloride i.e., an increase of 1.2 folds was observed with respect to control.

3.5.2. Salts

All the tested salts were found to supplement enzyme activity but ferrous sulphate decreased bacterial growth. Ferrous sulfate and calcium sulfate showed almost similar enzyme activity. Manganese sulfate containing medium showed maximum cellulase activity of 5.1 IU/L and demonstrated an increase of 3.6 folds as that of control.

3.5.3. Substrate concentration

Supplementation of CMC in the growth medium exhibited an improvement in both biomass and enzyme activity. 1% CMC showed enzyme activity of 3.8 IU/L. From above experiments glucose, ammonium chloride, manganese sulfate and CMC were found to increase enzyme activity as illustrated by Fig. 4.

An experiment for validation of these results was carried out that exhibited 31.5 IU/L enzyme activity that demonstrated an increase of 22.5 folds in enzyme activity as compared to control using one factor approach (1.4 IU/L). Therefore, these variables were selected for optimization using response surface methodology and to study their cumulative effect on enzyme production by the selected bacterial isolate.

3.6. Optimization of enzyme production by response surface methodology (RSM)

The cellulase production medium was further optimized by analyzing the interaction effects of glucose, ammonium chloride, manganese sulfate and concentration of the substrate CMC using CCD design. A total of 21 experiments were performed with four independent variablesat three levels (−1, 0 and +1). Based on the model summary, quadratic was selected as best fit as compared to other models as evident from Table 2.

Based on the model summary provided in Table 2, quadratic model in comparison to other models was suggested as the best fit. Fitness of model and its adequacy was studied by ANOVA as summarized in Table 3. The higher F-value of 262.47 for the model makes it significant which implies the maximum variation in response and can be explained by the model equation. In this case, A, C, D, AC, A^2 and C^2 are significant model terms. The lack of fit is insignificant indicating that the model was a good fit. Moreover, the goodness of fit was further assured by Fit statistics i.e., determination coefficient (R^2), predicted R^2 and adjusted R^2. In this case, R^2 was found to be 0.9984 explaining that 99.84% of experimental data was well-matched. High values of predicted R^2 (0.8594) and adjusted R^2 (0.9946) i.e., the difference of less than 0.2 exhibited high correlation between predicted and experimental values. The adequate precision value was found to be 50.12 indicating an adequate signal i.e., a satisfactory signal to noise ratio. Therefore, the predicted model can be used to navigate the design space. Different criteria three-dimensional response surface curves were plotted for enzyme activity responses obtained in CCD design to study interaction between four selected variables and to determine the optimum concentration of each for maximum enzyme activity and are given as Fig. 5. The quadratic effect of glucose, manganese sulfate and substrate CMC was significantly contributing to cellulase production in *Aneurinibacillus aneurinilyticus* strain BKT-9 (P < 0.0001). Based
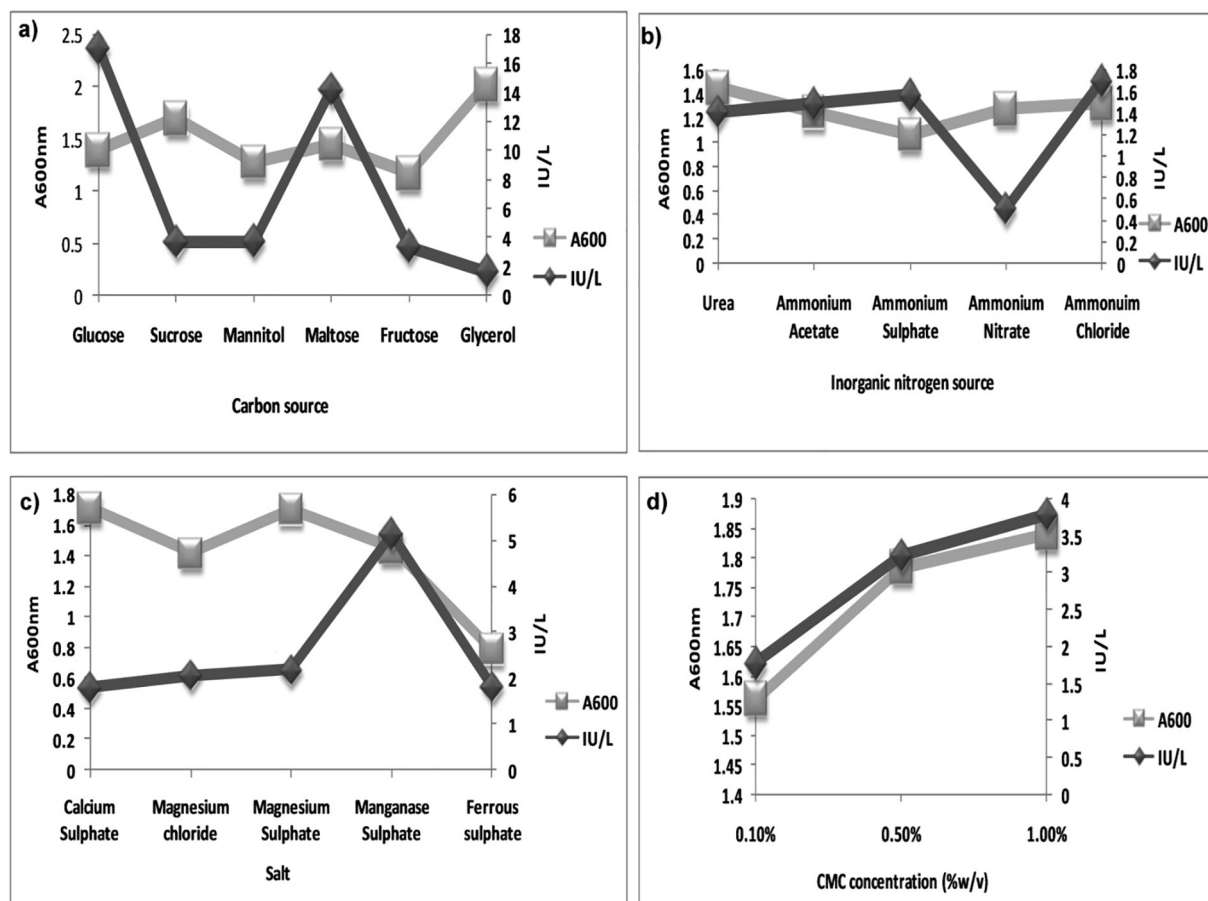

Fig. 4. Optimization of enzyme production by one factor approach: Bacterial growth (A600nm) *vs.* enzyme production (IU/L) under different (a) Carbon sources; (b) Inorganic nitrogen sources; (c) Salt sources; and (d) CMC concentration.

Table 2
Sequential Model Sum of Squares.

Source	Sum of Squares	Df	Mean Square	F-value	p-Value	
Mean *vs* Total	76392.91	1	76392.91			
Linear *vs* Mean	2423.06	4	605.76	7.85	0.0011	
2FI *vs* Linear	209.51	6	34.92	0.3404	0.9001	
Quadratic *vs* 2FI	1019.94	4	254.99	256.53	<0.0001	Suggested
Cubic *vs* Quadratic	4.54	2	2.27	6.35	0.0574	Aliased
Residual	1.43	4	0.3571			
Total	80051.39	21	3811.97			

Table 3
ANOVA analysis of CCD response variables.

Source	Sum of Squares	Coefficient Estimate	Df	Mean Square	Standard Error	95% CI Low	95% CI High	VIF	F-value	p-Value
Model	3652.51		14	260.89					262.47	*<0.0001*
Intercept		69.36			0.42	68.31	70.40			
A-carbon Source	559.06	9.94	1	559.06	0.42	8.92	10.97	2.41	562.44	*<0.0001*
B-Inorganic Nitrogen	0.5705	−0.32	1	0.5705	0.41	−1.34	0.70	2.41	0.57	*0.4774*
C-Salt	444.45	5.70	1	444.45	0.27	5.04	6.36	1.0000	447.14	*<0.0001*
D-Substrate	140.56	−4.98	1	140.56	0.41	−6.01	−3.96	2.41	141.41	*<0.0001*
AB	2.26	−0.82	1	2.26	0.54	−2.17	0.51	2.41	2.28	*0.1820*
AC	173.30	−4.65	1	173.30	0.35	−5.52	−3.79	1.0000	174.35	*<0.0001*
AD	15.30	−2.15	1	15.30	0.54	−3.49	−0.80	2.41	15.39	*0.0078*
BC	4.20	−0.72	1	4.20	0.35	−1.59	0.13	1.0000	4.23	*0.0855*
BD	13.50	−2.02	1	13.50	0.54	−3.36	−0.68	2.41	13.59	*0.0103*
CD	0.9431	0.34	1	0.9431	0.35	−0.52	1.21	1.0000	0.94	*0.3676*
A^2	544.33	−6.04	1	544.33	0.25	−6.67	−5.40	1.01	547.62	*<0.0001*
B^2	0.6286	−0.20	1	0.63	0.25	−0.83	0.42	1.01	0.63	*0.4568*
C^2	520.27	−5.90	1	520.27	0.25	−6.53	−5.27	1.01	523.42	*<0.0001*
D^2	46.62	−1.77	1	46.62	0.2579	−2.40	−1.14	1.01	46.91	*0.0005*
Residual	5.96		6	0.9940						
Lack of Fit	4.54		2	2.27					6.35	*0.0574*
Pure Error	1.43		4	0.3571						
Cor Total	3658.48		20							

on the response, a quadratic, non-linear polynomial equation in terms of actual factors was developed:

$$\text{Enzyme Activity} = -76.77767 + 11.84114A + 27.44603B + 230.18490C + 20.93737D - 0.612208AB - 5.17141AC - 0.955006AD - 12.08075BC - 13.45795BD + 3.43348CD - 0.298038A^2 - 2.27879B^2 - 147.50966C^2 - 7.06530D^2$$

where A = Glucose, B = Ammonium chloride, C = magnesium sulfate and D = CMC.

3.7. Experimental validation of the statistical model for cellulase production

Verification of the predicted model was performed by using selected solution for optimization having concentration of these independent variables as: glucose (11.1 g/L), ammonium chloride (0.77 g/L), manganese sulfate (0.49% *w/v*) and CMC (0.5% *w/v*). The maximum cellulase activity was found to be 75.99 IU/L, which authenticated cellulase activity of 76.370 IU/L as predicted by RSM. The enzyme production was scaled up to 1Litre flask level containing 400 mL of the RSM optimized media and the enzyme activity obtained was 83.1092 IU/L. Comparative account of cellulase production has been shown in Fig. 6.

4. Discussion

Microorganisms from different environments produce several extracellular enzymes for bioconversion of lignocellulosic materials to simple sugars (Leo et al., 2019). Many cellulose degrading microbes have been isolated from diverse environments like ani-mal waste, gut, soil and water sources (Leo et al., 2019; Premalatha et al., 2015) and have been considered eco-friendly alternative to hazardous chemical processing of lignocellulosic materials but still are insufficient to meet industrial demands (Premalatha et al., 2015). Moreover, bacteria are considered over fungi due to their more tolerance to harsh industrial conditions (Mathews et al., 2015). Therefore, incessant efforts are being made to explore different environments for the isolation of bacterial strains with potential cellulolytic activities and the current study was attempted for same intentions. In this study a Dal Lake bacterial isolate BKT-9 was selected as a potent and safe strain for cellulase production on the basis of biosafety assessment and antibiotic susceptibility tests. Biosafety assessment helps to rule out presence of virulence phenotype or antibiotic resistance which is generally not acceptable among industrial candidate strains It is reported that infections caused by spread of antibiotic resistant bacteria causes up to two-fold higher rates of adverse results in contrast to similar infections caused by susceptible strains. These adverse results may be clinical or economic and include disease severity, strain virulence or host vulnerability increases (Friedman et al., 2016). The isolate BKT-9 was found Gram +ve under microscopic examination and identified as *Aneurinibacillus aneurinilyticus* on the basis of 16S rRNA gene sequence homology. The isolate didn't show any hydrolytic zone on blood agar, gelatinase or DNase tests, hence considered safe. Also, it was found susceptible to all the tested antibiotics (Gupta and Malik, 2007). Further, there is least possibility of virulence factors in this environmental isolate as no *Aneurinibacillus* sp. has been isolated in alliance with animal or plant infections (Sanders et al., 2010; Shida et al., 1996).

The isolate BKT-9 was observed to show maximum growth and cellulase activity in YT medium at 30 °C for 36 h incubation under
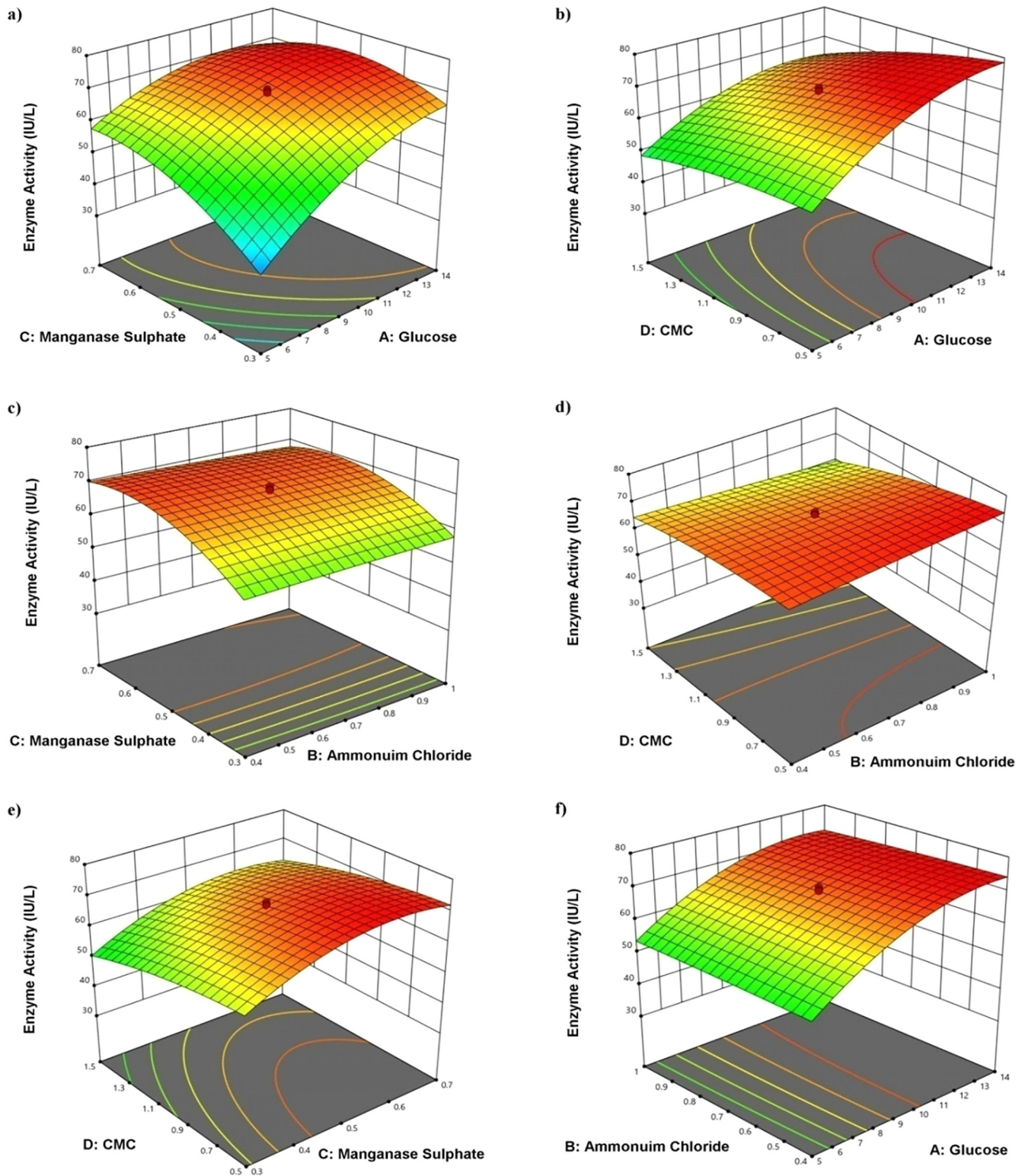
Fig. 5. Three dimensional response surface plots showing effects of variables and its interaction on cellulase activity: (A) showing the effect magnesium sulphate *vs* glucose; (B) CMC *vs* glucose; (C) magnesium sulphate *vs* ammonium chloride; (D) CMC *vs* ammonium chloride; (E) CMC *vs* magnesium sulphate and (F) ammonium chloride *vs* glucose on cellulase yield.

aerobic conditions at 150 rpm. Both growth and enzyme activity was found to enhance on supplementation of 0.1% NaCl. Therefore, these factors with YT medium were used as fixed parameters for cellulase production. *Aneurinibacillus aneurinilyticus* isolate BKT-9 shows optimal growth at pH (7–10), temperature (20–60 °C) and NaCl (0.1–10%) for growth. Earlier also, it has been reported that

yeast extract enhances growth and cellulase production in *Bacillus* sp. (Shajahan et al., 2017; Shida et al., 1996). Asem and coworkers has reported different incubation time for different strains of *Aneurinibacillus* for example, *A. aneurinilyticus* DBT87 and *Aneurinibacillus sp.* DBT14 require 120 h and 48 h incubation time respectively for maximum cellulase (FPase ~ 0.5 U/mL) activity. An
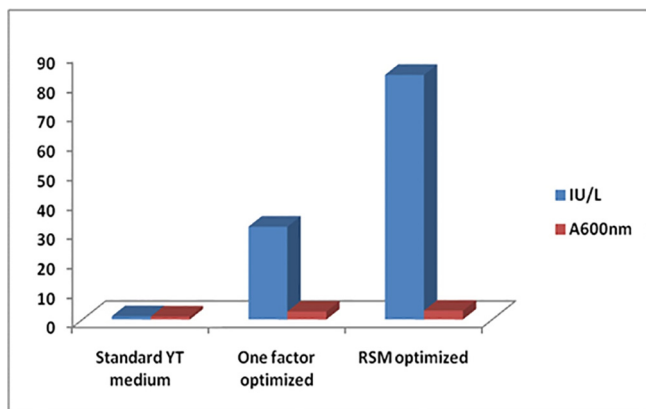
Fig. 6. Comparative account of cellulase production in *Aneurinibacillus aneurinilyti-cus* strain BKT-9 on unoptimized (standard YT medium), one factor optimized and RSM optimized medium.

optimum pH 7 for optimized growth as well as enzyme activity was reported in previous studies also (Asem et al., 2017; Raj et al., 2006). The growth characteristics revealed that the lag phase was observed up to 4 h only, logarithmic growth was observed from 8 h to 36 h of incubation followed by a decline in growth after 40 hr. Optimum growth as well as cellulase activity was observed at 36 h incubation i.e. during late log phase. The cellulolytic activity of any bacterial isolate depends on the sources and amount of cel-lulosic materials in its environment i.e., cellulase is an inducible enzyme (Islam and Roy, 2018). In this study, optimization of pro-duction medium supplements was carried out for carbon, inor-ganic nitrogen and salt sources and substrate concentration (CMC) using one factor analysis approach. Among carbon sources, glucose exhibited highest production as compared to other carbon sources used in the study. These results were in accordance with the previous studies that report glucose as best carbon source for cellulase production (Raj et al., 2006; Sethi et al., 2013). Among five inorganic nitrogen sources used in the study four were found to enhance enzyme activity in order of ammonium chloride, ammo-nium sulphate, ammonium acetate and urea. Several inorganic nitrogen sources including ammonium chloride, ammonium sul-phate and ammonium hydrogen carbonate enhance cellulase activ-ity (Sethi et al., 2013; Sharma et al., 2015; Tabssum et al., 2018). Stimulation of cellulase activity by ammonium sulphate as nitro-gen source has been reported earlier and attributed to its possible utilization in protein synthesis (Sethi et al., 2013). All the salt sources tested in this study had positive effect on enzyme activity; however, $FeSO_4$ had a negative effect on the bacterial growth. The highest activity was found with $MnSO_4$. It may be due to the acti-vation, stability, stimulation or inhibition of cellulase production by metal salts and possible utilization of sulfate in the protein syn-thesis (Pereira et al., 2017; Sethi et al., 2013; Usharani, 2010). CMC improved both growth as well as cellulase production which are in accordance with earlier reports highlighting that CMC generally induces cellulase production and acts as preferred substrate for endoglucanase production (Lucas et al., 2001; Thakkar and Saraf, 2014). The one factor analysis enhanced enzyme production by about 22 folds with respect to control.

RSM was used to optimize composition of cellulase production medium and the components were selected by one factor one fac-tor analysis. CMC has been reported as a positive inducer of cellu-lase production (Deka et al., 2011; Singh et al., 2013). A set of experiments consisting of 16 non-centre points and 5 centre points were carried out, selecting glucose, ammonium chloride, man-ganese sulfate and CMC as independent variables to improve cellu-lase production. The maximum production under optimized conditions was found about 52 times higher than that before opti-

mization i.e. with respect to control. The 3D plots evidently illus-trate the effect of these independent variables on production improvement. Glucose was found to be the most important con-tributor in enhancing cellulase activity followed by manganese sul-fate and CMC, whereas ammonium chloride exhibited least contribution. In presence of glucose, concentration of CMC > 0.5% *w/v* had a negative effect on enzyme activity whereas manganese sulfate at 0.5% *w/v* concentration enhances cellulase activity. This demonstrates the significance of nutrients in medium and these results harmonize with earlier reports on different bacterial strains (Premalatha et al., 2015). For validation, the experiment with opti-mized concentrations of these four independent medium variables provided by post RSM evaluation were carried out in addition to the four parallel experiments from actual design with maximum cellulase activity (>70 IU/L) and the results were compared. The predicted and actual enzyme activity was found to be in close proximity, therefore proved the validity of model. In this manner, RSM built up a methodology for obtaining optimum concentrations of medium parameters to get maximum cellulase production (83.1 IU/L) that was approximately 60 folds higher than that of the unoptimized (1.4 IU/L) YT medium.

Cellulases offer prospective of converting waste cellulosic mate-rial into foods to meet rapidly increasing population and have been the subject of intense research. Microbes provide an eco-friendly solution for the treatment and management of industrial wastes to battle the environmental threats as a key requirement for sus-tainable development (Bennet et al., 2002; Karigar and Rao 2011; Saxena et al., 2020). In this regard, further studies are required for scale-up of cellulase at an industrial level and utilization in var-ious industrial processes as microbial cellulases have shown their potential application in various industries including agriculture, biofuel production, brewing, food and feed industry, laundry, pulp and paper and textile (Kuhad et al., 2011). Microbial cellulase pro-duction is inducible, different cellulosic substrates could be tested in the production medium either in the form of purified cellulose (CMC, filterpaper, avicel, etc.) or as lignocellulosic biomass (agro-wastes) for further activity enhancement. Due to presence of both cellulose and hemicelluloses in lignocellulosic biomass, its utiliza-tion as substrate in cellulase production is reported to enhance production more efficiently than the pure cellulose as substrate. Moreover, the effect of different pretreatment methods (physical, chemical and biological) can be tested to improve microbial cellu-lase production (Sharma et al., 2019). To enhance the microbial efficiency for cellulase production various techniques (mutations, recombination, consortium studies, nanotechnology and metage-nomics) can be substituted for strain improvement and enhance-ment of cellulase competence (Leo et al., 2019) in *A. aneurinilyticus* BKT-9. Moreover, this Himalayan ecosystem may be further exploited for industrially beneficial microorganisms as currently no study is available on its microbial potential of biotech-nological use.

5. Conclusion and future prospects

In the present study, one factor based production optimization and RSM based statistical optimization have resulted in approxi-mately 60 folds higher cellulase activity in the *Aneurinibacillus aneurinilyticus* strain BKT-9. However, further studies are required to scale-up cellulase production for utilization of the strain at an industrial level. Future perspectives of the study include molecular characterization, purification and kinetic studies on free and immobilized enzymes. As microbial cellulase production is induci-ble, different cellulosic substrates could be tested in the production medium either in the form of purified cellulose or as lignocellulosic biomass (agrowastes) for further activity enhancement.

Acknowledgements

Declaration of Competing Interest

The authors have confirmed that there is no conflict of interest.

References

Allardyce, B.J., Linton, S.M., Saborowski, R., 2010. The last piece in the cellulase puzzle: the characterisation of beta-glucosidase from the herbivorous gecarcinid land crab Gecarcoidea natalis. J. Exp. Biol. 213 (Pt 17), 2950–2957.

Asem, D., Leo, V.V., Passari, A.K., Tonsing, M.V., Joshi, J.B., Uthandi, S., Hashem, A., Abd Allah, E.F., Singh, B.P., 2017. Evaluation of gastrointestinal bacterial population for the production of holocellulose enzymes for biomass deconstruction. PLOS ONE 12 (10).

Bennet, J.W., Wunch, K.G., Faison, B.D., 2002. Use of Fungi Biodegradation. ASM Press, Washington, DC.

Berg, B., Hofsten, B.V., Pettersson, G., 1972. Growth and cellulase formation by Cellvibrio fulvus. J. Appl. Bacteriol. 35 (2), 201–214.

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18 (5), 355–383.

Deka, D., Bhargavi, P., Sharma, A., Goyal, D., Jawed, M., Goyal, A., 2011. Enhancement of cellulase activity from a new strain of bacillus subtilis by medium optimization and analysis with various cellulosic substrates. Enzyme Res. 2011, 1–8.

Ding, S.-Y., Xu, Q., Crowley, M., Zeng, Y., Nimlos, M., Lamed, R., Bayer, E.A., Himmel, M.E., 2008. A biophysical perspective on the cellulosome: new opportunities for biomass conversion. Curr. Opin. Biotechnol. 19 (3), 218–227.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4), 783.

Friedman, N.D., Temkin, E., Carmeli, Y., 2016. The negative impact of antibiotic resistance. Clin. Microbiol. Infect. 22 (5), 416–422.

Gunst, R.F., Myers, R.H., Montgomery, D.C., 1996. Response surface methodology: process and product optimization using designed experiments. Technometrics 38 (3), 285.

Gupta, H., Malik, R.K., 2007. Incidence of virulence in bacteriocin-producing enterococcal isolates. Le Lait 87 (6), 587–601.

Irfan, M., Mushtaq, Q., Tabssum, F., Shakir, H.A., Qazi, J.I., 2017. Carboxymethyl cellulase production optimization from newly isolated thermophilic Bacillus subtilis K-18 for saccharification using response surface methodology. AMB Express 7 (1).

Islam, F., Roy, N., 2018. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC Res. Notes 11 (1).

Jarvis, M., 2003. Cellulose stacks up. Nature 426 (6967), 611–612.

Karigar, C.S., Rao, S.S., 2011. Role of microbial enzymes in the bioremediation of pollutants: a review. Enzyme Res.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr. Microbiol. 57 (5), 503–507.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16 (2), 111–120.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547–1549.

Kuhad, R.C., Gupta, R., Singh, A., 2011. Microbial cellulases and their industrial applications. Enzyme Res.

Lee, Y.-J., Kim, B.-K., Lee, B.-H., Jo, K.-I., Lee, N.-K., Chung, C.-H., Lee, Y.-C., Lee, J.-W., 2008. Purification and characterization of cellulase produced by Bacillus amyloliquefaciens DL-3 utilizing rice hull. Bioresour. Technol. 99 (2), 378–386.

Leo, V.V., Zothanpuia, Lalthafala, Ramesh, N., Singh, B.P., 2019. Microorganisms as an efficient tool for cellulase production: availability, diversity, and efficiency. In: New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, pp. 45–61.

Liang, Y.-L., Zhang, Z., Wu, M., Wu, Y., Feng, J.-X., 2014. Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of china and optimization of cellulase production by Paenibacillus terrae ME27-1. Biomed. Res. Int. 2014, 1–13.

Lucas, R., Robles, A., García, M.T., Alvarez de Cienfuegos, G., Gálvez, A., 2001. Production, purification, and properties of an endoglucanase produced by the hyphomycetechalara (synthielaviopsis) paradoxa CH32. J. Agric. Food. Chem. 49 (1), 79–85.

Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nyström, Å., Pettersen, J., Bergman, R., 1998. Experimental design and optimization. Chemometr. Intell. Lab. Syst. 42 (1–2), 3–40.

Mathews, S.L., Pawlak, J., Grunden, A.M., 2015. Bacterial biodegradation and bioconversion of industrial lignocellulosic streams. Appl. Microbiol. Biotechnol. 99 (7), 2939–2954.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31 (3), 426–428.

Parajo, J.C., Alonso, J.L., Lage, M.A., Vazquez, D., 1992. Empirical modeling of eucalyptus wood processing. Bioprocess. Eng. 8 (3–4), 129–136.

Pereira, J.d.C., Giese, E.C., Moretti, M.M.d.S., Gomes, A.C.d.S., Perrone, O.M., Boscolo, M., da Silva, R., Gomes, E., Martins, D.A.B., 2017. Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. In: Enzyme Inhibitors and Activators. InTech.

Premalatha, N., Gopal, N.O., Jose, P.A., Anandham, R., Kwon, S.-W., 2015. Optimization of cellulase production by Enhydrobacter sp. ACCA2 and its application in biomass saccharification. Front. Microbiol. 6.

Raj, A., Chandra, R., Reddy, M.M.K., Purohit, H.J., Kapley, A., 2006. Biodegradation of kraft lignin by a newly isolated bacterial strain, Aneurinibacillus aneurinilyticus from the sludge of a pulp paper mill. World J. Microbiol. Biotechnol. 23 (6), 793–799.

Ratnakomala, S., Fahrurrozi, Yopi, 2019. Enhancement of Cellulase (CMCase) production from marine actinomycetes Streptomyces sp. Bse 7–9: Optimization of fermentation medium by Response Surface Methodology. IOP Conf. Ser.: Earth Environ. Sci. 251, 012005.

Saha, S., Roy, R.N., Sen, S.K., Ray, A.K., 2006. Characterization of cellulase-producing bacteria from the digestive tract of tilapia, Oreochromis mossambica (Peters) and grass carp, Ctenopharyngodon idella (Valenciennes). Aquac. Res. 37 (4), 380–388.

Sanders, M.E., Akkermans, L.M.A., Haller, D., Hammerman, C., Heimbach, J.T., Hörmannsperger, G., Huys, G., 2010. Safety assessment of probiotics for human use. Gut Microbes 1 (3), 164–185.

Saravanan, P., Muthuvelayudham, R., Viruthagiri, T., 2013. Enhanced production of cellulase from pineapple waste by response surface methodology. J. Eng. 2013, 1–8.

Saxena, G., Kishor, R., Bharagava, R.N., 2020. Application of microbial enzymes in degradation and detoxification of organic and inorganic pollutants. In: Bioremediation of Industrial Waste for Environmental Safety. Springer, Singapore, pp. 41–51.

Sethi, S., Datta, A., Gupta, B.L., Gupta, S., 2013. Optimization of cellulase production from bacteria isolated from soil. ISRN Biotechnol. 2013, 1–7.

Shajahan, S., Moorthy, I.G., Sivakumar, N., Selvakumar, G., 2017. Statistical modeling and optimization of cellulase production by Bacillus licheniformis NCIM 5556 isolated from the hot spring, Maharashtra, India. J. King Saud Univ. – Sci. 29 (3), 302–310.

Sharma, A., Choudhary, J., Singh, S., Singh, B., Kuhad, R.C., Kumar, A., Nain, L., 2019. Cellulose as potential feedstock for cellulase enzyme production: versatility and properties of various cellulosic biomasses. In: New and Future Developments in Microbial Biotechnology and Bioengineering, Elsevier, pp. 11–27.

Sharma, A., Tewari, R., Soni, S.K., 2015. Application of statistical approach for optimizing CMCase production by Bacillus tequilensis S28 strain via submerged fermentation using wheat bran as carbon source. Int. J. Biol. Biomol. Agric. Food Biotechnol. Eng. 9 (1), 76–86.

Shida, O., Takagi, H., Kadowaki, K., Komagata, K., 1996. Proposal for Two New Genera, Brevibacillus gen. nov. and Aneurinibacillus gen. nov. Int. J. Syst. Bacteriol. 46 (4), 939–946.

Singh, S., Moholkar, V.S., Goyal, A., 2013. Optimization of carboxymethylcellulase production from Bacillus amyloliquefaciens SS35.3. Biotech 4 (4), 411–424.

Sreena, C.P., Sebastian, D., 2018. Augmented cellulase production by Bacillus subtilis strain MU S1 using different statistical experimental designs. J. Genet. Eng. Biotechnol. 16 (1), 9–16.

Srinubabu, G., Raju, C.A.I., Sarath, N., Kumar, P.K., Rao, J.V.L.N.S., 2007. Development and validation of a HPLC method for the determination of voriconazole in pharmaceutical formulation using an experimental design. Talanta 71 (3), 1424–1429.

Tabssum, F., Irfan, M., Shakir, H.A., Qazi, J.I., 2018. RSM based optimization of nutritional conditions for cellulase mediated Saccharification by Bacillus cereus. J. Biol. Eng. 12 (1).

Thakkar, A., Saraf, M., 2014. Application of statistically based experimental designs to optimize cellulase production and identification of gene. Natural Prod. Bioprospect. 4 (6), 341–351.

Tomme, P., Warren, R.A.J., Gilkes, N.R., 1995. Cellulose hydrolysis by bacteria and fungi. In: Advances in Microbial Physiology Volume 37, Elsevier, pp. 1–81.

Usharani, B.M., 2010. Production and characterization of protease enzyme from Bacillus laterosporus. Afr. J. Microbiol. Res. 4 (11), 1057–1063.

Yang, W., Meng, F., Peng, J., Han, P., Fang, F., Ma, L., Cao, B., 2014. Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. Electron. J. Biotechnol. 17 (6), 262–267.

Zhang, Y.-H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng. 88 (7), 797–824.