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OPTIMIZATION OF ENDOGLUCANASE PRODUCTION FROM THERMOPHILIC STRAIN OF *BACILLUS LICHENIFORMIS* RT-17 AND ITS APPLICATION FOR SACCHARIFICATION OF SUGARCANE BAGASSE

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Abstract

Thermostable cellulases are required for a variety of commercial processes. *Bacillus* is a house of thermostable proteins. Screening of indigenously isolated strains of bacteria revealed the promising production of cellulase by a strain, RT-17, at 50ºC. The strain was identified on the basis of biochemical and molecular characteristics as *B. licheniformis.* The factors affecting cellulase production from *B. licheniformis* RT-17 were evaluated for their significant effect using Plackett Burman Design and were optimized by employing Box-Behnken Design. The model predicted 9.808 IU/ml of endoglucanase (EG) under optimum conditions of 50°C; 10% inoculum size; pH 5; and 1% peptone in fermentation medium. Practically, a titer of 9.128 IU/ml was obtained, showed the validity of the model. The enzyme preparation from *B. licheniformis* RT-17 was applied in combination with xylanase and pectinase preparations from indigenous yeasts for the hydrolysis of sugarcane bagasse (SCB). A higher degree of synergy (7.1 folds) was observed when yeast pectinase was used with bacterial cellulase for the hydrolysis of alkali treated SCB. Whereas, the degree of synergy was lower when bacterial cellulase was mixed with yeast xylanase. The study revealed the possibility of utilization of combination of yeast and bacterial enzymes for biomass saccharification.

Key words: Endoglucanase, *B. licheniformis*, Box Behnken Design, Synergistic effect, Sugarcane-bagasse.

Introduction

Endoglucanase (EG; 3.2.1.4) is an essential enzyme to degrade the most abundant biopolymer, cellulose. It cleaves β-1-4 linkages present in cellulose and releases oligosaccharides and provides new ends for further enzymatic activity. It is more active on amorphous cellulose compared to crystalline cellulose. Mostly, its activity is manifested by its action on Carboxymethyl cellulose (CMC), and therefore, it is also referred as CMCase (Sadhu *et al.,* 2013).

EG from 13 different glycosyl hydrolase (GH) families have been reported from many prokaryotic and eukaryotic microorganisms (Yennamalli *et al.,* 2013). In some of the organisms such as in *Trichoderma reesei*, as many as five isozymes of EG have been reported (Lynd *et al.,* 2002) that act synergistically to degrade cellulosic biomass efficiently. EGs from different organism exhibit diversified structural and biochemical features. Mostly fungal EG share acidic pI and perform optimally under acidic conditions (Yoon *et al.,* 2007), whereas, many bacterial EGs require alkaline pH for their optimum activities (Ogawa *et al.,* 2007). Additionally, several reports describe heat stable EGs from bacteria, particularly from *Thermomonospora* and *Bacillus.* Thermostable EGs have potential applications in biofuel industry as their use can reduce the cost of cooling process and higher rates of reactions can be achieved. Thermostability can be attributed to the property of protein to withstand and to perform its function at elevated temperature. Although the exact mechanism of thermostability remains unknown, however, it is believed that presence of certain amino acids, hydrophobicity and compactness render a protein thermostable. The rules governing thermostability in EGs have been discussed particularly (Yennamalli *et al.,* 2013).

Higher growth rate and production of diversified glycosyl hydrolases render *Bacillus* as an ideal organism for the production of EGs. Among various *Bacillus* species, *B. licheniformis* isolated from bovine rumen was found to be producer of cellulase having ability to degrade cellulose at high temperature under microaerophilic conditions (Fujimoto *et al.,* 2011). Likewise, halophilic, alkaliphilic and solvent-resistant cellulase have been reported from the strains of *Bacillus subtilis* (Asha & Sakthivel, 2014) and *B. vallismortis* (Gaur & Tiwari, 2015) isolated from agricultural soil. Balasubramanian & Simoes (2014) have described thermostable cellulase from *Bacillus pumilus* S124A that can degrade a variety of substrates including agricultural wastes. Sadhu *et al.* (2013) reported about the production of detergent stable EG from a strain of *Bacillus* that can hydrolyze a variety of lignocellulosic (LC) waste materials.

The advancement in the technology to convert LC mass to biofuel is suffered with the production cost of cellulase enzyme and lack of stability of the enzyme preparation as required to be compatible with thermal pretreatment processes (Yennamalli *et al.,* 2013). Sugarcane bagasse (SCB) is an abundantly present LC mass which is generated after extracting juice from sugarcane. Sugarcane is mainly cultivated in South American and Asian countries (Kim & Dale, 2004). The crop is the main source of sugar for the world. It is estimated that 15 million tons of SCB is generated in Pakistan (Arshad & Ahmed, 2016) which remains under-utilized. With 50-75% fermentable sugars and higher moisture content, SCB can be an excellent raw material for many fermentation industries. It can also be subjected to enzymatic hydrolysis to release glucose and xylooligosaccharides (Xue *et al.,* 2016) for subsequent conversion into biofuel, lactic acid, methane, hydrogen and other valuable products (Juhasz *et al.,* 2005). The heterogeneity of SCB limits its efficient saccharification,

therefore, enzyme cocktails comprised of fungal preparations have been studied (Arias *et al.,* 2016; da Silva *et al.,* 2016). The mixture of cellulase and hemicellulases not only exhibit additive effect, rather synergistic effects have also been observed. It is believed that the studies on synergistic effect of cellulase and xylanase can be used to design cellulosome for single step bioconversion of a variety of LC biomass (Bhattacharya *et al.,* 2015).

Although, it seems imperative that the cellulolytic enzymes acting on different sites can show synergistic behavior (such as exo-endo synergy), however, there is little understanding available about mechanism of synergistic action. The synergy between enzymes from two different organisms has also been reported; whereas, variable degrees of synergism by different enzymes are exhibited on different substrates (Kostylev & Wilson 2012). Therefore, it is important to study the effect of combination of different enzymes from different organisms on the saccharification of crude LC substrates. This particularly becomes crucial when most of the pretreatment methods fail to remove hemicellulose portion completely and hence the residual part may interfere with cellulolytic action (Bura *et al.,* 2009). Subsequently, a synergistic action can be observed when cellulase enzyme is mixed with hemicellulase preparation.

In this study, indigenously isolated bacterial strains were tested for their ability to produce cellulase at higher temperature and the production of one of the promising strain was optimized using statistical design. The cellulase preparation from RT-17 was used along with yeast enzyme preparation to hydrolyze sugarcane-bagasse. The utmost possible literature survey did not turn up any study where a mixed preparation of bacterial and yeast enzymes was utilized for the bioconversion of SCB.

Materials and Methods

Isolation of aerobic thermoduric cellulolytic bacteria: The bacterial strains were isolated from various soil samples which were collected from a site in a local garden where gardeners were used to burn grass and other plant material. The samples were transferred to mineral salt medium (MSM) described by Mandels & Weber (1969) supplemented with 0.5% Carboxymethyl cellulose (CMC) and kept in a shaking incubator at 50ºC and 150 rpm for 72 h. An aliquot (5 ml) from this flask was subcultured to another flask containing MSM with CMC (MSM-CMC) and incubated under the same conditions for 72 h. After another round of sub-culturing, the contents were serially diluted for the isolation using MSM-CMC agar plates at 50ºC. The isolated strains were maintained on Nutrient agar and MSM-CMC agar slants. Stock cultures were preserved in 50% glycerol.

Screening of the isolated strains for the production of cellulase and other enzymes: The isolates were cultivated at 37º and 50ºC in MSM-CMC agar to check the presence of growth and secretion of cellulase. The growth was monitored visually and cellulase activity was detected by adopting iodine staining method. The presence of halo around the colonies was indicative of cellulase activity. The strains showed cellulase activity at 50ºC were further studied for the ability to produce cellulase at temperature 60º and 70ºC. In yet another set of experiments, the cellulase activity was screened in cell-free culture supernatant (CFCS). The strains were cultivated in MSM-CMC broth at 37º and 50ºC and the CFCS was added to wells made in CMC-agar plates. The plates were kept at 37º and 50ºC for 2 h and halos around the wells were visualized by iodine method.

The cellulolytic isolates were also studied for their ability to produce other industrially important enzymes including xylanase, pectinase, amylase, protease and lipase as described by Sohail *et al.* (2009).

Cellulase assay: The culture was transferred to MSM-CMC by inoculating a single colony and incubated at 50ºC until an OD_{600} of 0.6 was obtained. The inoculum (0.5 ml) was transferred to a 5 ml of fresh MSM-CMC and incubated in a shaking incubator at 50ºC for 24 h at 150 rpm. The CFCS obtained after centrifugation was assayed for endoglucanase (EG), β-glucosidase (BGL) and Filter paperase (FPase) activities as described by Sohail *et al.* (2016).

Identification of the isolates: The isolates were identified on the basis of standard microbiological and biochemical protocols. The identification of the most promising strain was further confirmed by sequencing DNA *gyr* B gene after Huang *et al.* (2012). The genomic DNA was extracted and the gene was amplified using primers Blich-F1 (5'-AKACGGAAGTGACGGGAAC-3') and Blich-R1 (5'-AGAAACTTTTCRAGCGCTT-3'). The band correspond to 600 bp was purified and was sequenced. The sequence was subjected to BLAST using NCBI tool and the strain was identified. The sequence was submitted to GenBank with an accession number KY347821. Phylogenetic tree was constructed using MEGA 6 software.

Production of yeast enzymes: Yeast strains AA-15 and MK-162 were obtained from the culture collection of the Department of Microbiology, University of Karachi, and maintained on Yeast extract agar. The strain MK-162 was cultivated in xylan containing MSM for 2 days at 25ºC (Kumari *et al.,* 2017). Whereas, the strain AA-15 was grown on pectin containing MSM medium for 2 days at 30ºC. The cell-free culture supernatant (CFCS) was obtained after centrifugation and assayed for xylanase and pectinase activity as mentioned by Sohail *et al.* (2009).

Statistical optimization of cellulase production by the strain RT17: The strain RT-17 was cultivated in the MSM with the modifications (Table 1) in a given set of conditions. The conditions were varied to study their effect on EG production. Six factors were screened for their significance at two levels by Plackett-Burman design (PBD) using Minitab 17 software. Four factors were optimized by Box-Behnken Design at three levels (Table 2).

Table 1. List of factors studied for their effect on cellulase production by *B. licheniformis* RT-17 using Plackett Burman design.

Type of factors	Factors	Low (-1) level	High (+1) level
Chemical factors	pH	5	7
	Peptone concentration in media	0.5%	1%
	Substrate concentration in media	0.5%	1%
Physical factors	Temperature	50ºC	60ºC
	Incubation time	24 hrs.	48 hrs.
Biological factor	Inoculum size	5%	10%

Table 2. List of factors studied for their optimum effect on cellulase production by *B. licheniformis* RT-17 using Box Behnken design.

Type of factors	Factors	Low (-1) level	Middle (0) level	High (+) level
Physical factors	Temperature	50ºC	55ºC	60ºC
Biological factor	Inoculum size	5%	7.5%	10%
Chemical factor	pH	5	6	7
	Peptone concentration in media	0.5%	0.75%	1%

Alkali pretreatment of SCB: Sugarcane-bagasse (SCB) was obtained locally, dried and grind to 100 mesh size. It was pretreated with alkali. Powdered SCB was loaded in 1% (w/v) sodium hydroxide (NaOH) at the rate of 50 ml/g at room temperature for 24 h. The slurry was washed repeatedly with tap water to neutralize the pH, and then oven dried at 60ºC. After autoclaving, it was stored in screw capped bottles, until used.

Hydrolysis of commercially purified substrates and SCB: Hydrolysis of different substrates (CMC, pectin, xylan, untreated SCB and alkali treated SCB) by the yeast or bacterial enzymes (cellulases, xylanase and pectinase) was carried out in eight different sets. In five of these sets, the enzymes were used separately to hydrolyse all the substrates. Whereas, in the remaining three sets, cellulase produced by *Bacillus licheniformis* RT-17 was used in combination with the yeast pectinase (by the strain AA-15) and/ or with yeast xylanase (by the strain MK-162). The enzymes were loaded in accordance to their activities (IU/mL). For cellulases, the amount was adjusted to 10 IFPU (International Filter Paperase Unit) per gram of substrate, whereas, for pectinase and xylanase, the adjusted amount was 0.5 units/g and 5 units/g of substrate, respectively.

Saccharification of SCB was carried out in 100 ml flasks containing the required amount of enzyme with 0.25g substrate along with 0.2% (w/v) sodium-azide and the final volume of 25 ml was made up with sodium citrate buffer pH 4.8. The reaction mixture was incubated at 35ºC for 48 to 52 h. with shaking at 100 rpm. Aliquots were drawn at different time intervals and assayed for reducing sugars by DNS method (Miller, 1959).

The degree of synergy (DS) of xylanase of MK-162 and pectinase of AA-15 on cellulase of RT-17 was calculated after Song *et al.* (2016) by using the equation:

$$DS = Y_{1+2}/ (Y_1+Y_2)$$

where, Y_1 is the amount of reducing sugars released by the activity of bacterial cellulase and Y2 is the amount of reducing sugars released by the activity of yeast xylanase or pectinase. Y_{1+2} is the amount of reducing sugars released by the activity of combination of the two enzymes.

Results and Discussion

Aerobic cellulolytic fungi and bacteria offer various advantages to obtain cellulase on commercial scale, therefore, isolation and screening of cellulase producing microorganisms from different habitats is routinely carried out in search of getting enzymes with novel properties (Sharma *et al.*, 2016) such as thermostability. Thermostable cellulases are applied in a variety of setting, for instance, in the processes where lignocellulosic biomass is pretreated by a combination of thermal and chemical methods. Although the probability of getting thermophilic strains is higher when specialized habitats are explored, however, thermophilic or thermoduric strains can also be isolated from mesophilic habitats. In this study, twenty six isolates were obtained from different soil samples; of which 19 strains were found to be cellulolytic by plate screening method (Data not shown). The isolates were screened for the production of cellulase at temperatures 37, 50 and 60ºC by spot inoculating the cultures on MSM-CMC agar plates followed by iodine staining (Data not shown). All the isolates showed clearance of more than 10 mm at temperature 37 or 50ºC. The size of the zone decreased significantly at 60ºC. The extracellular cellulase production was investigated by cultivating the strains in MSM-CMC broth at 37º and 50ºC and the cell-free culture supernatant (CFCS) was screened for cellulase activity by agar-well diffusion method at different temperatures from 37-70ºC (Data not shown). The zone of clearance by the CFCS from 37ºC cultivation showed smaller zones compared to the CFCS from 50ºC cultivation in the plates screened at temperature 37-60ºC. Whereas, CFCS from some of the isolates retained their cellulolytic activity even at 70ºC.

The strain RT-17 elaborated more cellulase production at 50ºC than at 37ºC (Data not shown). Sadhu *et al.* (2013) reported about the stability of cellulase from a *Bacillus* sp. at 50ºC that decreased significantly at 60 and 70ºC. The inactivity of cellulase at higher temperature has also been reported for the preparations from psychrophiles. Jain & Krishnan (2017) reported that more (52%) bacterial isolates were found to be positive for extracellular enzymes at 4ºC than at 20ºC (41%).

All the bacterial strains were also screened for the ability to produce other industrially important enzymes. It was observed that proteolytic and lipolytic activities were commonly present as most of the isolates were found to be positive for these two activities (Data not shown). Whereas, 13 xylanolytic and 8 amylolytic strains were detected. Pectinase activity was not present in any of the strains. It is noteworthy that 13 of the strains were capable of producing three enzymes i.e. amylase, protease and lipase and can be used for fermentation processes in future studies. In an earlier study, xylanolytic activity was most dominant among actinomycetes isolated from Colorado soil, whereas, pectinase production was least observed depolymerizing activity (Yeager *et al.,* 2017). Contrary to this finding, Jain & Krishnan (2017) found the least number of isolates as xylanolytic or cellulolytic; It can also be attributed to the environment (a glacier) from which the isolation was carried out.

Based on the zones of clearance by CFCS and spot cultures, seven isolates were selected for quantification of extracellular cellulase activity. The strain RT-6 showed the highest EG activity while the highest BGL activity was exhibited by the strain RT-4 (Table 3). A balanced preparation was produced by the strain RT-17 i.e. >7 IU/ml of both, EG and BGL along with >2 IFPU.

Table 3. Production of endoglucanase (EG), β-glucosidase (BGL) and filter paperase (IFPU) activity by the strains of *B. licheniformis* in shake flask experiments.

Strains	Enzyme activity (IU/ml)		
	EG	BGL	IFPU
RT-3	7.44	5.22	2.10
RT-4	3.50	10.90	1.66
RT-5	3.03	6.56	1.20
RT-6	8.21	4.63	0.94
RT-16	6.90	3.40	1.43
RT-17	7.25	7.75	2.23
RT-18	5.52	3.67	1.44

The isolates were identified by following Bergey's Manual of Systematic Bacteriology and all the biochemical tests were found similar (Data not shown) to the members of *Bacillus licheniformis* group, except for catalase test. *B. licheniformis* is reported as catalase positive. Whereas the strains studied did not show any catalase activity. This might indicate a variant of *B. licheniformis* strains. *Bacillus* is known as a house of thermostable proteins and various workers have described thermophilic strains of this genus. Sadhu *et al.* (2013) isolated a thermostable cellulase producing *Bacillus* sp. from cow-dung. Annamalai *et al.* (2013) reported about the production of thermostable and alkali-stable cellulase from *Bacillus halodurans* Cas 1.

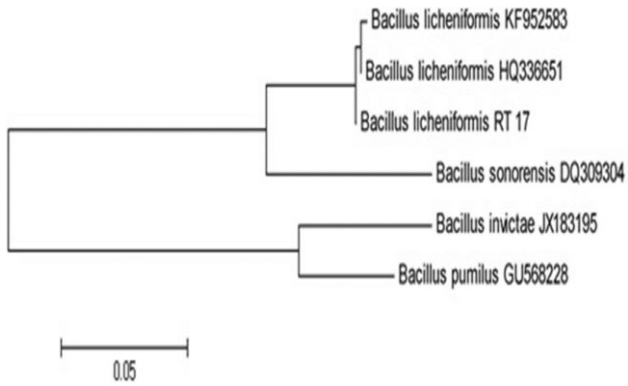

Fig. 1. Phylogenetic tree of *B. licheniformis* RT-17.

The most promising strain (RT 17) was further identified as *Bacillus licheniformis* on the basis of DNA gyrase B gene. The gene sequence was submitted to GenBank with an accession number KY347821. The phylogenetic analysis of the strain showed a close relatedness with the strains HQ336651 and KF952583 of *B. licheniformis* (Fig. 1).

The production of cellulase from *B. licheniformis* RT 17 was optimized using statistical design, initially by Plackett-Burman design (PBD) and then by Response Surface Methodology (RSM). Annamalai *et al.* (2014) adopted a similar approach of using PBD and RSM for the optimization of cellulase production from *B. carboniphilus* CAS 3.

Six different factors were evaluated in 12 runs for their significant effect on cellulase production by PBD. The Regression Equation in Uncoded Units was EG = -5.8 + 0.065 temperature + 0.317 pH + 0.87 substrate conc -0.0074 incubation time + 0.396 inoculum size + 2.42 peptone.

All the experiments were performed in triplicate and the average titers were taken as response. Analysis of the response after experiments as proposed by PBD by Normal plot (Fig. 2a) and Pareto chart (Fig. 2b) of the standardized effects clearly illustrated that none of the factors was significant for the EG production. The analysis was also carried out for the other two responses (BGL and FPase production) and showed that inoculum size and incubation period were affecting significantly the production of FPase activity. However, an increase in EG production was noted in some of the experimental runs of PBD. It was observed (Table 4) that when a 10% inoculum was added to MSM fortified with 0.5% CMC and 1% peptone with pH adjusted to 5.0 and cultivated at 60ºC for 24 h., it yielded the highest titers of EG (9.76 IU/ml) and IFPU (2.72 IU/ml). In a recent study, Yahya *et al.* (2016) utilized PBD for the optimization of amylase production from *A. tubingensis* SY 1 and did not get any significant factor affecting the process, but an increase in the enzyme production was reported. For robust processes, PBD may not indicate significant factors, nonetheless, an improvement in the production is observed (Mesko *et al.*, 1996). On the contrary, Shajahan *et al.* (2016) analyzed seven factors affecting cellulase production by *B. licheniformis* NCIM 5556 using a 12 run based PBD and observed significant positive effects of CMC, $CaCl_2$, temperature and Tween 20.

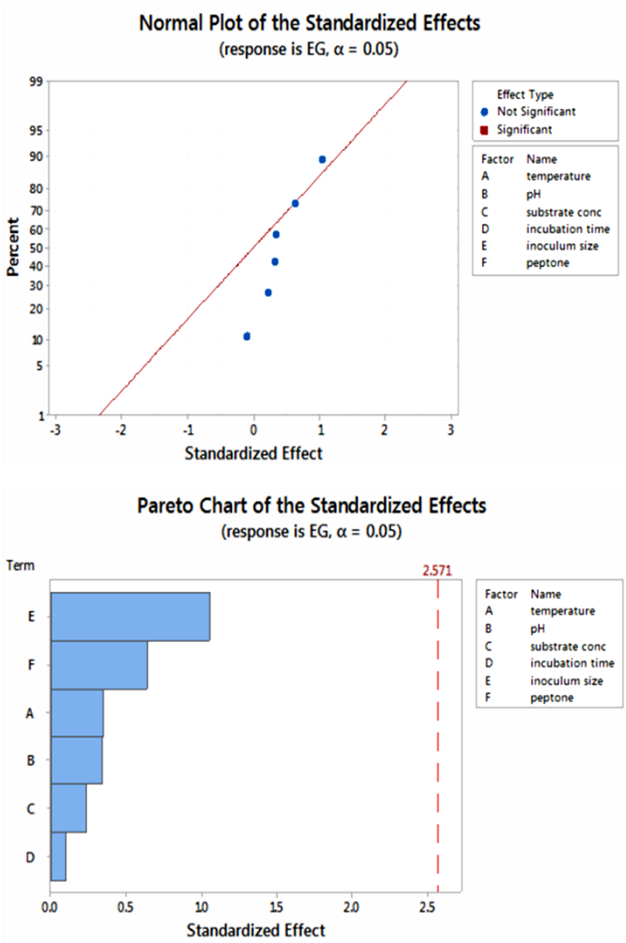

Fig. 2. Analysis of PBD (a) Normal effect of factors on EG production (b) Pareto Chart.

Based on careful sifting of responses obtained for experimental runs in PBD, four factors (including temperature, inoculum size, pH and media) were shortlisted to optimize further using a popular Response Surface Methodology (RSM) tool, namely Box Behnken Design (BBD). In BBD, along with high and low levels of factors (used for PBD), a mid-value was also introduced and these levels were coded as +1, 0, -1. The model proposed 25 experimental runs (Table 5) with a combination of different levels of each factor in search of optimum conditions for maximum enzyme yield and also for studying factor interaction and its effect on enzyme production.

Enzyme titers obtained by BBD experiments were analyzed by ANOVA. This analysis showed that the model generated for EG gave S-value of 0.195 and R^2 value of 95.06% (Table 6). This R^2 value indicated that the total variation of 95.06% in the model was attributed to the factors and only about 4.94% of the total variation could not be explained by the model. It indicates that the model generated for EG is significant. The model p-value was less than 0.05 with an F-value of 45.45 also supported it. Further analysis showed that the linear effect of temperature, inoculum size and peptone was significant while the effect of pH was not significant. The data showed that increase in inoculum size to 10% at a temperature 50°C can generate IU/ml as high as 9.5 (Fig. 3). The production at high temperature can be exploited at large scale to get heat stable enzymes with a minimum chance of contamination of production media.

The effect of interaction of variables on EG production was studied against two independent variables by keeping the other independent variable as constant. It was studied by taking the assistance of contour plots (Fig. 4). Interaction of temperature with pH and peptone was not found significant thus low titers of EG were obtained. Interaction of pH and inoculum size was found to be statistically significant when the inoculum size was kept 10%, thus, giving an EG activity of 9.5 IU/ml. The interactive effect of temperature and inoculum size was also found to be significant. Main effects indicate the increase in inoculum size resulted in the increased enzyme production. Increase in peptone concentration also had positive effect on EG production while the enzyme production was not affected by the increase in pH and temperature.

Table 4. Plackett Burman experimental design and responses in the form of endoglucanase (EG), β-glucosidase (BGL) and Filter paperase (IFPU) production.

Experimental factors							IU/ml		
Run Order	Temperature	pH	Substrate Concentration	Incubation time	Inoculum size	Peptone	EG	BGL	IFPU
1	60°C	5	1%	24 hrs.	55%	0.5%	4.44	2.22	1.85
2	60°C	7	0.5%	48 hrs.	5%	0.5%	5.27	5.52	2.11
3	50°C	7	1%	24hrs.	10%	0.5%	4.63	8.88	1.95
4	60°C	5	1%	48 hrs.	5%	1%	0.93	4.53	1.44
5	60°C	7	0.5%	48 hrs.	10%	0.5%	3.79	6.85	1.85
6	60°C	7	1%	24 hrs.	10%	1%	6.56	8.68	1.81
7	50°C	7	1%	48 hrs.	5%	1%	8.28	5.27	1.51
8	50°C	5	1%	48 hrs.	10%	0.5%	5.27	4.35	1.07
9	50°C	5	0.5%	48 hrs.	10%	1%	4.73	5.83	1.78
10	60°C	5	0.5%	24 hrs.	10%	1%	9.76	7.00	2.72
11	50°C	7	0.5%	24 hrs.	5%	1%	2.18	6.11	2.54
12	50°C	5	0.5%	24 hrs.	5%	0.5%	1.77	7.94	2.00

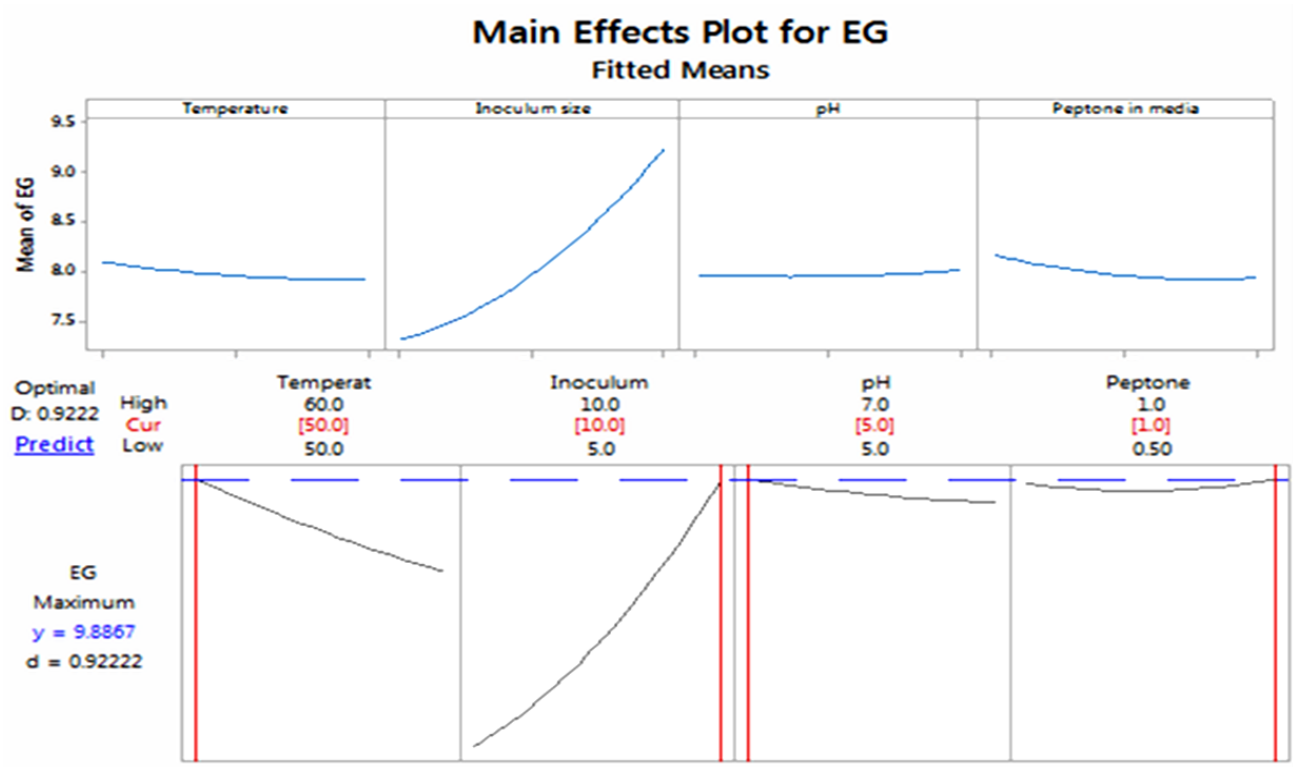

Fig. 3. Main effect of various factors on EG production by *B. licheniformis* RT-17.

Table 5. Box-Behnken experimental design for optimizing factors affecting endoglucanase (EG), β-glucosidase (BGL) and filter paperase (IFPU) production by *B. licheniformis* RT-17.

Run order	Experimental factors					Enzume activity (IU/ml)		
	Blocks	Temperature	Inoculum size	pH	Peptone in media	EG	BGL	IFPU
1	1	50°C	5%	6	0.75%	7.12	7.00	1.34
2	1	60°C	5%	6	0.75%	7.39	7.65	1.23
3	1	50°C	10%	6	0.75%	10.12	9.00	2.19
4	1	60°C	10%	6	0.75%	9.14	8.99	2.34
5	1	55°C	7.5%	5	0.5%	8.13	8.43	1.89
6	1	55°C	7.5%	7	0.5%	8.57	8.22	1.79
7	1	55°C	7.5%	5	1%	7.94	7.56	1.81
8	1	55°C	7.5%	7	1%	8.15	7.98	1.98
9	1	55°C	7.5%	6	0.5%	8.15	8.14	1.59
10	2	50°C	7.5%	6	0.5%	7.98	8.23	1.39
11	2	60°C	7.5%	6	0.5%	8.15	7.99	1.94
12	2	50°C	7.5%	6	1%	7.74	8.10	1.66
13	2	60°C	7.5%	6	1%	7.74	7.56	1.79
14	2	55°C	5%	5	0.75%	7.12	7.06	1.32
15	2	55°C	10%	5	0.75%	9.15	9.13	2.34
16	2	55°C	5%	7	0.75%	7.19	7.32	1.34
17	2	55°C	10%	7	0.75%	9.00	8.32	2.13
18	3	50°C	7.5%	5	0.75%	8.32	7.43	2.72
19	3	60°C	7.5%	5	0.75%	8.01	7.84	2.34
20	3	50°C	7.5%	7	0.75%	8.12	7.65	2.65
21	3	60°C	7.5%	7	0.75%	7.92	7.67	1.71
22	3	55°C	5%	6	0.5%	7.72	6.03	1.24
23	3	55°C	10%	6	0.5%	9.16	8.94	2.32
24	3	55°C	5%	6	1%	7.62	7.87	1.23
25	3	55°C	10%	6	1%	9.18	9.13	2.16

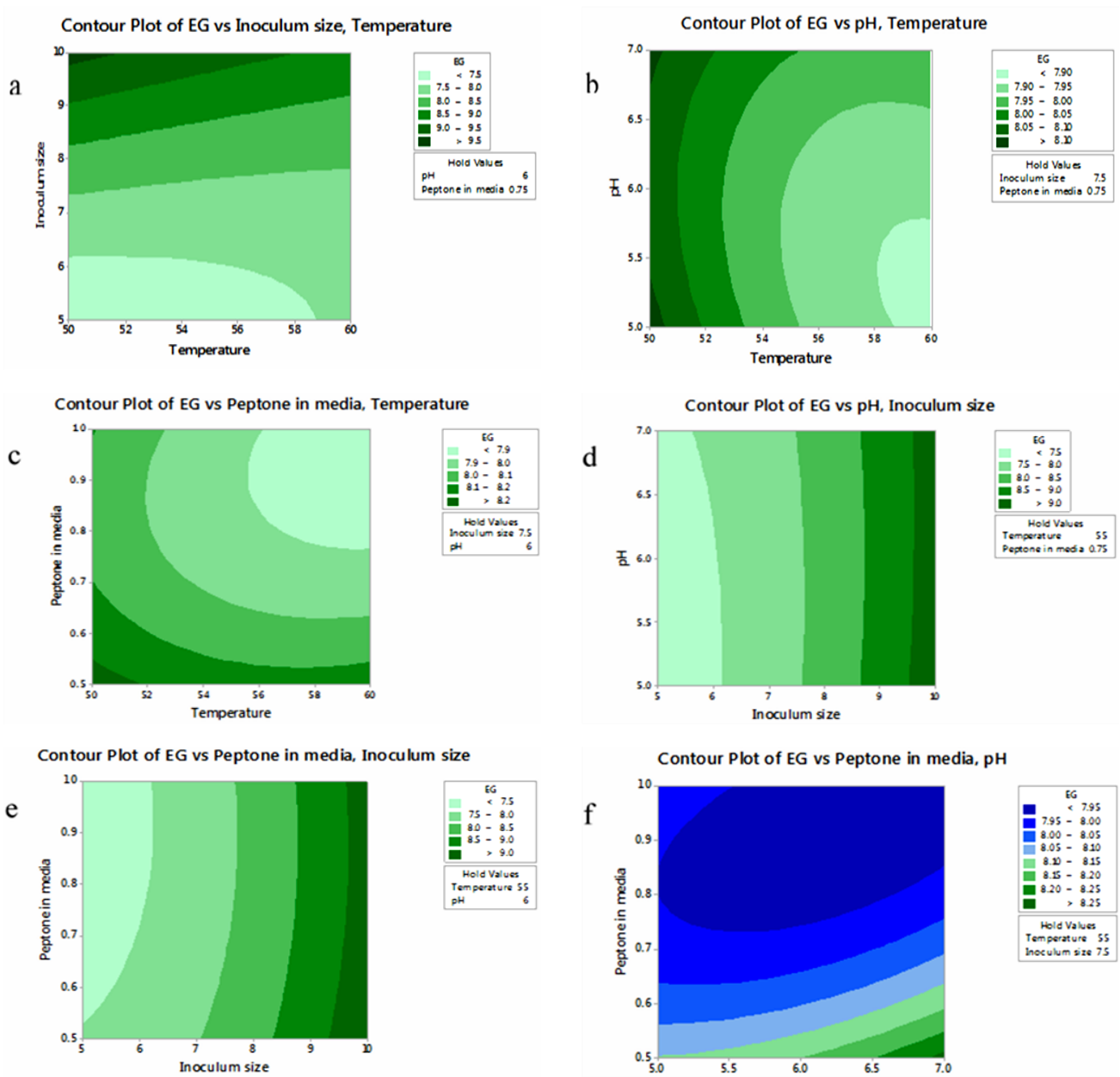

Fig. 4. Contour plots showing interactive effect of (a) Inoculum size, Temperature (b) pH, Temperature (c) Peptone, Temperature (d) pH, Inoculum size (e) Peptone, Inoculum size (f) Peptone, pH on EG production by *B. licheniformis* RT-17

Comparison of enzyme activity obtained after PBD and BBD revealed a slight increase in enzyme activities of EG and BGL. In PBD, maximum values of EG were 9.76 IU/ml and of BGL were 8.88 IU/ml whereas in BBD, highest obtained values of EG and BGL were 10.12 IU/ml and 9.13 IU/ml, respectively.

Analysis of variance was also performed for BGL and IFPU (data not shown) but the model generated was found to be insignificant giving R^2 values of less than 90%. Hence they were not proceeded for response optimization.

After analyzing the results for BBD for EG and knowing that this design is significant, a response optimization experiment was performed. In this experiment the software initially analyzed all the factors and their effects on response. It then proposed an experimental design for the maximum EG production while taking into consideration all the factors. The optimization results obtained were confirmed by studying the cellulase production using the optimum conditions obtained by the model i.e. temperature (50°C), inoculum size (10%), pH (5) and peptone concentration in fermentation medium (1%). Under these optimal conditions, cellulase titers of 9.808 IU/ml was predicted and a real value of 9.128 IU/ml was obtained by performing the experiment under these conditions, which is close to the predicted level. Hence it indicated the validity of the model.

The ability of cellulase from RT-17 was evaluated for the hydrolysis of sugarcane bagasse (SCB). The heterogeneity of SCB requires activity of hemicellulases for its complete degradation. A mixture of cellulase and hemicellulase can saccharify SCB more efficiently compared with the single enzyme preparation. Although, the hemicellulolytic potential of

the yeasts has not been explored widely, however, two of the yeasts isolated from environmental samples showed higher productivity of xylanase and pectinase. The unidentified strains were labelled as MK-162 (producing xylanase) and AA-15 (producing pectinase). The possible synergistic effect of the yeast xylanase and pectinase on cellulase of RT-17 for the hydrolysis of SCB was investigated. Since the enzymes from the different organisms had different temperature optima, therefore, the hydrolysis of SCB was carried out at 35ºC to avoid any denaturation of the yeasts' enzymes. The amount of reducing sugars released (µg/ml.h) after enzyme action (of yeasts and bacteria) on different substrates was taken as a measure of saccharification (Table 7). The data showed that when the cellulase preparations from bacteria RT-17 was used separately to hydrolyze untreated SCB, it produced 6.9 µg/ml.h. Whereas, alkali pretreated SCB was hydrolysed at the rate of 3 µg/ml.h. Hydrolysis of commercially available cellulose was achieved with 3 µg/ml.h of reducing sugars by the bacterial cellulase. It showed that the enzyme was more active towards the crude LC substrate.

The xylanase preparation from MK-162 was found to be acting synergistically with the cellulase of *B. licheniformis* RT-17. The highest degree of synergy (DS) was found when CMC was used as substrate, whereas, DS was 1.71 and 1.86, when untreated and alkali treated SCB were saccharified, respectively. Song *et al.* (2016) observed a 1.4-2.3 DS between cellulase and xylanase for the bioconversion of corncob powder, corn straw and rice straw and obtained up to 19.5 mg/ml of reducing sugars. The synergistic effect of bacterial xylanase or fungal cellulase has been reported for the bioconversion of pretreated corn-stover (Gao *et al.*, 2010).

The combination of pectinase preparation from AA-15 and cellulase preparation of RT-17 was found most promising for the hydrolysis of alkali-treated SCB. However, this degree was reduced to 1.96, when the xylanase preparation of MK-162 was also included in this preparation. Synergy between fungal and bacterial cellulase and hemicellulases have not been studied extensively (Bhattacharya *et al.*, 2015). Therefore, further studies are required to understand the mechanism of synergy between yeast and bacterial enzyme and for their subsequent utilization in commercial processes.

Table 6. Analysis of variance of BBD for EG production by *B. licheniformis* RT-17.

Source	DF	Adj. SS	Adj. MS	F-value	P-value
Model	22	38.1057	1.7321	45.45	0.000
Blocks	8	1.2764	0.1595	4.19	0.001
Linear	4	34.2858	8.5715	224.90	0.000
Temperature	1	0.2783	0.2783	7.30	0.009
Inoculum size	1	33.5473	33.5473	880.22	0.000
pH	1	0.0221	0.0221	0.58	0.450
Peptone	1	0.4382	0.4382	11.50	0.001
Square	4	1.3244	0.3311	8.69	0.000
Temp.*Temp.	1	0.0008	0.0008	0.02	0.886
Inc. size*Inc. size	1	0.6152	0.6152	16.14	0.000
pH*pH	1	0.0003	0.0003	0.01	0.925
Peptone*Peptone	1	0.0211	0.0211	0.55	0.461
2-Way interaction	6	1.2873	0.2146	5.63	0.000
Temp*inc. size	1	1.1700	1.1700	30.70	0.000
Temp.*pH	1	0.0094	0.0094	0.25	0.621
Temp.*peptone	1	0.0217	0.0217	0.57	0.454
Inc. size*pH	1	0.0390	0.0390	1.02	0.316
Inc. size*peptone	1	0.0106	0.0106	0.28	0.600
pH*peptone	1	0.0366	0.0366	0.96	0.331
Error	52	1.9818	0.0381		
Total	74	40.0875			

Model summary: S = 0.195223; R-sq. = 95.06%; R-sq. (Adj.) = 92.96%; R-sq. (Pred.) = 89.88%

Table 7. Rate of hydrolysis of different substrates by cellulase preparation of *B. licheniformis* RT-17 (Cell of RT-17) and synergistic effect of xylanase preparation from MK-162 (Xyl of MK-162) and pectinase preparation from AA-15 (Pect of AA-15).

Substrates	Cell of RT-17	Synergy with		
		Xyl of MK-162	Pect of AA-15	Xyl of MK-162 & Pect of AA-15
Untreated SCB	6.9	1.71	1.39	1.39
Alkali treated SCB	3	1.86	7.1	1.96
CMC	3	2.5	3.96	1.8
Pectin	14	1.02	0.9	4.61
Xylan	6	1.6	1.28	1.6

*The values represent average of triplicate with significant standard deviation

Conclusion

Statistical tools namely Plackett-Burman Design and Box Behnken Design were found to be appropriate for the optimization of Endoglucanase (EG) production from a thermophilic strain of *B. licheniformis*. The EG preparation was mixed with yeast pectinase and xylanase and was applied for the saccharification of sugarcane bagasse. A higher degree of synergy was noted between yeast pectinase and bacterial EG for the saccharification of bagasse.

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