Statistical optimization of cellulase production and its efficacy in hesperidin extraction from orange peel and bioethanol production from rice straw by simultaneous saccharification and fermentation

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Abstract

Cellulolytic enzymes are of great interest for the hydrolysis of cellulosic biomass and their demand is increasing intensely due to their potential applications in second generation bioethanol production. In this study, cellulase production from Bacillus cereus KG10 was explored and the production media and cultural conditions were optimized through Box-Behnken design. Under optimized conditions, the cellulase activity was improved to 0.51 U/mL. The purity and the molecular mass of the enzyme (55.1 kDa) were confirmed by HPLC and MOLDI-TOF MS respectively. Additionally, cellulase mediated extraction of hesperidin was performed to prove extraction efficiency by ESI-MS. Likewise, the cellulase efficiency in bioconversion of lignocellulosic biomass was also confirmed towards improving the ethanol yield.

The results showed that higher amounts of reducing sugar 73.2 and 89.3 % were explored from cellulase mediated hydrolysis of rice straw than that from acid-alkali mediated hydrolysis. A high ethanol yield was also achieved in media added with cellulase treated rice straw (38.1 g/L) than that of acid-alkali treated rice straw (27.4 g/L) through simultaneous saccharification and fermentation (SSF) process using cellulase from B. subtilis KG10 and Saccharomyces cerevisiae. Thus, the cellulase from B. cereus KG10 could be a potential candidate in various industrial sectors.

Keywords: Cellulase, statistical optimization, hesperidin, ethanol, simultaneous saccharification and fermentation.

Introduction

Recently, the green bioprocessing technologies have emerged to substitute the toxic and extravagant chemical processes increasing our environmental safety. Thus, this inventiveness increased the utility of enzymes in industrial sectors as they require less or no toxic metal ions for their biocatalytic functions[7]. Further, highly thermophilic enzymes are highly demanded due to their potential to withstand the extreme and harsh conditions of industrial processing.

Presently, cellulose, the second largest enzymes in industrial processing including food, laundry, textiles, paper as well as animal feed[4] contributes to about 8% of the industrial enzymes and its demand is continuously increasing[10].

Microbial cellulases are synthesized during their growth on cellulosic materials. Generally, fungi, bacteria and actinomycetes are involved in cellulase production. But bacteria are frequently employed for fast growth rate and higher rate of enzyme production[23]. Although, various bacterial genera such as Bacillus, Cellulomonas, Rummminococcus, Clostridium, Alteromonas etc. have been reported for cellulase activity, Bacillus is the predominant amongst others. Various *Bacillus* sp. such as *B. brevis*[31], *B. amyloliquefaciens* DL-3[16], *B pumilus* BPCRI 6[14], *B. pumilus* S124A[3], *B. subtilis* K-18[13], *B. subtilis*[9], *B. subtilis* BY-3[20], *Bacillus* sp.[24,28] are involved in cellulase production.

In addition, *B. licheniformis*[12] also has been reported for cellulase activity. Various factors including physical (pH, temperature, aeration, agitation) and media components (carbon, nitrogen, minerals, inducers and additives) can influence enzyme productivity and yield. Further, these factors are major key parameters to improve the yield and reduce the production cost. For screening the various physical and media components, one variable at a time (OVAT) approach is mostly used by research people. But, this is a time-consuming process with a lack of facility to evaluate the relationship of parameters[5].

A statistical-based approach has been employed to overcome these problems and to produce fast and most reliable results. In this study, Box Behnken design was used to evaluate the interaction of the most significant variables towards enhancing the cellulase production from *B. subtilis* KG10.

Further, we also investigated the efficiency of cellulase to extract hesperidin from orange peel and to improve bioethanol production by increasing the bioconversion of sugars to ethanol.

Table 1
Experimental range and levels of three independent variables for cellulase production from *B. subtilis* KG10 by BBD

Code	Variables	Range and levels		
		-1	**0**	**1**
X_1	Inoculum size (%)	1	2	3
X_2	NaCl (%)	0.25	0.5	0.75
X_3	Yeast Extract (%)	0.25	0.5	0.75

Material and Methods

Organism and cultural conditions: Cellulase-producing bacteria *B. subtilis* KG10 isolated from the virgin forest of Kovai Kutralam, Coimbatore, India was used in this study[26]. The isolate was maintained in 5% glycerol stock.

The seed culture was prepared by inoculating the culture into 10 mL of nutrient broth and grown at 37 °C in a shaking incubator for 24 h.

Experimental design and optimization study: The most significant variables (inoculum size, NaCl and yeast extract) in cellulase production were screened from OVAT method[27]. Hence, these variables were selected to evaluate their optimal level for enhanced production of cellulase from *B. subtilis* KG10 using a response surface Box-Behnken design. The levels of each variable are given in table 1 and totally 15 runs were carried out. The second-order non-linear polynomial equation (1) was used to fit the data value:

$$Y=\beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (1)$$

where Y is the predicted response, β_0 is the constant, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients and β_{12}, β_{13}, β_{23} are the cross-product coefficients.

The statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert Software. Subsequently, the feasibility and grid searches were performed to locate the composition of optimum formulations. The 3-D response surface plots were drawn using this software. Eight optimum check points were selected by intensive grid search performed over the entire experimental domain to validate the chosen experimental design and polynomial equations. The formulations corresponding to these checkpoints were prepared and evaluated for various response properties.

The resultant experimental data of response properties were compared with that of the predicted values. Regression plots between the observed and predicted values of the response properties were drawn.

Statistical analysis: This experimental design was obtained from MINITAB software and Design expert version 9. Data presented on the average of three replicates (±SD) are obtained from three independent experiments.

Partial purification and characterization of cellulase: The culture-free supernatant (CFS) obtained by centrifuging (at 10000 rpm and 4 °C) the 24 h old broth culture of *B. subtilis* KG10 grown under optimized conditions was purified. The CFS was precipitated overnight at 4 °C with 60 % ammonium sulphate and the precipitates were recovered by centrifuging at 10000 rpm for 10 min. The pellet was re-suspended in a minimum amount of 100 mM phosphate buffer (pH 7.0) and dialysed in the same buffer with frequent buffer exchange. The dialysate was dissolved in the same buffer and assayed for cellulase activity.

Cellulase assay: Cellulase activity of *B. subtilis* KG10 was measured using carboxymethylcellulose as substrate as described by Sreena and Sebastian[32]. One mL of the enzyme solution was added with an equal amount of 0.05 M sodium citrate buffer, pH 5.0 and incubated at 40 °C for 30 min. The released reducing sugar was measured by the standard DNS method. Glucose was used as a standard to plot the calibration curve. One unit of cellulase activity is the amount of enzyme required to release 1µmol of glucose per minute.

Bacterial growth and cellulase production kinetics: To demonstrate the influence of bacterial growth in cellulase production, the kinetics study was performed under optimized conditions. About 2% of bacterial isolate containing $2x\ 10^7$ CFU/mL was inoculated into an optimized medium and the bacterial growth and cellulase activity were measured every 2 h up to 48 h incubation.

Zymogram analysis: Zymogram assay was carried out in non-denature 12% SDS polyacrylamide gel added with 0.2% CMC. Electrophoretic separation was carried out at 4 °C at constant voltage (100 V) for 3.5 h. For cellulase activity, the stained gel was wash for 5 min (5 times) with a 50 ml wash buffer containing 0.1mM sodium succinate (pH 5.8) with 10 mM DTT.

Finally washed with the same buffer without DTT at 30 °C for 30 min. Gels containing CMC were stained by incubating in 0.1% congo red solution for 10-15 min at room temperature and destained using 1M NaCl until clear activity bands became visible and were photographed. The clear zone indicated the presence of cellulase activity[17].

HPLC analysis: The HPLC spectrum of the purified enzyme was obtained using the C18 column (Sodexo, 4.6 mm x 100 mm) using HPLC (Shimadzu, Japan). About 10 µL of the sample was injected using an automated syringe and

separated using the solvent system of acetonitrile-water (70:30) at a flow rate of 1.0 mL/min. A highly sensitive UV detector at 280 nm was used to detect the peak.

MALDI-TOF MS analysis: About 20 µL of trypsin digested enzyme solution was used for the MALDI-TOF MS analysis and recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA, time-of-flight spectrometer with a pulsed nitrogen laser, (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive high-mass mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetone and water along with 0.1% trifluoroacetic acid was used for obtaining the mass spectra.

For identification of the protein fragments, selected peptide masses were submitted to Mascot (http://www.matrixscience.com) for SwissProt database search.

Industrial applications of cellulase

In food industry- Hesperidin extraction from orange peel: Hesperidin, a bioflavonoid with antioxidant and anti-inflammatory potential, was extracted from air-dried sweet orange peels by both chemical method and cellulase mediated method.

Hesperidin extraction by the conventional method: Air-dried orange peel powder (80 g) was placed in a reflux condenser and 600 mL of petroleum ether was added and refluxed for 1.5 h. After filtration of the hot mixture through a Buchner funnel, the powder was allowed to dry at room temperature. The dried powder was returned to the flask and add 600 ml of methanol. The content was heated under reflux for 2 h and then the hot mixture was filtered. The filtrate was concentrated with a distillation column, leaving a syrup residue crystallized from dilute acetic acid (6%) to yield crude hesperidin[15].

Cellulase mediated extraction of hesperidin: In the cellulase mediated method, 0.1 IU/mL concentration of cellulase was added with orange peel powder mixed with 400 mL of 50 mM phosphate buffer (pH 7.0) and incubated at 37 °C in a shaking incubator for 8 h. The lyophilized powder was used for extraction as followed by the conventional method.

The crude hesperidin was added to chloroform. The white crystalline hesperidin was then filtered through a Buchner funnel. Pure hesperidin has a melting point of 248-250 °C. Biologically derived hesperidin was confirmed using ESI mass spectrometry (Thermo Finnigan, ESI MS, Madison, USA).

In bioethanol industry- Saccharification of paddy straw using cellulase

Combined (acid-alkali) pretreatment of rice straw: About 50 g of rice straw was chipped and put into 1L Erlenmeyer flask containing 500 ml of 50 mM phosphate buffer (pH 7.0). The chopped rice straw was subjected to both chemical and biological pre-treatment. The chemical treatment was done by the combination of 2 % acid (sulfuric acid) and 2 % of sodium hydroxide[38].

Enzymatic (cellulase) hydrolysis: The pre-treated rice straw (100 g/L) was suspended in 25mL of 0.1M sodium acetate buffer (pH 5.5). This was added with a cellulase enzyme solution containing 2g/L of total protein concentration. The total suspension was incubated at 28 °C and 120 rpm for 96 h. The hydrolysate was obtained by filtering the suspension and the content of reducing sugar was analyzed by DNSA method[21].

Chemical composition of rice straw: The compositions such as cellulose, hemicellulose[40], lignin[39], protein[19] and reducing sugar[21] of rice straw before pre-treatments and after pre-treatments were analyzed by their respective protocol.

Simultaneous saccharification and fermentation (SSF): SSF was carried out in 250 mL bottle along with 100 g/L of pre-treated (separate bottles for chemically and biologically treated) rice straw suspension and 50 mL of nutrient broth (pH 5.5) containing 5 g/L of yeast extract, 7.5 g/L of $(NH_4)_2SO_4$, 3.5 L of KH_2PO_4, 0.75 g/L of $MgSO_4 \cdot 7H_2O$ and 1.0 g/L of $CaCl_2 \cdot 2H_2O$. The broth was inoculated with *Saccharomyces cerevisiae* (0.6 OD_{590}) and a cellulase enzyme solution containing 2.0 g/L of total protein. The whole suspension was incubated in a shaking incubator (100 rpm) at 28 °C for 48 h. Then the broth culture was filtered and the supernatant was subjected to estimation of reducing sugar and ethanol content by DNSA and $K_2Cr_2O_7$ method respectively[36].

Results and Discussion

The cellulolytic activity of bacterial strains majorly depends on the source of the isolate and the availability of bio-waste in the environment. *B. subtilis* KG10 used in this study was isolated, characterized and screened for cellulolytic activity[26]. In the present industrial scenario, the demand for cost-effective bioprocessing is increasing day-by-day. There is no specific medium for enzyme production by different microorganisms. Each strain has its own specific physiological and nutritional requirements for growth and enzyme production.

Thus a designing of medium components and culture conditions may enhance enzyme production. The culture medium components and physical conditions are optimized by OVAT method and screened for the most significant variables for cellulase production[27]. In the present study, the appropriate model, BBD, was used to fit the data for finding optimum level and their interactions.

Table 1 depicted the numerical value of three independent variables, inoculum size (X_1), yeast extract (X_2) and NaCl (X_3) for cellulase production by BBD. Maximum cellulase activity (0.51 U/mL) was achieved in run no. 11. The

predicted values of the cellulase enzyme were obtained ranging from 0.194 to 0.493 U/ml of cellulase production of *B. subtilis* KG10 (Table 2). Multiple regression analysis was applied for the experimental data obtained and the result was incorporated into 2nd order polynomial as follows:

$$Y = -0.782 + 0.343.X_1 + 1.552.X_2 + 2.087.X_3 - 0.087.X_1^2 - 1.707.X_2^2 - 2.147.X_3^2 + 0.100.X_1X_2 - 0.050.X_1X_3 + 0.120.X_2X_3 \quad (2)$$

Table 2
BBD showing the response of cellulase production as influenced by inoculum size (X1), NaCl (X2) and yeast extract (X3) with predicted and observed values of response and residuals

Trails	X_1	X_2	X_3	Cellulase production (U/ml)		Residual value
				Observed Value	Predicted Value	
1	2	0.75	0.75	0.24	0.261250	- 0.0212500
2	2	0.75	0.25	0.31	0.296250	0.0137500
3	3	0.75	0.50	0.37	0.372500	- 0.0025000
4	2	0.25	0.25	0.28	0.258750	0.0212500
5	3	0.25	0.50	0.26	0.270000	- 0.0100000
6	1	0.50	0.25	0.24	0.263750	- 0.0237500
7	1	0.75	0.50	0.29	0.280000	0.0100000
8	3	0.50	0.25	0.32	0.331250	- 0.0112500
9	1	0.50	0.75	0.25	0.238750	0.0112500
10	3	0.50	0.75	0.28	0.256250	0.0237500
11	2	0.50	0.50	0.51	0.493333	0.0166667
12	2	0.50	0.50	0.49	0.493333	- 0.0033333
13	1	0.25	0.50	0.28	0.277500	0.0025000
14	2	0.25	0.75	0.18	0.193750	- 0.0137500
15	2	0.50	0.50	0.48	0.493333	- 0.0133333

Table 3
Estimated model coefficients by multiple linear regressions for cellulase production from *B. subtilis* KG10 in BBD

Term	Coefficient	T-value	P- value
Constant	-0.782	- 6.067	0.002
Individual effect			
X_1	0.343	5.220	0.003
X_2	1.552	5.901	0.002
X_3	2.087	7.940	0.001
Quadratic Effect			
X_1^2	-0.087	-6.442	0.001
X_2^2	-1.707	-7.928	0.001
X_3^2	-2.147	-9.972	0.000
Interactive Effcet			
X_1X_2	0.100	1.934	0.111
X_1X_3	-0.050	-0.967	0.378
X_2X_3	0.120	0.580	0.587

(R^2) Residual square S= 0.02585; R^2= 97.6%; R^2 (adjusted) = 93.3

Table 4
Analysis of variance (ANOVA) for the factorial design of cellulase production from *B. subtilis* KG10 in BBD

Source	DF	Seqss	Adjss	AdjMs	F	P>F
Regression	9	0.14	0.14526	0.015	25.56	0.0012
Residual error	5	0.04724	0.04724	0.009448		
Total	14	0.13977				

DF- Degree of freedom, Seq.ss-sum of squares, Adjss-Adjusted sum of square, AdjMs – Adjusted mean square, F- F value, P- probe

The coefficient of the regression model and the linear, quadratic and combined effects of these variables are listed in table 3. The significance of each coefficient was determined by the student's t-test and the p-values are given in table 3. The smaller p- value of this study indicated the significance of the corresponding coefficient. The p values are 0.001 for X_3, X_1^2, X_2^2, X_3^2 and 0.002 for X_1 and 0.003 for X_2, indicating their significance in cellulase production (Table 3). The regression equation indicates the R^2 value as 0.976. The closer R^2 to 1 indicates the stronger model terms and the experiment predicts better response[18].

The R^2 value also indicates proportionality between observed and predicted values of the response. The adjusted R^2 for cellulase production was 0.933 revealing the goodness of fit of the proposed experiment run. The statistical significance of the study was analyzed by ANOVA and the F value (25.56) suggested the significance of the model (Table 4).

Contour plots for cellulase activity were plotted against any two independent variables by keeping the third variable at constant (zero level) and a total of three response surfaces were attained (Fig. 1a-c). Cellulase activity increases with an increase in yeast extract about 0.53% (Fig. 1a and b) and the same trend was observed for the variable inoculum size and NaCl. The cellulase activity increases with an increase in inoculum size about 2.21% and beyond their optimum level, reduces the cellulase activity. This finding is correlated with Shankar and Isaiarasu[30] who reported that 2% of inoculum size showed maximum production of cellulase from *B. pumilus* EWBCMI than others (1, 3, 5 and 7%).

A dispearate result was reported by Afzal et al[1] who depicted that 4% of inoculum size increased the cellulase production from *B. cereus* MRLBI whereas Ray et al[29] reported that 3% inoculum increased the cellulase production from *Bacillus* sp. and *B. subtilis* BY-2. These results indicated that the requirement of inoculum size is specifically based on the strain and is varied from each strain.

Validation of the model: The adequacy of the statistical model was verified by performing all the experiments in triplicate. The maximum cellulase activity anticipated by the model under the optimized conditions (2.21% inoculum size, 0.5% NaCl and 0.53% of yeast extract) was 0.493 U/mL. This predicted value was in good agreement with the observed value (0.51 U/mL), revealing the validity of the model.

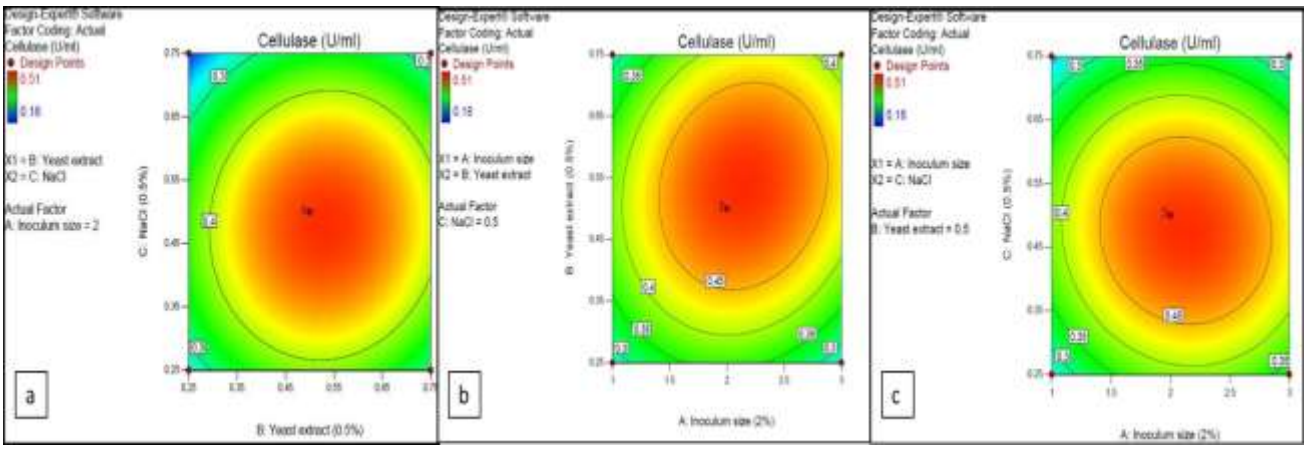


Fig. 1: Contour plot showing interactive effect of variables on cellulase production. (a) Yeast extract *vs* NaCL; (b) Inoculum size *vs* yeast extract; (c) Inoculum size *vs* NaCl

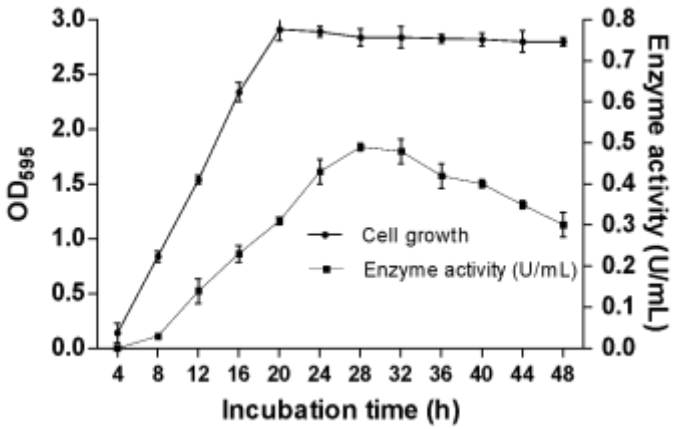


Fig. 2: Kinetics study of cell growth and activity of cellulase produced from *B. subtilis* KG10 produced under optimized conditions at various time points till 48 h. Each data indicates mean ±SD, N=3

Growth and cellulase production kinetics: The growth curve analysis revealed that growth was logarithmically increased after 6 h and maintained up to 28 h and thereafter the growth reached a statue line whereas the cellulase activity was slowly increased after 8 h only and maximum activity was observed at 26th h (0.49 U/mL) and thereafter activity was slowly reduced to 0.30 U/mL at 48 h (Fig. 2). This result revealed that growth and cellulase activity were dependent on each other. A similar trend of results was reported by Sreena and Sebastian[32].

Zymogram activity of cellulase: In zymogram gel, a single and distinct yellow band of CMC digestion was observed after being stained with congo red solution indicating the presence of cellulase (Fig. 3). A similar pattern of activity was reported by Sriariyanun et al[33], Listyaningrum et al[17]and Dar et al[8].

HPLC analysis: The HPLC spectrum of the purified enzyme was obtained using the C18 column. A single peak at the retention time of 4.25 min appeared using 280 nm as detector wavelength (Fig. 4) confirming the purity of the enzyme. The peak area was 332501 with 100% area coverage. The result agreed with the report of Bai et al[2] who documented the HPLC spectrum of cellulase with the same peak area at the retention time of 4.20 min.

The trypsin digested cellulase solution was injected into the MALDI-TOF mass spectrometer. The obtained mass spectra were used to search for the best match in the SwisProt database and revealed that the purified enzyme matched with the existing database match of *B. subtilis* 1, 4 endoglucanse (cellulase) with the molecular mass of 55.1 kDa with the protein score of 172 (Fig. 5). The protein score is 10* Log (P), where P is the probability that the observed match is a random event. A protein score greater than 49 is significant ($p<0.05$). The sequence similarity of the searched enzyme mass matched with the database of *Bacillus subtilis* endoglucanase with 55.1kDA. This result has coincided with our previous studies of SDS-PAGE analysis of cellulase from *B. subtilis* KG10 as it showed molecular weight of ~56 kDa[27].



Fig. 3: Zymogram analysis of cellulase from *B. subtilis* KG10 on non-dentaure 12% SDS polyacrylamide gel added with 0.2% CMC

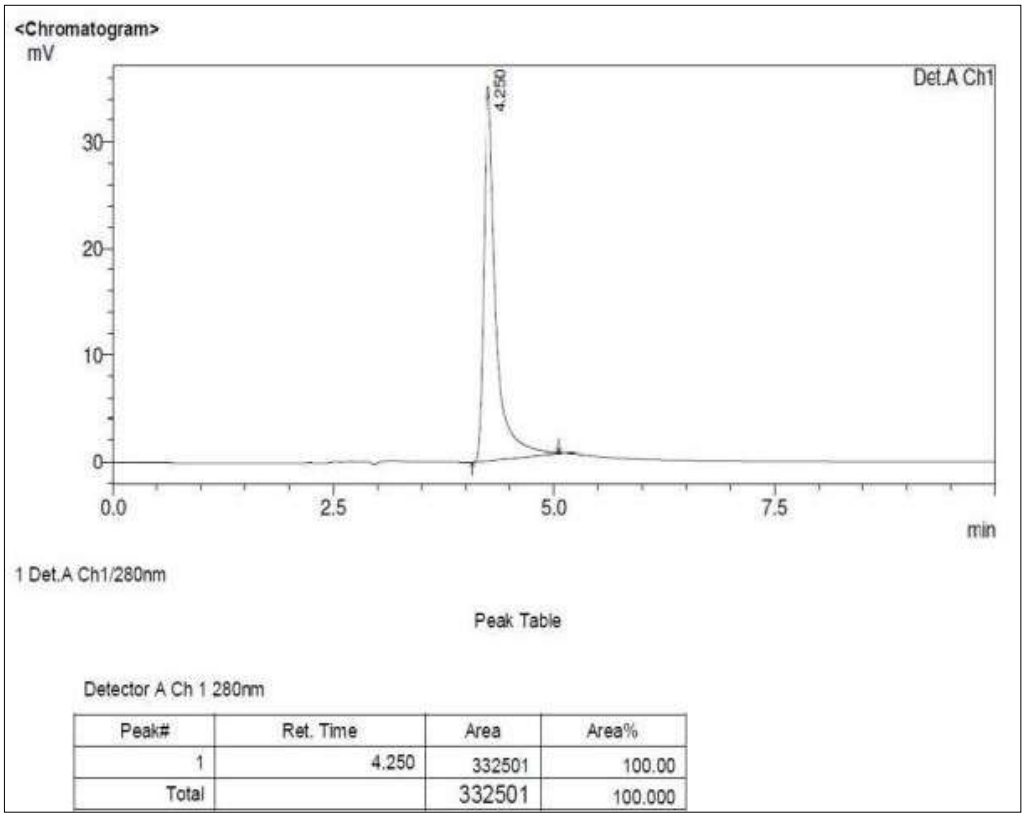


Fig. 4: HPLC profile of cellulase from *B. subtilis* KG10 showed purity of the enzyme.

Further, more than 62% of sequence coverage was observed in the mascot search result confirming that the purified enzyme belongs to the class of cellulase enzyme (Fig. 6). The MALDI-TOF mass spectrum clearly revealed the mass of the enzyme (55.1 kDa) and source taxa belongs to the *B. subtilis.* The trypsin digested MALDI-TOF mass spectrum is one of the best analytical methods to identify the unknown protein from the mixture of protein sources using the 1D and 2D gel electrophoresis method.

Similar proteomic studies have been conducted to identify the unknown protein from known sources such as soybean protein[22] and eukaryotic keratinocyte cytoplasmic membrane[11].

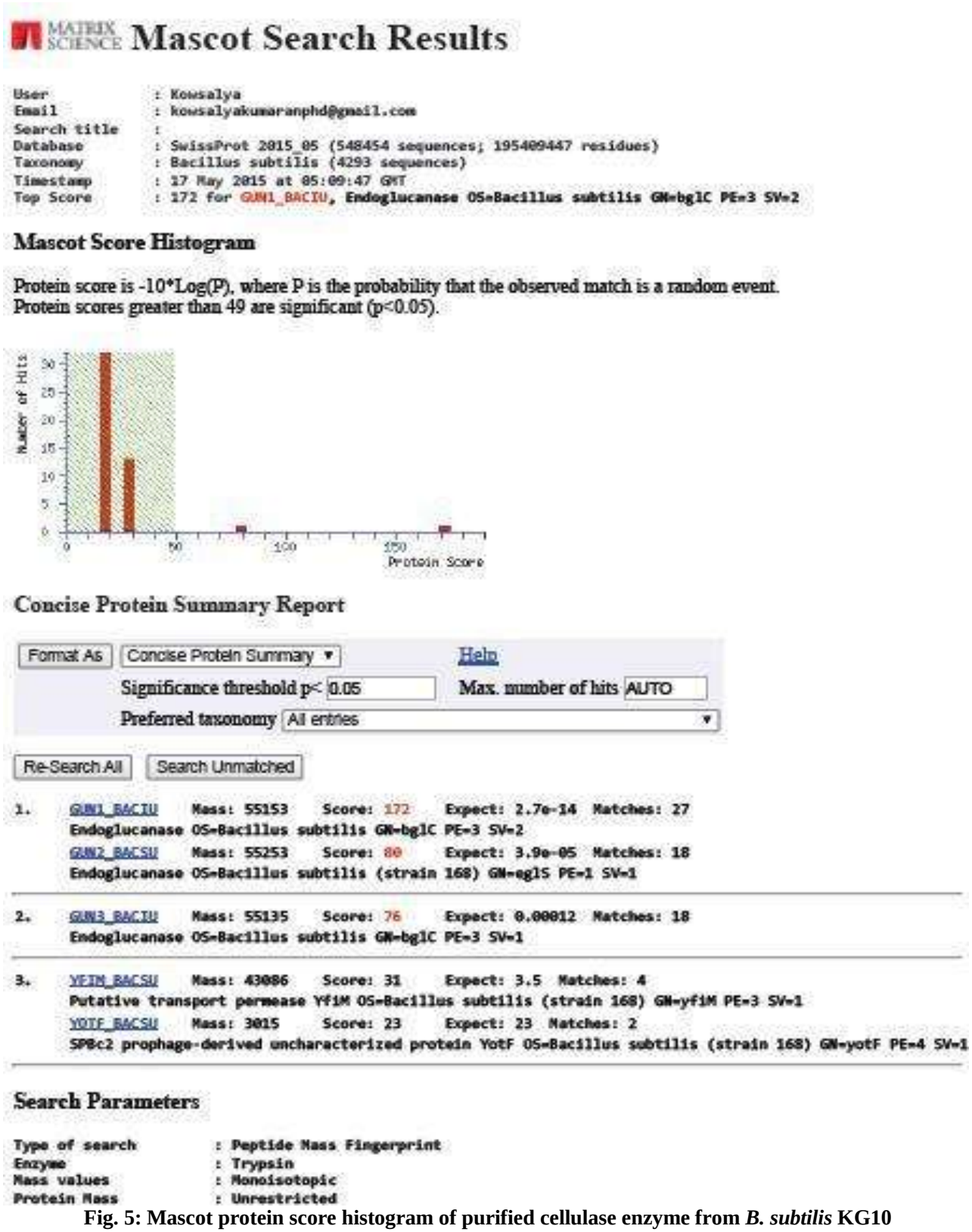


Fig. 5: Mascot protein score histogram of purified cellulase enzyme from *B. subtilis* KG10

MATRIX SCIENCE **MASCOT Search Results**

Protein View: GUN1_BACIU

Endoglucanase OS=Bacillus subtilis GN=bglC PE=3 SV=2

Database:	SwissProt
Score:	172
Expect:	2.7e-14
Nominal mass (M_r):	55153
Calculated pI:	8.12
Taxonomy:	Bacillus subtilis

Sequence similarity is available as an NCBI BLAST search of GUN1_BACIU against nr.

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Mass values searched:	78
Mass values matched:	27

Protein sequence coverage: 62%

Matched peptides shown in *bold red*.

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  1 MKRSISIFIT CLLIAVLTMG GLLPSFASAA GTKTPVAKNG QLSIKGTQLV
 51 NRDGKAVQLK GISSHGLQWY GDFVNKDSLK WLRDDWGITV FRAAMYTADG
101 GYIDNPSVKN KVKEAVEAAK ELGIYVIIDW HILNDGNPNQ NKEKAKEFFK
151 EMSSLYGNTP NVIYEIANEP NGDVNWKRDI KPYAEEVIPV IRKNDPDNII
201 IVGTGTWSQD VNDAADDQLK DANVMYALEF YAGTHGQSLR DKANYALSKG
251 APIFVTEWGT SDASGNGGVF LDQSREWLNY LDSKNISWVN WNLSDKQESS
301 SALKPGASKT GGWPLTDLTA SGTFVRENIR GTKDSTKDVP ETPAQDNFTQ
351 EKGVSVQYKA GDGRVNSNQI RPQLHIKGNG NATVDLKDVT ARYWYNVNNK
401 GQNFDCDYAQ NGCGNLTHKF VTLEKPKQGA DTYLELGFKT GTLSPGASTG
451 NIQLRLHNDD WSNYAQSGDY SFFQSNTFKT TKKITLYSQG KLIWGTEPN
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Unformatted sequence string: 499 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Peptide
39 - 45	759.4359	758.4286	758.4286	-0.0000	0	K.NGQLSIK.G
46 - 52	787.4308	786.4235	786.4348	-0.0113	0	K.GTQLVNR.D
46 - 52	787.4421	786.4348	786.4348	0.0000	0	K.GTQLVNR.D
56 - 60	558.3609	557.3536	557.3537	-0.0001	0	K.AVQLK.G
61 - 76	1807.8762	1806.8689	1806.8689	-0.0000	0	K.GISSHGLQWYGDFVNK.D
84 - 92	1108.5421	1107.5348	1107.5349	-0.0001	0	R.DDWGITVFR.A
93 - 109	1772.8159	1771.8086	1771.8087	-0.0001	0	R.AAMYTADGGYIDNPSVK.N
114 - 120	717.3777	716.3704	716.3704	-0.0000	0	K.EAVEAAK.E
147 - 150	570.2922	569.2849	569.2849	-0.0000	0	K.EFFK.E

Fig. 6: The mascot search results of protein view of purified cellulase enzyme from *B. subtilis* KG10. Protein sequence coverage of 62% indicated identity or extensive homology

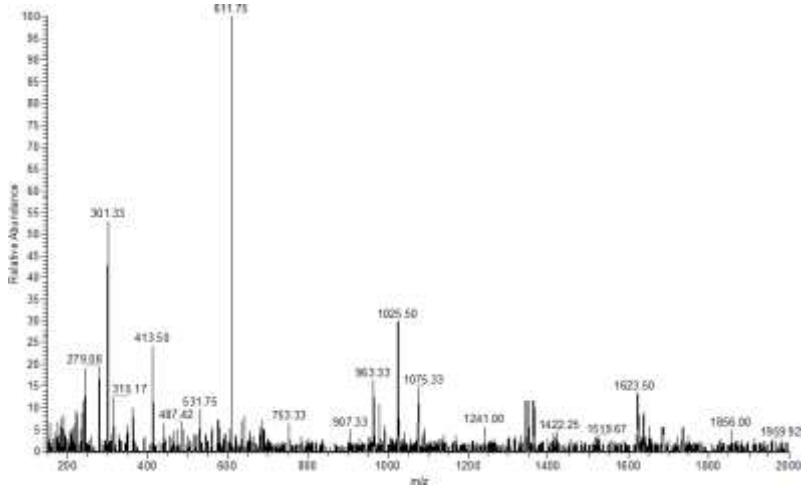


Fig. 7: ESI-MS confirmation of hesperidin extracted *via* cellulase mediated method

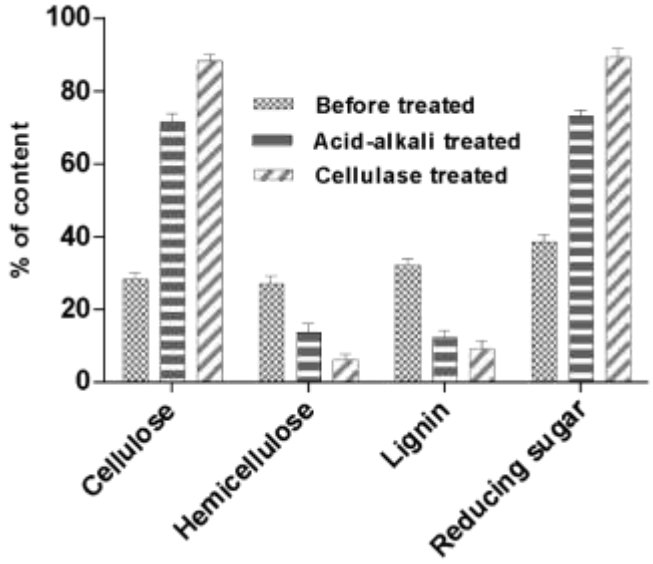


Fig. 8: Composition of rice straw before and after hydrolysis (acid-alkali and cellulase enzyme mediated). The data of the experiment was based on the mean value of three independent experiments

Applications of cellulase in the food industry

Bio-extraction of naturally and economically viable components from complex materials: Nowadays, modern medication is utilizing bioactive natural products such as hesperidin, beta carotene, astaxanthin, pregnan glycosides, rutin etc. Among them, hesperidin and beta carotene extraction from orange peel and sweet potato respectively is quite difficult and inefficient. Hesperidin, a flavanone-type is found abundantly in the peel of orange peel and other citrus fruits. Hesperidin is an antioxidant that enhances the action of vitamin C (ascorbic acid) to lower cholesterol levels[34].

The cellulase-mediated extraction showed a higher yield of hesperidin (59.1 mg/g) than the conventional method (18.4 mg/g). The overall bio-extraction ability was calculated and shown as 3.2 times better than the conventional method. The current result is concurrent with results of Stanisic et al[34] who stated that the conventional method of extraction yield is comparatively not so good when compared to any other specialized methods especially the green method. Similarly, Lahmer et al[15] reported that purification of hesperidin from orange peel yielded through conventional methods showed significant yield but less than the cellulase mediated bio-extraction. This method can be utilized for the commercial isolation of hesperidin from orange peel for pharmaceutical formulations.

Further, the ESI-MS showed the clear hesperidin spectrum with characteristic features of fragment and the m/z of 301 molecular ion peak in the negative ion mode (Fig. 7) confirming the presence of the bioactive compound, hesperidin. However, Lahmer et al[15] utilized spectroscopic analysis like UV, IR and NMR to confirm the presence of hesperidin and to elucidate the functional groups of extracted hesperidin from orange peel.

Saccharification and fermentation of rice straw using cellulase for bioethanol production: Bioethanol is one of

the most significant renewable fuels to replace fossil-derived fuels. Rice straw is an abundant agricultural byproduct in India and it was used as the carbon source for bioethanol production performed by SSF using *S. cerevisiae* MTCC 463 and cellulase from *B. subtilis* KG10.

Chemical composition of rice straw before and after pre-treatments: The pre-treatment of rice straw is essential to remove lignin and to produce ethanol efficiently by saccharification and fermentation. Alkali treatment is the most effective and profitable method to remove lignin, to fiberize and to easily break the crystal structure of cellulose[35]. In the current study, both chemical (acid and alkali method) and enzymatic hydrolysis were performed for efficient ethanol production. The lignin content decreased in both [chemical (12.4%) and enzymatic (9.3%) hydrolysis] than in the native form of rice straw (32.2%) indicating that both pretreatments reduced the lignin content. A similar trend of results was reported by Takano and Hoshino[36].

Cellulose content increased from 28.3% to 71.5 and 88.2 % after acid-alkali and enzyme treatment respectively whereas hemicellulose was reduced from 27.1% to 13.7 and 6.3 % due to chemical and enzyme-mediated hydrolysis respectively and this reduction may be due to partial solubilization of xylan by alkali treatment[6]. Reducing sugar in the alkali pre-treated rice straw is increased from 38.6% to 73.2% and increased to 89.3% by enzymatic hydrolysis (Fig. 8). According to Oberoi et al[25], alkali (NaOH) pre-treatment of rice straw resulted in the higher climb of cellulose and decrease of hemicellulose content. In this study, a pronounced increase in cellulose and decrease in hemicellulose content suggested great attainment of biomass making best use of these polysaccharides in biomass for ethanol production.

Ethanol production by SSF: Ethanol production was separately achieved from 100 g/L of alkali-treated and enzyme-treated rice straw by SSF using *S. cerevisiae*. After 8 h incubation, the reducing sugar content of alkali-treated and enzyme-treated substrate material was found to be 24.2 and 29.6 g/L respectively. After 48 h incubation, the reducing sugar was reduced to 0.7 and 0.3 g/L respectively indicating more than 70% of reducing sugar found at the initial stage consumed immediately by the yeast cells and was converted to ethanol. The ethanol content reached to 27.4 and 38.1 g/L at 48 h from acid-alkali treatment and cellulase treated rice straw (Fig. 9).

Takano and Hoshino[36] reported that about 13.6 g/L glucose and 9.7 g/L of other disaccharides were produced during the initial stage of cultivation and the subsequent fermentation produced 28.9 g/L of ethanol after 48 h incubation indicating that a high amount of ethanol was produced in the current study using rice straw hydrolyzed with cellulase produced from *B. subtilis* KG10.

Conclusion

The utilization of agricultural biomass especially rice straw for producing biofuel is one of the eco-friendly and effective methods in biofuel technology. In this study, cellulase-producing *B. subtilis* KG10 was used for bioethanol production. Thus, the cellulase production was improved (0.51 U/mL) under optimized culture conditions (2.21% inoculum size, 0.5% NaCl and 0.53% of yeast extract). Additionally, the purity of the enzyme was confirmed by HPLC. MOLDI-TOF mass spectrum confirmed their molecular weight (55.1 kDa). Further, the enzyme was checked for its ability to extract hesperidin from orange peel employed in the food and pharmaceutical industries. The cellulase-mediated extraction of hesperidin was confirmed by ESI-MS and was found to be 1.67 times higher than the conventional extraction method.

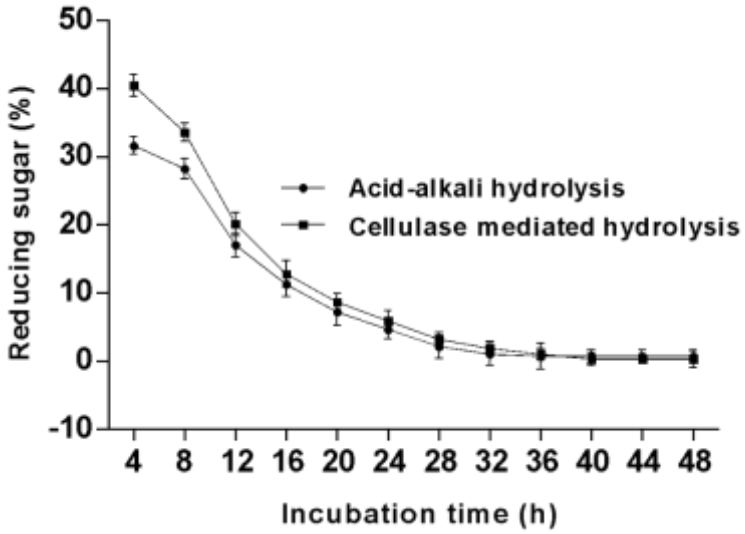


Fig. 9: Reducing sugar content of fermentation media added with rice straw (acid and alkali treated and cellulase treated) at different time points.

The study also presented a promising result of SSF using cellulase and *S. cerevisiae* for improved ethanol production from enzyme hydrolyzed rice straw. The present study concluded that cellulase from *B. subtilis* KG10 could be a prospective tool in various industrial sectors due to versatile appeal.

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References

1. Afzal I., Shah A., Makhdum Z., Hameed A. and Hasan F., Isolation and characterization of cellulase producing *Bacillus cereus* MRLB1 from soil, *Minerva. Biotecnol*., **24**, 101–109 **(2012)**

2. Bai S., Kumar M., Kumaran M., Kalaichelvan P. and Mani R., Cellulase Production by *Bacillus subtilis* isolated from Cow Dung, *Arch. Appl. Sci. Res*., **4**, 269–279 **(2012)**

3. Balasubramanian N. and Simões N., *Bacillus pumilus* S124A carboxymethyl cellulase; a thermo stable enzyme with a wide substrate spectrum utility, *Int. J. Biol. Macromol*., **67**, 132–139 **(2014)**

4. Bhat M.K. and Bhat S., Cellulose degrading enzymes and their potential industrial applications, *Biotechnol. Adv.,* **15**, 83–620 **(1997)**

5. Brijwani K., Oberoi H.S. and Vadlani P.V., Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran, *Process Biochem*., **45**,120–128 **(2010)**

6. Carvalheiro F., Duarte L. and Gírio F., Hemicellulose biorefineries: A review on biomass pretreatments, *J. Sci. Ind. Res*., **67**, 849 **(2008)**

7. Comfort D.A., Chhabra S.R., Conners S.B., Chou C.J., Epting K.L., Johnson M.R., Jones K.L., Sehgal A.C. and Kelly R.M., Strategic biocatalysis with hyperthermophilic enzymes, *Green Chem.,* **6**, 459–465 **(2004)**

8. Dar M., Pawar K., Rajput B., Rahi P. and Pandit R., Purification of a cellulase from cellulolytic gut bacterium, *Bacillus tequilensis* G9 and its evaluation for valorization of agro-wastes into added value byproducts, *Biocatal. Agric. Biotechnol*., **20**, 101219 **(2019)**

9. Deka D., Das S.P., Sahoo N., Das D., Jawed M., Goyal D. and Goyal A., Enhanced Cellulase Production from *Bacillus subtilis* by Optimizing Physical Parameters for Bioethanol Production, *ISRN Biotechnol.,* **2013**, 965310 **(2013)**

10. Gaur R. and Tiwari S., Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07, *BMC Biotechnol.,* **15**, 19 **(2015)**

11. Gonnet F., Lemaître G., Waksman G. and Tortajada J., MALDI/MS peptide mass fingerprinting for proteome analysis: identification of hydrophobic proteins attached to eucaryote keratinocyte cytoplasmic membrane using different matrices in concert, *Proteome Sci.,* **1**, 2 **(2003)**

12. Gupta M., Sharma M., Singh S., Gupta P. and Bajaj B.K., Enhanced Production of Cellulase from *Bacillus licheniformis* K-3 with Potential for Saccharification of Rice Straw, *Energy Technol.,* **3**, 216–224 **(2015)**

13. Irfan M., Mushtaq Q., Tabssum F., Shakir H.A. and Qazi J.I., Carboxymethyl cellulase production optimization from newly isolated thermophilic Bacillus subtilis K-18 for saccharification using response surface methodology, *AMB Express*, **7**, 29 **(2017)**

14. Kotchoni O.D., Shonukan O.O. and Gachomo W.E., *Bacillus pumilus* BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression, *African J. Biotechnol.,* **2**,140–146 **(2003)**

15. Lahmer N., Belboukhari N., Cheriti K. and Sekkoum K., Hesperidin and Hesperitin preparation and purification from citrus sinensis peels, *Der. Pharma. Chem.,* **7**, 1–4 **(2015)**

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

17. Listyaningrum N., Sutrisno A. and Wardani A., Characterization of thermostable cellulase produced by *Bacillus* strains isolated from solid waste of carrageenan, IOP. Conf. Ser. Earth. Environ. Sci., **131**, 12043 **(2018)**

18. Liu H.L., Lan Y. and Cheng Y.C., Optimal production of sulphuric acid by *Thiobacillus thiooxidans* using response surface methodology, *Process Biochem*., **39**,1953–1961 **(2004)**

19. Lowry O.H., Rosebrough N.J., Farr A. and Randall R.J., Protein measurement with the folin Phenol Reagent, *J. boil. Chem.,* **193**, 256–275 **(1951)**

20. Meng F., Ma L., Ji S., Yang W. and Cao B., Isolation and characterization of *Bacillus subtilis* strain BY-3, a thermophilic and efficient cellulase-producing bacterium on untreated plant biomass, *Lett. Appl. Microbiol.,* **59**, 306–312 **(2014)**

21. Miller G.L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, *Anal. Chem*., **31**, 426–428 **(1959)**

22. Mooney B.P. and Thelen J.J., High-throughput peptide mass fingerprinting of soybean seed proteins: automated workflow and utility of UniGene expressed sequence tag databases for protein identification, *Phytochemistry,* **65**, 1733–1744 **(2004)**

23. Nagendran S., Hallen-Adams H.E., Paper J.M., Aslam N. and Walton J.D., Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus Amanita bisporigera, based on the secretome of Trichoderma reesei, *Fungal Genet. Biol*., **46**, 427–435 **(2009)**

24. Nandimath A.P., Kharat K.R., Gupta S.G. and Kharat A.S., Optimization of cellulase production for *Bacillus* sp. and *Pseudomonas* sp. soil isolates, *African J. Microbiol. Res*., **10**, 410–419 **(2016)**

25. Oberoi H.S., Babbar N., Sandhu S.K., Dhaliwal S.S., Kaur U., Chandha B.S. and Bhargav S., Ethanol production from alkali-treated rice straw via simultaneous saccharification and

fermentation using newly isolated thermotolerant *Pichia kudriavzevii* HOP-1, *J Ind. Microbiol. Biotechnol*., **39(4)**, 557-566 **(2012)**

26. Ramalingam K. and Ramasamy G., Isolation, screening and characterization of cellulase producing *Bacillus subtilis* KG10 from virgin forest of Kovai Kutralam, Coimbatore, India, *Res. J. Biotechnol*., **8(6)**, 28–34 **(2018)**

27. Ramalingam K. and Ramasamy G., Optimization, purification and characterization of thermostable cellulase from *Bacillus subtilis* KG10 isolated from virgin forest of Kovai Kutralam, Coimbatore, India, *Int. J Pharma. Bio. Sci.*, **5**, B432–B448 **(2014)**

28. Rawat R. and Tewari L., Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3, *Extremophiles*, **16**, 637–644 **(2012)**

29. Ray A., Bairagi A., Ghosh K. and Sen S.K., Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut, *Acta Ichthyol, Piscat*., **37**, 47–53 **(2007)**

30. Shankar T. and Isaiarasu L., Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions, *Middle-East J. Sci. Res*., **8(1)**, 40-45 **(2011)**

31. Singh V.K. and Kumar A., Production and purification of an extracellular cellulase from *Bacillus brevis* vs-1, *IUBMB Life*, **45**, 443–452 **(1998)**

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

33. Sriariyanun M., Tantayotai P., Yasurin P., Pornwongthong P. and Cheenkachorn K., Production, purification and characterization of an ionic liquid tolerant cellulase from *Bacillus* sp. isolated from rice paddy field soil, *Electron. J. Biotechnol*., **19**, 23–28 **(2016)**

34. Stanisic D., Liu L.H.B., Dos Santos R.V., Costa A.F., Durán N. and Tasic L., New Sustainable Process for Hesperidin Isolation and Anti-Ageing Effects of Hesperidin Nanocrystals, *Molecules*, **25**, 4534 **(2020)**

35. Taherzadeh M.J. and Karimi K., Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review, *Int. J. Mol. Sci.*, **9**, 1621–1651 **(2008)**

36. Takano M. and Hoshino K., Bioethanol production from rice straw by simultaneous saccharification and fermentation with statistical optimized cellulase cocktail and fermenting fungus, *Bioresour. Bioprocess. Tech.*, **5**, 16 **(2018)**

37. Vijayaraghavan P. and Prakash S.G.V., Purification and characterization of carboxymethyl cellulase from *Bacillus* sp. isolated from a paddy field, *Polish J. Microbiol.*, **61**, 51 **(2012)**

38. Wang Q., Hu J., Shen F., Mei Z., Yang G., Zhang Y., Hu Y., Zhang J. and Deng S., Pretreating wheat straw by the concentrated phosphoric acid plus hydrogen peroxide (PHP): Investigations on pretreatment conditions and structure changes, *Bioresour. Technol*., **199**, 245-257 **(2016)**

39. Wottitz C., Pasquali C.E. and Herrera H.A., Thermal decomposition of Algarrobo Negro (Prosopis nigra). Wood and its main components: Lignin, alpha cellulose and hemicelluloses "A" and B", *Lat. Am. Appl. Res.*, **31**, 37–40 **(2001)**

40. Xiao B., Sun X.F. and Sun R., Chemical, structural and thermal characterizations of alkali-soluble lignins and hemicelluloses and cellulose from maize stems, rye straw and rice straw, *Polym. Degrad. Stab*., **74**, 307–319 **(2001)**.

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