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Cellulase Production from *Bacillus subtilis* SV1 and Its Application Potential for Saccharification of Ionic Liquid Pretreated Pine Needle Biomass under One Pot Consolidated Bioprocess

Parushi Nargotra, Surbhi Vaid and Bijender Kumar Bajaj *

School of Biotechnology, University of Jammu, Bawe Wali Rakh, Jammu 180006, India; parushi11nargotra@gmail.com (P.N.); sur.vaid@gmail.com (S.V.)

* Correspondence: bajajbijenderk@gmail.com; Tel.: +91-941-9102-201; Fax: +91-191-2456534

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Abstract: Pretreatment is the requisite step for the bioconversion of lignocellulosics. Since most of the pretreatment strategies are cost/energy intensive and environmentally hazardous, there is a need for the development of an environment-friendly pretreatment process. An ionic liquid (IL) based pretreatment approach has recently emerged as the most appropriate one as it can be accomplished under ambient process conditions. However, IL-pretreated biomass needs extensive washing prior to enzymatic saccharification as the enzymes may be inhibited by the residual IL. This necessitated the exploration of IL-stable saccharification enzymes (cellulases). Current study aims at optimizing the bioprocess variables viz. carbon/nitrogen sources, medium pH and fermentation time, by using a Design of Experiments approach for achieving enhanced production of ionic liquid tolerant cellulase from a bacterial isolate *Bacillus subtilis* SV1. The cellulase production was increased by 1.41-fold as compared to that under unoptimized conditions. IL-stable cellulase was employed for saccharification of IL (1-ethyl-3-methylimidazolium methanesulfonate) pretreated pine needle biomass in a newly designed bioprocess named as "one pot consolidated bioprocess" (OPCB), and a saccharification efficiency of 65.9% was obtained. Consolidated bioprocesses, i.e., OPCB, offer numerous techno-economic advantages over conventional multistep processes, and may potentially pave the way for successful biorefining of biomass to biofuel, and other commercial products.

Keywords: ionic liquid stable cellulase; *Bacillus subtilis* SV1; response surface methodology; pine needle biomass; ionic liquid pretreatment; one pot consolidated bioprocess

1. Introduction

Ever-increasing world demand of energy, fast depleting fossil fuel reserves, and climate change issues have motivated investigations for potential renewable sources of energy [1]. Among different alternatives, lignocellulose biomass (LB) may be one of the most appropriate renewable resources for the production of energy/biofuels [2]. LB is composed of cellulose, hemicellulose and lignin in a densely compact form which in fact poses a major hurdle in its conversion into simple fermentable sugars that, in turn, can be used for production of biofuels/chemicals and other commercial products [3]. Extensive pretreatments are required for disrupting the recalcitrance of LB and to make the cellulose accessible to saccharifying enzymes [4,5]. However, most of the pretreatment approaches are expensive, tedious, energy intensive, and need harsh conditions like high temperature, pressure, extreme pH, usage of hazardous chemicals, can cause sugar loss and may produce microbial inhibitors [6] that may be detrimental to the fermentation microorganisms [7]. Therefore, there is a

need for development of environmentally-benign pretreatment methods that may be executed under ambient process conditions.

Pretreatment of LB with ionic liquid(s) may represent a relatively novel and efficient approach for reducing recalcitrance of biomass; this approach proposes several merits over the traditional pretreatment methods such as it can be accomplished under ambient process conditions, does not require high temperature/pressure/extremes of pH or harmful chemicals, does not produce inhibitors, does not cause sugar loss, and, finally, it is cost and energy efficient [7,8]. However, the IL-pretreated biomass needs ample washing prior to enzymatic hydrolysis for removal of residual IL as the latter is considered as a strong inhibitor of enzymes that are used for saccharification. However, extensive washing of IL-pretreated biomass leads to loss of sugar, wastage of water and consequential escalated generation of effluent, and this overall undermines the efficacy of ILs as LB pretreatment agents [5]. The washing step could be obviated should the ionic liquid tolerant saccharification enzymes viz. cellulases and others are available. Considering that the techno-economic sustainability and success of the IL based pretreatment approach is substantially determined by the availability of IL-stable cellulases, extensive research attempts are being undertaken for IL-stable cellulases [9]. Several microorganisms have been reported to produce cellulases that are tolerant towards ionic liquids, viz. *Bacillus subtilis* [4], *Paenibacillus tarimensis* [10], *Pseudoalteromonas* sp. [11], and from metagenomic sources [12]. Availability of ionic liquid tolerant cellulases may potentially be used for developing novel consolidated bioprocesses such as "one pot consolidated bioprocess" (OPCB). In OPCB, unit operations like pretreatment and saccharification, or pretreatment, saccharification, and fermentation are executed in a single reaction vessel. OPCB offers several advantages over a conventional multi-operational strategy such as cost effectiveness, high product recovery, and no or minimal sugar loss, among others. [13]. Consolidated bioprocesses may be operated at different levels like pretreatment and enzymatic saccharification of LB or pretreatment, enzymatic saccharification and sugar fermentation in a single vessel, either using single microorganism or microbial consortium [14–16].

The high cost of saccharifying enzymes viz. cellulases, xylanases and others is another hurdle for the success of LB-biofuel technology. Microbial sources are generally used for saccharifying enzymes production [17]. High cost is incurred due to usage of expensive carbon/nitrogen sources for growth of microorganisms for enzyme production [5]. The enzyme production cost may be reduced by usage of agro-residues as carbon/nitrogen sources for microbial growth and enzyme production [18,19]. Furthermore, the bioprocess optimization may enhance enzyme yield and overall process economy [20]. Design of Experiments (DoE) based optimization offers several advantages over the conventional one-variable-at-a-time (OVAT) optimization strategy [19]. One of the most commonly used DoE approaches is response surface methodology (RSM). RSM represents an effective and proficient tool for elucidation of processes involving multiple variables [21], and has been used extensively for cellulase production [19,20]. Nonetheless, limited studies have been done on DoE mediated optimization of process variables for producing ionic liquid tolerant cellulases [4,5].

Exploration of new LB resources for potential production of biofuels/chemicals has been a continuous process [6], and pine needle biomass (PNB) may represent an important feedstock [22]. In coniferous forests, accumulation of leaves of pine trees (pine needles) on soil causes multifaceted problems viz. destroys the nutrient dynamics of soil, affects the decomposition/mineralization of organic matter and the flora/fauna of soil; tannins released from pine needles may inhibit the growth of various beneficial soil microbes [5,22,23], and, finally, the dried heaps of pine needles may risk forest fires [22,23]. Pine needles are mainly composed of polysaccharides (cellulose, hemicelluloses) which can be hydrolysed into simple sugars, that in turn may be microbially fermented into valuable products of commercial importance like biofuel, biomaterials, energy, and other products (biorefining) [5,22,23]. Thus, PNB may be exploited as a resource that might help not only mitigate the problems associated with pine needle accumulation but might also realize the "valorization of waste". Rare reports are available on usage of PNB as feedstock for production of biofuels /chemicals [5].

The current study aimed at DoE-based optimization for the production of IL-stable cellulase from *Bacillus subtilis* SV1 using agroindustrial residues as substrates, and its prospective for saccharification of PNB through OPCB.

2. Results and Discussion

2.1. Cellulolytic Bacteria

Primary screening showed that all the five bacterial isolates D1, J2, L10, SV1 and SV29 exhibited cellulolytic activity (Figure 1a). Secondary (quantitative) screening indicated that bacterial isolate SV1 produced maximum cellulase (CMCase) titre (2.201 IU/mL ± 0.06) after 72 h of fermentation, and was followed by isolates SV29 (1.574 IU/mL ± 0.035), L10 (1.115 IU/mL ± 0.06), D1 (1.102 IU/mL ± 0.05) and J2 (0.862 IU/mL ± 0.06) (Figure 1b). Of all the five bacterial isolates, D1, L10 and SV29 displayed maximum growth after 72 h, and showed a direct relationship between growth and cellulase production. However, partial association between growth and cellulase production was exhibited by the isolates J2 and SV1 [5] (Figure 1c). The cellulase from all the isolates was also analyzed for its FPase activity from 24 to 72 h. Bacterial isolate SV29 exhibited maximum FPase activity after 72 h (0.233 IU/mL ± 0.0035) (Figure 1d). Microbial cellulases have got vast application potential in various industries [19]. Diverse ecological habitats have been explored for the isolation of several cellulolytic bacteria like *Bacillus subtilis* MS 54 [19], *Bacillus licheniformis* K-3 [20], *Bacillus subtilis* G_2 [5], *Paenibacillus terrae* ME27-1 [24].

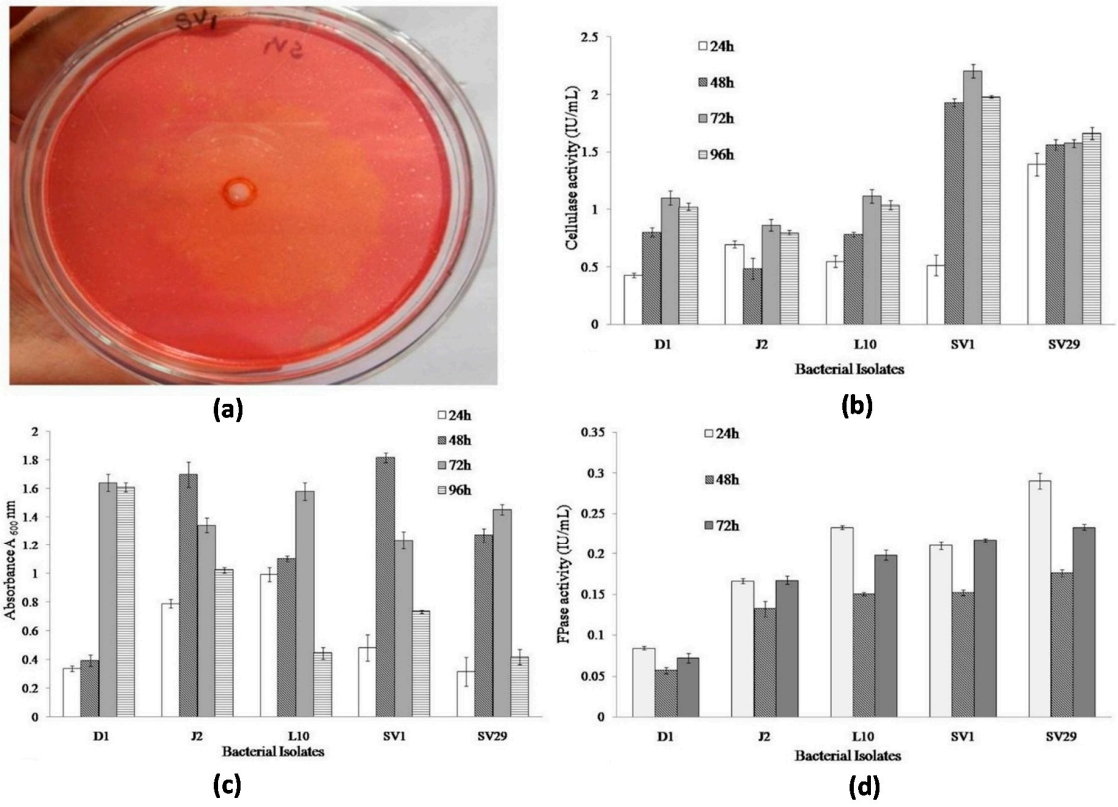

Figure 1. Cellulolytic activity of bacterial isolate SV1 (**a**); CMCase activity (**b**); Growth profile (**c**); and FPase activity (**d**) of the bacterial isolates under submerged fermentation.

2.2. IL Stability of Cellulase

Crude cellulase produced from all the bacterial isolates was examined for its stability/tolerance towards IL 1-ethyl-3-methylimidazolium methanesulfonate (EMIMS, 5%, *v*/*v*). Cellulase from SV1 exhibited maximum stability (residual activity, 161.6%) and retained highest activity after 72 h of

prolonged exposure to the IL, EMIMS (5%, v/v) followed by SV29 (115%), J2 (54.8%), L10 (53.1%) and D1 (18%) (Figure 2a). The IL-stability of cellulase from bacterial isolate SV1 was further examined with higher concentrations of EMIMS (10%–50%, v/v). The cellulase from bacterial isolate SV1 exhibited 98.12% residual activity in 50% EMIMS after 4 h of incubation while it showed substantial residual activity of 72.9%, 69.52%, 65.71%, and 88.93% in 10%, 20%, 30% and 40% EMIMS, respectively. The enzyme retained 70.14% residual activity even after 48 h of incubation with EMIMS. However, after 72 h of incubation, the activity decreased considerably (Figure 2b). Thus, cellulase of bacterial isolate SV1 exhibited excellent stability towards IL.

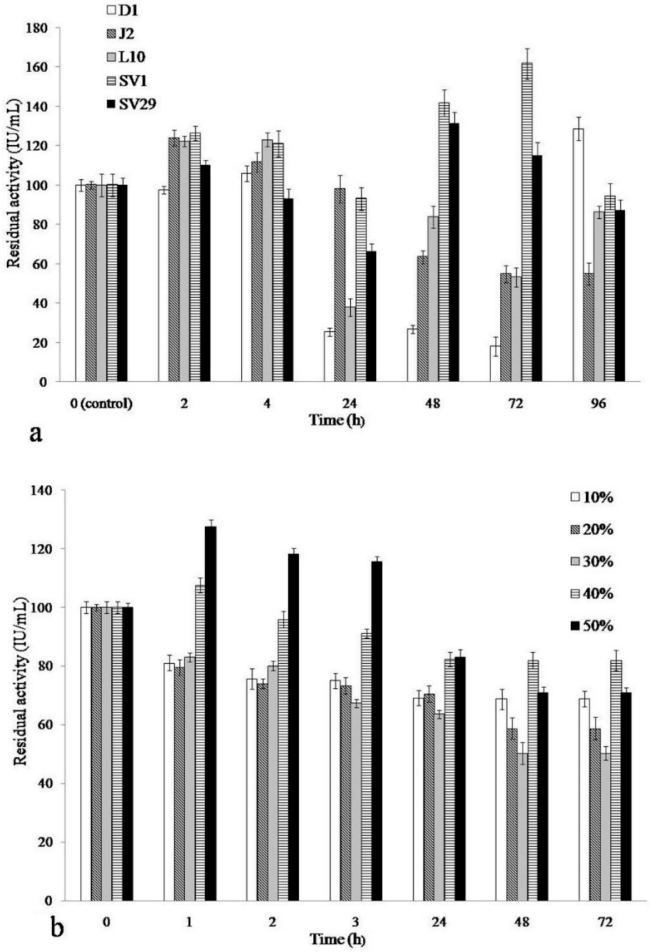

Figure 2. Stability of cellulase from the bacterial isolates against ionic liquid, 1-ethyl-3-methylimidazolium methanosulfonate (**a**); stability of cellulase of bacterial isolate SV1 at different concentrations of 1-ethyl-3-methylimidazolium methanosulfonate (**b**).

The potential of IL-stable cellulases may be exploited for biorefining of LB. IL-stable cellulases have been reported from several microorganisms. *B. subtilis* I-2 cellulase exhibited high stability (activity retention 93%–98%) after 72 h with 1-ethyl-3-methylimidazolium methanesulfonate (EMIMS) [4]. IL tolerant cellulase from *B. subtilis* G_2 showed 95%–100% stability at 20%–50% 1-ethyl-3-methylimidazolium methanosulfonate after 72 h of exposure [5]. Similarly, activity of cellulase Hu-CBH1 from heat tolerant haloalkalaiphilic archaeon *Halorhabdus utahensis* remained unchanged or even slightly stimulated in the presence of 20% [EMIM]Ac [9]. *Fusarium oxysporum* cellulase BN showed quite high and long-term stability in the presence of [Emim][DMP] and [Emim][MtSO4] [15]. Thus, ionic liquid stability of cellulases from different microorganisms varies with different types of ionic liquids (Table 1).

Table 1. Ionic liquid stability of cellulases from different microorganisms.

Microorganism	Ionic Liquid (IL)	IL Concentration (%)	Stability (Residual/Relative Activity, %)	Reference
Bacillus subtilis I-2	1-ethyl-3-methylimidazolium methanesulfonate	10	93–98	4
Bacillus subtilis G_2	1-ethyl-3-methylimidazolium methanesulfonate (EMIMS) 1-butyl-3-methylimidazolium chloride 1-ethyl-3-methylimidazolium bromide 1-ethyl-3-methylimidazolium acetate 1-butyl-3-methylimidazolium trifluoro methanesulfonate	20–50	95–100	5
Halorhabdus utahensis	1-Ethyl-3-methylimidazolium acetate ([Emim]Ac) 1-ethyl-3-methylimidazolium chloride ([Emim]Cl 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) 1-allyl-3-methylimidazolium chloride	20	100 (Remained unchanged) Slightly increased Slightly increased 100 (remained unchanged)	9
Pseudoalteromonas sp.	1-ethyl-3-methylimidazolium Methanesulfonate 1-ethyl-3-methylimidazolium bromide 1-ethyl-3-methylimidazolium acetate 1-butyl-1-methylpyrrolidinium trifluromethanesulfonate 1-butyl-3-methylimidazolium trifluoromethanesulfonate 1-butyl-3-methylimidazolium trifluoromethanesulfonate	20	59 67 93.47 80.2 74.69 73.2	11
Fusarium oxysporum BN	1-ethyl-3-methyl-imidazolium dimethylphosphate 1-ethyl-3-methyl-imidazolium methylphosphonate 1-ethyl-3-methylimidazolium Phosphinate	10	93 More than 84 More than 74	15
***Bacillus subtilis* SV1**	**1-ethyl-3-methylimidazolium methanesulfonate**	**10–50**	**72.9–98.12**	**Present study**

2.3. Identification of IL-stable Cellulase Producing Bacterium

The bacterial isolate SV1 that produced cellulase which exhibited substantial IL-stability was examined on the basis of morphological, microscopic, and 16S rDNA sequence analysis. Bacterial isolate SV1 showed rapid growth on nutrient as well as CMC agar plates, and formed slimy, off-white, irregular colonies (Figure 3a). The bacterial isolate SV1 was Gram-positive, rod shaped (bacillus), and had spore-forming ability. The bacterial isolate SV1 possessed potential capability of hydrolyzing starch, xylan, gelatin, casein and triglycerides (Figure 3b). The phylogenetic study of isolate SV1 based on 16S rDNA sequence analysis showed its highest homology with several other *Bacillus subtilis* strains available in the GenBank database (Figure 3c,d). Hence, this isolate is one of the strains of *Bacillus subtilis*, and designated as *Bacillus subtilis* SV1. The sequence was submitted to GenBank under accession number *KU871117.*

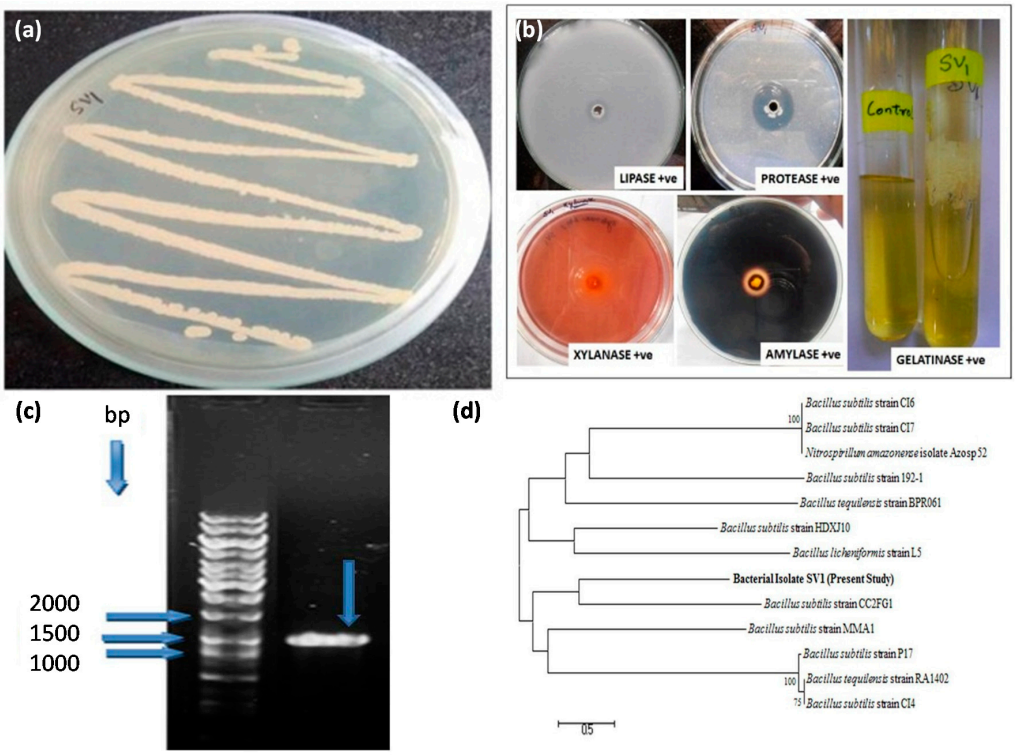

Figure 3. Identification of bacterial isolate SV1. Plate culture of isolate SV1 on nutrient agar (**a**); hydrolytic potential of bacterial isolate SV1 (**b**); PCR-amplified amplicon of 16S rDNA sequence from isolate SV1 (**c**); phylogenetic homology analysis of 16S rDNA sequence from isolate SV1 (**d**).

2.4. DoE Based Optimization of Cultural and Environmental Variables for Cellulase Production

DoE based optimization approach not only surmounts the limitations of the OVAT approach but represents an efficient tool for enhancing product yield by optimization of process variables [17,19]. In the present study, cultural and environmental variables were optimized using response surface methodology (RSM) to boost the production of ionic liquid stable cellulase from *B. subtilis* SV1. Central composite design (CCD) of RSM was applied for determining the optimum levels of the selected independent variables, i.e., wheat bran (A), spirulina powder (B) and medium pH (C) and incubation time (D), and the interactions between the variables. Based on the design, 30 experimental runs were executed, and the corresponding responses are presented in Table 2. ANOVA was performed (Table 3) and a polynomial equation was obtained (Equation 1) in which cellulase yield (Y, response) is presented as a function of various variables. The polynomial equation obtained after multiple regression analysis was as follows:

$$\begin{aligned} \text{Response Y (Cellulase production)} &= 1.77 + 0.32A + 0.17B + 0.25C + 0.093D + 0.12A^2 \\ &- 0.025\,B^2 + 9.688E - 004\,C^2 - 0.012\,D^2 + 0.031\,AB - 0.13\,AC + 0.079\,AD - 0.14\,BC + \\ &0.081\,BD + 0.17\,C \end{aligned} \quad (1)$$

The equation shows the variation of response (cellulase yield) as a function of various variables, i.e., wheat bran (A), spirulina powder (B) and medium pH (C) and incubation time (D).

Table 2. Experimental and predicted response for CMCase production from *B. subtilis* SV1 based on RSM-designed experiments for optimization of medium and environmental variables.

Runs	Experimental Variables *				Response (Enzyme Activity, IU/mL)	
Run number	A	B	C	D	Experimental	Predicted
1	1.5	1.5	5	72	0.66	0.64
2	1.5	3.0	9	72	1.97	2.18
3	2.25	2.25	7	48	1.89	1.77
4	1.5	1.5	9	72	2.31	2.02
5	2.25	2.25	7	48	1.82	1.77
6	1.5	3.0	9	24	1.80	1.66
7	2.25	2.25	7	96	1.85	1.91
8	1.5	1.5	5	24	1.26	1.11
9	2.25	2.25	7	48	1.19	1.77
10	3.0	1.5	9	24	2.05	1.98
11	2.25	3.75	7	48	1.99	2.02
12	3.75	2.25	7	48	2.29	2.91
13	3.0	3.0	9	72	3.04	2.79
14	1.5	3.0	5	72	1.70	1.36
15	2.25	0.75	7	48	1.08	1.33
16	1.5	1.5	9	24	1.98	1.81
17	2.25	2.25	11	48	2.25	2.28
18	1.5	3.0	5	24	1.63	1.51
19	3.0	1.5	5	72	1.90	1.64
20	3.0	3.0	5	72	2.21	2.49
21	3.0	3.0	5	24	2.43	2.32
22	3.0	3.0	9	24	1.81	1.95
23	3.0	1.5	9	72	2.25	2.50
24	2.25	2.25	7	0	1.32	1.54
25	2.25	2.25	7	48	1.90	1.77
26	2.25	2.25	3	48	1.02	1.28
27	2.25	2.25	7	48	1.97	1.77
28	3.0	1.5	5	24	1.89	1.80
29	0.75	2.25	7	48	1.26	1.62
30	2.25	2.25	7	48	1.87	1.77

*A—Wheat bran (%, *w*/*v*), *B—Spirulina powder (%, *w*/*v*), *C—pH, *D—Incubation time (h).

Table 3. Results of ANOVA for cellulase production by *B. subtilis* SV1 based on RSM designed experiments for medium and environmental variables *.

Source	Sum of Squares	DF	Mean Squares	F Value	Prob > F	Significance
Model	6.64	14	0.47	5.18	0.0015	Significant
A	2.49	1	2.49	27.23	0.0001	Significant
B	0.71	1	0.71	7.78	0.0138	Significant
C	1.50	1	1.50	16.35	0.0011	Significant
D	0.21	1	0.21	2.26	0.1533	-
A^2	0.41	1	0.41	4.51	0.0507	-
B^2	0.017	1	0.017	0.19	0.6694	-
C^2	2.574×10^{-5}	1	2.574×10^{-5}	2.813×10^{-4}	0.9868	-
D^2	3.727×10^{-3}	1	3.727×10^{-3}	0.041	0.8428	-
AB	0.016	1	0.016	0.17	0.6859	-
AC	0.27	1	0.27	2.97	0.1051	-
AD	0.099	1	0.099	1.08	0.3146	-
BC	0.30	1	0.30	3.33	0.0879	-
BD	0.11	1	0.11	1.15	0.3007	-
CD	0.45	1	0.45	4.93	0.0422	Significant
Residual	1.37	15	0.092			-
Lack of fit	0.95	10	0.095	1.14	0.4714	Not significant
Pure Error	0.42	5	0.084			-
Cor Total	8.01	29				-

*A—Wheat bran (%, *w*/*v*); B—Spirulina powder (%, *w*/*v*); C—pH; D—Incubation time (h).

The model F-value of 5.18 implies the model is significant. The chance of getting this high model F-value due to noise is quite low (0.15%). The significance of the model terms is shown by probability > F < 0.05. Probability > F < 0.4714 shows that lack of fit is not significant which in turn implies the strength and sturdiness of the model. Based on p value ($p < 0.05$), A, B, C, and CD were found to be the significant model terms. A low value of standard deviation (0.30) and high coefficient of determination, R-square (0.8286), point towards robustness of the model that has a reasonably good predictability. Adequate precision of 10.587 indicates an adequate signal for the present results.

The interactive effects of independent variables were investigated by analyzing the 3-D response surface plots. The interaction between wheat bran and pH (AC), and spirulina powder and pH (BC) had a negative impact on the cellulase production (Figure 4a,b) whereas the interaction between medium pH and incubation time (CD) was found to have a positive significance for enzyme production as depicted in the 3-D response plot (Figure 4c). It is obvious from the graph that increasing pH as well as incubation time led to enhanced response (cellulase yield). Figure 4d shows the perturbation plot. Perturbation plot explains the increase or decrease in the response when the value of each variable is changed keeping other variables constant with respect to the chosen reference point. When the variable A (wheat bran) was changed from the reference point, it had the maximum positive effect on the response among all the variables whereas variable D (incubation time) had the least effect on the response. This is also substantiated from the equation generated by the design (Equation I). It showed the maximum value of A (+0.32 A) and the least value of D (+0.093).

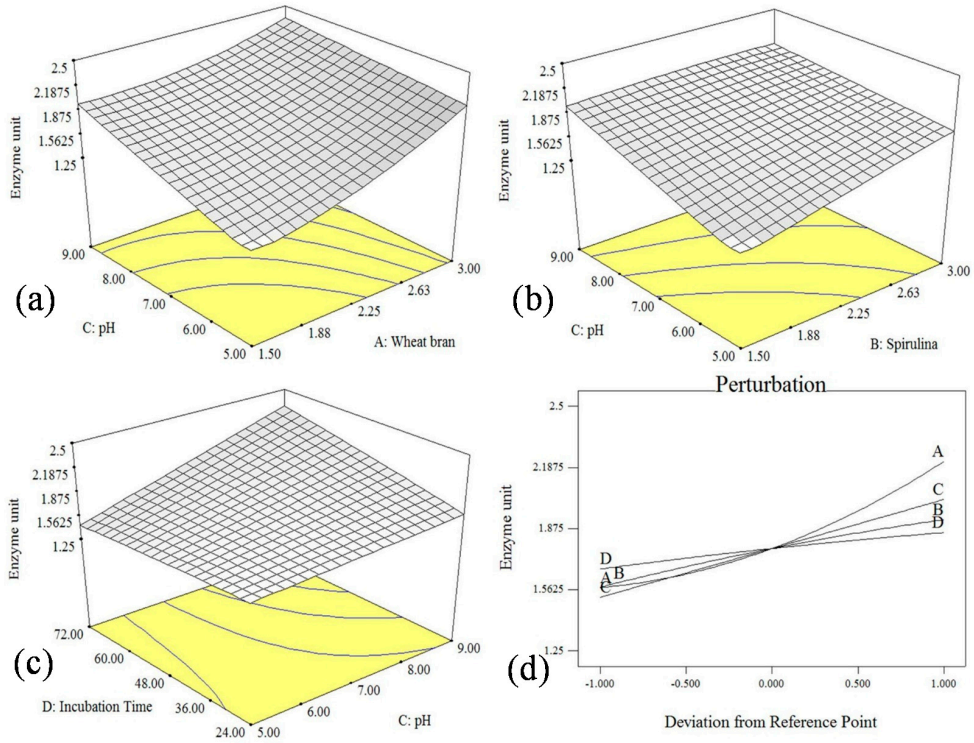

Figure 4. Response surface plots showing the interactions between different variables for cellulase production from *B. subtilis* SV1, wheat bran and medium pH (**a**); spirulina powder and pH (**b**); and pH and incubation time (**c**); perturbation plot showing effect of individual variables (**d**).

The validation of the statistical model was done by using point prediction tool of RSM in which the optimum value of all the four variables, i.e., wheat bran, 3.0 g; spirulina powder, 3 g; medium pH 9 and incubation time, 72 h was determined and the experiment was conducted. The close proximity of observed (CMCase yield of 3.11 IU/mL) and predicted responses (3.10 IU/mL) validated the model. DoE based optimization enhanced cellulase yield by 1.41-fold as compared to that under unoptimized

conditions (2.201 IU/mL). One of the major targets for bioprocess development is to achieve enhanced product yield which in turn may contribute substantially towards improving the overall economy of the process; optimization of process variables by DoE, especially the response surface methodology, has quite often been used [18,19]. Though RSM based-optimization of cellulase production has been attempted by several researchers, there are only a few reports on the optimization for production of IL-stable cellulase using RSM. IL-stable cellulase production from *Bacillus subtilis* I-2 [4] was increased by 4.1 and from *B. subtilis* G_2 [5] was increased by 2.66 fold by sequential RSM based optimization of medium and environmental variables. Similarly, many *Bacillus* spp. have been reported to yield enhanced cellulase due to RSM mediated optimization of variables [18–21].

2.5. Some properties of B. subtilis SV1

The *B. subtilis* SV1 cellulase exhibited activity over a broad temperature range (4–90 °C) with an optimal temperature of 45 °C. The cellulase activity at different pH (4–10) showed its optimal activity at pH 10 indicating its alkaline behavior (Figure 5a,b). Similar to the current results, cellulase from *B. aquimaris* [25] and *Pseudomonas fluorescens*, *B. subtilis*, *E. coli* and *Serratia marscens* [26] showed alkaline behavior and showed optimum activity at 40–45 °C [25,26]. The cellulase from *B. subtilis* G_2 showed its optimum activity at 45 °C and pH 7 [5]. The optimal temperature of cellulase from *B. subtilis* YJ1 was 60 °C and but exhibited a slightly acidic behavior in contrast to the present study [27]. Thermostability of enzyme depends on molecular interactions which impart a high degree of stabilization due to various forces like hydrophobic and electrostatic interactions, hydrogen, disulphide or other covalent bonding [5,19,20]. Deviations from optimum pH may change the native 3-dimensional structure of enzyme which may lead to alterations in substrate/cofactor/coenzyme binding, and hence decrease or cause total loss of activity [5].

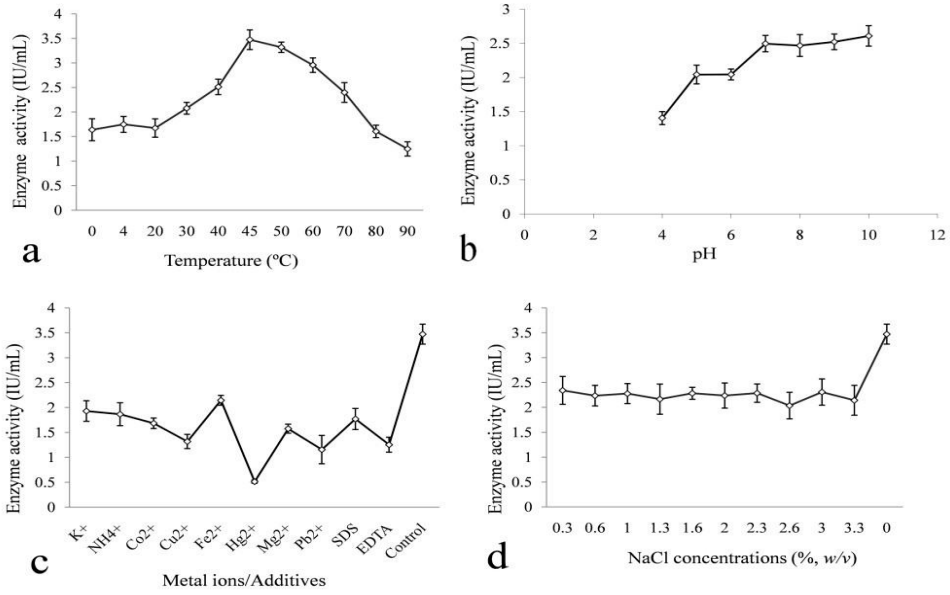

Figure 5. Biochemical properties of cellulase from *B. subtilis* SV1, effect of temperature (**a**); pH (**b**); metal ions/additives (**c**); and sodium chloride on activity of cellulase (**d**).

Each of the metal ions/additives showed an inhibitory effect on cellulase activity. Among all the metal ions/additives, Fe^{2+} had least inhibitive effect while Hg^{2+} had a maximum inihibitory effect on enzyme activity (Figure 5c). Similar to the present study, the activity of cellulase from *B. subitlis* YJ1 was inhibited by Hg^{+}, Cd^{2+}, Fe^{2+}, Fe^{3+} and SDS [27]. *B. vallismortis* RG-07 cellulase was slightly inhibited by Cu^{2+} and Zn^{2+} but Hg^{2+} and Mn^{2+} strongly inhibited cellulase [28]. The Cu^{2+} and Co^{2+} may inhibit cellulase by competing with other cations that might be associated with enzyme resulting in

decreased activity. The published reports suggest that Hg^{+} might cause inhibition of activity due to its interactions/binding with –SH or –COOH group of amino acids, or interactions with tryptophan [28]. Metal ions can have a profound effect on the enzyme activity, and can stimulate or hamper activity by multiple mechanisms [5,19].

Salt tolerance of cellulase has been reported to be very much related with IL-stability of the enzyme [11]. Salt tolerance of cellulase was examined by including different concentrations of NaCl (0.3%–3.3%) in enzyme assay reaction mixture. Though cellulase activity decreased in the presence of NaCl, over a range of NaCl concentrations, enzyme activity remained almost constant (66.4%). The results show that cellulase from *B. subtilis* SV1 has considerable salt tolerance (halotolerance) (Figure 5d). Contrary to the present study, cellulase from *Marinimicrobium* sp. LS-A18 retained more than 88% residual activity at 0%–25% NaCl concentrations [29] while cellulase from *B. flexus* NT showed 70% residual activity at 15% NaCl concentration [30]. Halotolerant cellulases are hypothesized to be good candidates that may exhibit good IL-stability due to their adaptation to high salinity [11,12]. Halotolerant enzymes show structural modification like the prevention of protein aggregates' formation through electrostatic repulsion due to the presence of too much charged acidic amino acids on the surface imparting stability in ionic liquid [5,11].

2.6. Pretreatment and Enzymatic Saccharification under a One Pot Consolidated Bioprocess

OPCB allows the execution of multiple unit operations in a single vessel, thus enhancing the overall economy of the process [11]. In the present study, OPCB involved the pretreatment of pine needle biomass with IL (EMIMS) followed by in situ enzymatic saccharification of PNB by using IL-stable cellulase from *B. subtilis* SV1 in a single pot. It is apparent that IL-stability of saccharifying enzymes (cellulase) is mandatory for OPCB. After executing consolidated IL-pretreatment and enzymatic saccharification in one vessel i.e., OPCB, reducing sugar yield was determined to assess the efficacy of the process. Pine needle biomass was pretreated with different IL-concentrations and an increase in sugar yield was observed from 0.149 g/g to 0.200 g/g with the increasing IL concentration from 10% to 50%. The sugar yield in control I was 0.125 g/g (non-pretreated biomass) (Figure 6).

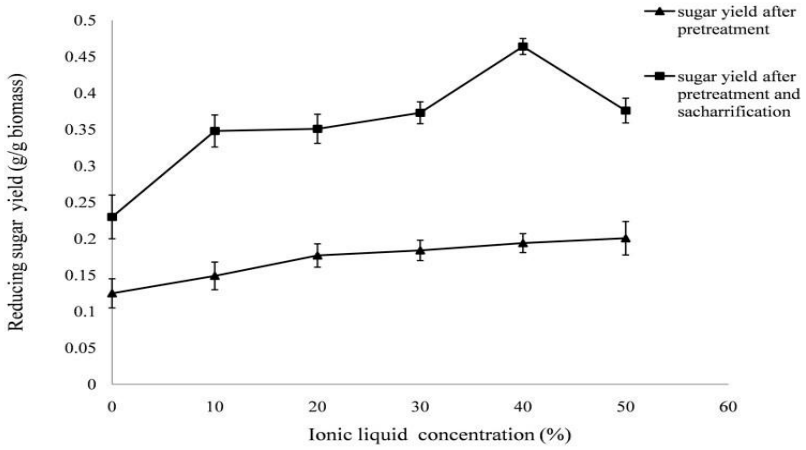

Figure 6. Reducing sugar yield obtained after IL pretreatment and enzymatic saccharification of pine needle biomass using cellulase from *B. subtilis* SV1 (3.1 IU/mL) under one pot consolidated bioprocess. Control shows sugar yield obtained by direct cellulase treatment, i.e., without IL pretreatment.

After pretreatment, enzymatic saccharification was carried out using cellulase from *B. subtilis* SV1 in the same vessel and reducing sugar yield was estimated. A maximum of 0.464 g/g pine needle biomass of reducing sugar yield was observed which was 2.01-fold higher than control II (0.230 g/g pine needle biomass) (Figure 6) at 40% EMIMS concentration. Thus, saccharification efficiency of 65.9% was obtained for PNB under OPCB. From the results, it is inferred that pretreatment

resulted in a higher degree of delignification and made cellulose accessible to cellulase and hence yielded higher sugar as compared to the control. OPCB may be commercially vital due to its huge economic benefits [14], but a consolidated process involving application of ionic liquids for LB pretreatment and in situ enzymatic saccharification has scarcely been reported [31]. One pot IL (1-ethyl-3-methylimidazolium acetate, [C2mim][OAc]) pretreatment following the enzymatic saccharification with IL stable cellulase was carried out for switch grass to attain 80% glucose and xylose liberation [31]. Ionic liquid coupled with HCl led to synergistic effects on sugar release from corn stover [32]. Pretreatment of sugarcane bagasse has been effectively done with a combination of ionic liquid and surfactant [33]. Similarly, an enhanced sugar release was observed from eucalyptus, rice straw and grass, but not from pine [34]. Ionic liquid pretreatment of sugarcane bagasse with pure 1,3-dimethylimadazolium dimethyl phosphate gave better results as compared to that obtained with aqueous solution of IL [35]. Pretreatment of rice straw with 20% cholinium lysine IL-water mixtures with subsequent hydrolysis liberated 81% and 48% glucose and xylose yield, respectively [36].

3. Materials and Methods

3.1. Chemicals and Media

Various media, reagents, and chemicals employed in the experiments for current research investigation were of high grade standard and purity, and were procured from suppliers like HiMedia Laboratories, Merck and Co., Ranbaxy Fine Chemicals and Sigma-Aldrich. Ionic liquid 1-ethyl-3-methylimidazolium methanesulfonate used in the current study was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Cellulase Producing Bacteria

Bacterial isolates used in this study were procured from culture collection of Fermentation Biotechnology Laboratory, School of Biotechnology, University of Jammu (Jammu, India). The isolates were examined primarily for cellulolytic activity by plate assay by using Congo red staining method [5,20]. Congo red stain binds specifically with β-1,4 linked glycosidic linkage in cellulose. Bacterial isolates which produce cellulase cleave β-1,4-glycosidic bonds in carboxymethyl cellulose (CMC) agar, and form a zone of clearance around the colonies. Bacterial isolates exhibiting considerable cellulase activity were subjected to secondary screening.

3.3. Submerged Fermentation for Cellulase Production

Cellulolytic bacterial isolates (D1, J2, L10, SV1 and SV29) selected on the basis of primary screening were subjected to submerged fermentation in shake flasks for production and quantification of cellulase activity. The bacterial isolates were grown under shaking (180 rpm) at 37 °C for 18 h in carboxymethyl cellulose-peptone-yeast extract (CPYE) broth to attain the required cell concentration (A600, 0.9), and then inoculated (2%, v/v) into the CPYE production medium [19]. Submerged fermentation was carried out at 37 °C under shaking (180 rpm). The samples withdrawn at varying time intervals (24, 48, 72 and 96 h) were centrifuged (Eppendorf centrifuge 5804R) at 10,000 × g for 10 min. The supernatant was considered as crude cellulase and assayed for activity. Carboxymethyl cellulase (CMCase, cellulase) and filter paperase (FPase) activities were determined using substrate carboxymethyl cellulose (CMC) and Whatman No. 1 filter paper as substrates, respectively [19]. The amount of reducing sugar released was measured spectrophotometrically (UV-1800, Shimadzu, Japan) by using dinitrosalicylic acid (DNSA) method [37]. One unit (IU) of CMCase and FPase was defined as the amount of enzyme which produced one µmole of glucose equivalent per mL per min under assay conditions. The growth profile of the bacterial isolates was measured spectrophotometrically (A_{600}).

3.4. IL Stability of Bacterial Cellulases

Cellulases produced from bacterial isolates were pre-incubated with IL 1-ethyl-3-methylimidazolium methanesulfonate (EMIMS, 5%, v/v) at room temperature. Samples were

withdrawn at various time intervals (1, 2, 3, 4, 24, 48, 72 and 96 h) and examined for residual activity. Cellulase from bacterial isolate that exhibited maximum stability against EMIMS for a prolonged time period was further investigated for its tolerance at higher concentrations of EMIMS i.e., 10%–50%.

3.5. Identification of the Selected Bacterium

Identification of the selected bacterial isolate SV1 that was capable of producing IL stable cellulase was done by studying the cultural, morphological, and microscopic characteristics. The identity of the bacterium was further confirmed by analyzing its 16S rDNA sequence and comparing it with that other sequences available in GenBank (National Center for Biotechnology Information) [5,20]. CMC agar plates were used for the examination of colony morphology of bacterial isolate SV1. Gram staining and endospore staining analysis was done for microscopic examination. The bacterial isolate SV1 was observed for its ability to produce various hydrolytic enzymes viz. lipase, amylase, xylanase, protease, and gelatinase [5,20]. Genomic DNA was extracted (Wizard Genomic DNA Preparation Kit, Promega Co., Madison, WI, USA) and PCR-amplified using universal 16S rDNA primers (forward primer 5′-AGTGTTTGATCCTGGCTCAG-3′, reverse primer 5′-CGGCTACCTTGTTACGACTTT-3′) for 16S rDNA sequence analysis [19]. The amplified product was eluted (Axygen DNA gel extraction kit, Union City, CA, USA) and sequenced (SciGenom Labs Pvt. Ltd., Chennai, India). BLAST analysis of the DNA sequence data was performed for closest homology. The neighbor-joining phylogenetic analysis was carried out with MEGA 6 software. Phylogenetic tree was constructed using MEGA 6 (http://www.megasoftware.net).

3.6. Optimization of Cultural and Environmental Variables for Cellulase Production

Cellulase production from SV1 was enhanced by optimizing the medium components and environmental variables by employing a central composite design of (CCD) of response surface methodology (RSM). The medium variables selected for optimization were crude carbon/nitrogen source viz. wheat bran (A) and spirulina powder (B), and environmental variables were medium pH (C) and incubation time (D). The maximum and minimum range of independent variables was selected based on the already published papers [5]. The variables investigated and full experimental plan are presented in Table 4. A total of 30 experiments were conducted and the results were analyzed using design of expert software version 6.0 (Stat-Ease, Inc., Minneapolis, MN, USA) (Table 5). The three-dimensional (3-D) response surface plots were used to understand the interaction between the variables and to analyze the optimum value of each parameter to maximize cellulase production. The regression equation gave an empirical model that related the measured response to the independent variables of the experiments. The statistical model was then validated in shake-flask experiments for cellulase production under the conditions predicted based on point prediction tool. The response values (Y) were measured as the average of triplicate experiments.

Table 4. Experimental range and levels of the medium and environmental variables used in RSM for cellulase production from *B. subtilis* SV1.

Study Type: Response Surface			**Experiments: 30**	
Initial Design: Central Composite			**Design Model: Quadratic**	
Response	**Name**	**Units**		
Y	Enzyme activity	IU/mL	Experimental values	
Factors	Name	Units	Lower	Higher
A	Wheat Bran	%, *w*/*v*	1.5	3
B	Spirulina powder	%, *w*/*v*	1.5	3
C	Medium pH	-	5	9
D	Incubation time	H	24	72

Table 5. RSM-designed experiments for medium and environmental variables for cellulase production from *B. subtilis* SV1.

Runs	Experimental variables *			
Run number	A	B	C	D
1	1.5	1.5	5	72
2	1.5	3.0	9	72
3	2.25	2.25	7	48
4	1.5	1.5	9	72
5	2.25	2.25	7	48
6	1.5	3.0	9	24
7	2.25	2.25	7	96
8	1.5	1.5	5	24
9	2.25	2.25	7	48
10	3.0	1.5	9	24
11	2.25	3.75	7	48
12	3.75	2.25	7	48
13	3.0	3.0	9	72
14	1.5	3.0	5	72
15	2.25	0.75	7	48
16	1.5	1.5	9	24
17	2.25	2.25	11	48
18	1.5	3.0	5	24
19	3.0	1.5	5	72
20	3.0	3.0	5	72
21	3.0	3.0	5	24
22	3.0	3.0	9	24
23	3.0	1.5	9	72
24	2.25	2.25	7	0
25	2.25	2.25	7	48
26	2.25	2.25	3	48
27	2.25	2.25	7	48
28	3.0	1.5	5	24
29	0.75	2.25	7	48
30	2.25	2.25	7	48

*A—Wheat bran (%, *w*/*v*), *B—Spirulina powder (%, *w*/*v*), *C—pH, *D—Incubation time (h).

3.7. Some Properties of IL-Stable Cellulase

The cellulase produced under optimized process was used for studying the effect of temperature and pH on the activity. Cellulase activity was assayed at different temperatures (4–90 °C) for deducing the effect of temperature on activity. Similarly, the effect of pH was realized by executing an activity assay at different pH by using buffers (50 mM) of appropriate pH: acetate buffer (pH 4–5), glycine-NaOH buffer (pH 6–8) and phosphate buffer (pH 9–10) [4].

For determining halotolerance of cellulase, an activity assay was executed in the presence of varying concentrations of sodium chloride (0.3%–3.0%).

The cellulase activity was assayed in the presence of several metal ions/additives viz. potassium chloride, ammonium chloride, cobalt chloride, copper chloride, ferrous sulphate, mercuric chloride, magnesium sulphate, lead acetate, sodium dodecylsulphate (SDS), and ethylenediaminetetraacetic acid (EDTA) at a final concentration of 1.66 mM. The activity without metal ion/additive was considered as control.

3.8. Pretreatment and Enzymatic Hydrolysis of PNB Using One Pot Consolidated Bioprocess (OPCB)

3.8.1. Pine Needle Biomass (PNB)

Pine needles used in the study were procured from forest area near Udhampur (Jammu, India). Pine needles were thoroughly washed with tap water and then air dried at 50 °C, and dry matter

content between 91% and 94% was obtained. The dried material was ground, and the fraction passing through a 4–5 mm sieve was collected and used for further experiments [23]. The powdered PNB was composed of (dry weight basis) holocellulose (64.12%), pentosan (14.12%) and lignin (27.79%) [23].

3.8.2. One Pot Consolidated Bioprocess

Pretreatment of PNB with 1-ethyl-3-methylimidazolium methanesulfonate (EMIMS) was carried out in order to partially remove lignin and disrupt the crystalline structure of cellulose. An appropriate quantity of dried and ground pine needles biomass was immersed in EMIMS at different concentrations (10%–50%, *w*/*v*). The contents were incubated at 70 °C under shaking at 180 rpm for 18 h, and then subjected to enzymatic hydrolysis in situ (same pot) using IL-stable cellulase preparation (at 311 IU/g of PNB). After addition of cellulase preparation the contents were incubated at 37 °C under shaking (180 rpm) for 24 h, following which reducing sugar was assayed. The untreated PNB was considered as control I, and enzymatically hydrolyzed untreated PNB was used as control II.

4. Conclusions

Bacillus subtilis SV1 is capable of utilizing agroindustrial residues as carbon and nitrogen sources for growth and IL-stable cellulase production. DoE based optimization of process variables appreciably enhanced cellulase production (1.41-fold). Furthermore, integration of IL based pretreatment and enzymatic saccharification in a single unit (OPCB) gave excellent results as indicated by saccharification efficiency of PNB. Further research on the molecular basis of IL stability of cellulase, and functional mechanisms of IL mediated reduction of LB recalcitrance, is underway in our laboratory. Process-scale-up and other parameters need further investigation for harnessing the full potential of OPCB to ultimately realize a sustainable, economically viable and highly efficient biorefinery process, i.e., conversion of biomass to biofuel (ethanol, butanol, etc.) and other products of commercial importance.

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Author Contributions: Bijender Kumar Bajaj conceptualized the research problem; Surbhi Vaid designed the experiments; Parushi Nargotra performed the experiments, and wrote the draft MS; Bijender Kumar Bajaj and Surbhi Vaid corrected the MS, analyzed and interpreted data.

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