Optimization of Pre-treatment Using RSM on Wheat Straw and Production of Lactic Acid Using Thermotolerant, Inhibitor Tolerant and Xylose Utilizing Bacillus Sonorenesis Strain DGS15

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Research Article

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Optimization of pre-treatment using RSM on wheat straw and production of lactic acid using thermotolerant, inhibitor tolerant and xylose utilizing Bacillus sonorenesis strain DGS15

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Abstract

Thermotolerant lactic acid producing bacteria, isolated from red soil of brick kiln was identified by 16S rRNA sequencing as Bacillus sonorenesis, which showed remarkable capability to ferment sugars of lignocellulosic biomass after pre-treatment, yielding 0.97 g/g lactic acid with overall productivity of 0.38 g L\(^{-1}\)/h. RSM was employed to optimize the sulphuric acid pre-treatment combined with dilute NaOH and hot water pre-treatment. Pretreated wheat straw biomass had 40.4% cellulose, 18.4% hemicellulose, 12.4% lignin and 28.2 g L\(^{-1}\) reducing sugar, while native wheat straw biomass had 36% cellulose, 25% hemicellulose, 20% total lignin, and 0.94 g L\(^{-1}\) reducing sugar. Scanning electron microscopy (SEM) revealed that the ordered and compact structure of wheat straw was destroyed upon pre-treatment. X-ray diffractogram (XRD) revealed 9.71% increase in crystallinity index (CrI) in pretreated biomass. FTIR spectrogram showed removal of lignin due to reduction of peak at 1640 cm\(^{-1}\) in pretreated biomass. Bacillus sonorenesis DGS15 is inhibitor tolerant [furfural (1.2 g L\(^{-1}\)) and HMF (2.4 g L\(^{-1}\))]. Furfural was consumed after 72 h of fermentation and HMF got accumulated with 3.75-fold increase in concentration in the fermentation broth. In terms of final concentration, yield, and fermentation duration, this
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is the best performance of DGS15 for lactic acid production utilizing xylose, glucose as the carbon source. All of these findings showed that the thermotolerant *Bacillus sonorensis* strain DGS15 is a novel, attractive candidate for producing lactic acid from lignocellulosic biomass.

**Keywords:** Lactic acid, wheat straw, RSM, sulphuric acid pretreatment, thermotolerant bacteria, xylose utilizing

# Introduction

Biomass is by far the largest source of energy, accounting for 1,150 million tons of oil equivalent and accounting for 79 percent of total energy supply. Researchers have been committed to exploring the synthesis of value-added chemicals and biofuels derived from lignocellulosic biomass due to the depletion of fossil fuels and environmental contamination [1]. Biofuels and value-added chemicals can be made from lignocellulosic biomass, which is a plentiful and inexpensive source of fermentable sugars. Wheat straw is a lignocellulosic material that can be utilised to make biofuels and other value-added products [2]. Lactic acid has wide applications in food, pharmaceutical, cosmetic, textile, fiber and feedstock industries and production of polylactic acid which is biodegradable and playing a key role in 3D printing industry [3]. Chemical synthesis can be used to produce racemic mixture of lactic acid, while microbial fermentation can yield an optically pure lactic acid using the desirable organisms [4]. Conventionally starchy materials are used for production of lactic acid; however, their high cost limits its industrial application. Lignocellulosic biomass being composed of cellulose (C6-sugars), hemicellulose (mainly C5-sugars) and lignin has been used for the large-scale production of several biobased products [5]. Because of the inflexible and difficult-to-degrade nature of the biomass cell walls, biomass pretreatments are critical steps in the low-cost bioconversion of cellullosic biomass to sugar. Pretreatments are employed to separate cellulose from amorphous lignin and hemicellulose. Chemical pretreatments with acid and alkali reagents have received a lot of attention due to their ease of use and effectiveness [6]. Plant cell walls, particularly their hemicellulose component, are hydrolyzed by acid pretreatments. In dilute and acidic states, H$_2$SO$_4$, HNO$_3$, and HCl are commonly employed for acid pretreatments [7–10]. In acidic conditions, solubilized hemicellulose can be transformed to xylose, α-monomer, and then over degraded in a severely acidic environment [11, 12]. Though glucose and xylose can be biologically transformed to a variety of biochemical building blocks, they can also be over degraded and converted to by-products such furfural and hydroxymethylfurfural (HMF) [8, 13]. As a result, an adequate acid concentration, reaction temperature, and other key variables must be determined experimentally in order to achieve selective hydrolysis using an acid reagent. Wheat straw was pretreated with dilute
sulfuric acid in a prior study, and the effects of the chemicals on the pre-
treatment were investigated. In addition, a statistical model and a computer
software were used to optimize the pretreatment to avoid overdegradation
of biomass [14]. Because xylose is derived from hemicellulose, lignin can be
chemically and biologically transformed into useful molecules such as organic
solvents, aromatic compounds, and fuel additives. By increasing enzyme acces-
sibility to cellulose, removing hemicellulose and lignin could greatly improve
enzyme digestibility. For effective saccharification and lignin isolation, a pre-
treatment with dilute sulfuric acid followed by aqueous ammonia would be
required. Thermotolerant lactic acid producing bacteria have attracted many
researchers because of its ability to ferment hydrolysates effectively at rela-
tively high temperature (50°C) enabling mass lactic acid production without
cooling expenses and carbon dioxide production [15]. The present work reports
isolation and characterization of a novel thermotolerant Bacillus sonorenesis
DGS15, and a statistical method and model were used to examine pretreatment
parameters and optimize the pretreatment of wheat straw using sulphuric acid.
Following statistical analysis, the crystallinity index (CrI) was measured using
an X-ray diffractometer (XRD), and the molecular structure of wheat straw
was investigated using a Fourier transform infrared (FTIR) analysis and the
structural changes were seen using Scanning Electron Microscopy (SEM). Fur-
ther, producing lactic acid from wheat straw hydrolysate using thermotolerant
DGS15.

Methods

Isolation of thermotolerant lactic acid bacteria

Samples from bakery waste, brick kiln, municipal corporation waste, degraded
bark of tree, hot water springs were collected from various locations of Patiala,
Punjab. Samples were inoculated in nutrient broth at 50°C for 24 hours under
shaking conditions (120 rpm) for isolation and enumeration of cultivable bacte-
ria. Screening of lactic acid producing bacteria was done using the method by
Ye et al. [16] and incubated at 50°C for 48 h. The strain with highest lactic acid
production was selected and biochemically characterized using different test
(gram staining, capsule staining, catalase, nitrate reductase, Voges- Proskauer
reaction, urease test, carbohydrate fermentation, urease, starch hydrolysis,
methyl red test) as described in Bergey’s Manual of Systematic Bacteriology
[17].

Molecular characterization of strain DGS15 by 16S
rRNA Analysis

Genomic DNA was isolated from bacterial strain DGS15 using DNA Extrac-
tion Solution (Cat No. 2120600021730) and 16S rRNA gene was ampli-
fied by polymerase chain reaction (PCR) by using primers 27 F (5’ -
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AGAGTTTGATCCTGGCTCAG - 3 ’ & 1392 R (5 ’ - GGTTACCTTGTTACGACTT - 3 ’). 16S rRNA gene sequencing of isolate DGS15 was done by Genei Laboratories Pvt Ltd and found to be 1466 bp long gene sequence. In order to identify the strain, sequences which were closely related to the 16S rRNA gene of DGS15 were downloaded from the NCBI website [16S ribosomal RNA sequences (Bacteria and Archaea) database] using an algorithm Basic Local Alignment Search Tool (BLAST). Identification of 16S rRNA sequence of DGS15 was confirmed using available DNA data in EzTaxon-e database (https://eztaxon-e.ezbiocloud.net/) introduced by Kim et al. [18]. Closely related sequences obtained were undergone CLUSTALW (pairwise alignment) and phylogenetic tree was constructed by MEGA X software, using neighbor-joining method with Kimura two-parameter distance model for calculating evolutionary distances [19]. Bootstrap analysis was performed with 1000 replications to assess the statistical support for the phylogenetic tree.

Feedstock and pretreatment process of biomass

Wheat straw was collected from nearby villages of Patiala, dried at 50°C and pulverized to particle size of 0.5mm. Wheat straw was treated with different concentrations of sulphuric acid according to Box Behnken Design and further was given alkali and hot water treatment to convert complex sugars into simple sugars by method explained by Peng et al [20]. Treated and native samples were characterized by Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) spectroscopy. Change in surface morphology of native, pretreated wheat straw was analyzed by SEM (JEOL JSM 6510-LV, USA) operated at an applied voltage of 10-15 KV. X-ray diffractometer (Panalytical XPert Pro) was used to study the crystallinity index of samples. Samples were irradiated with monochromatized Cu Kα radiation (1.5406 Å) and analyzed between scan range (2θ) 5° to 50° at voltage (45 kV) and current (40 mA). The crystallinity index (CrI) was calculated by Segal et al. [21] as follows:

\[
CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100
\]

where \(I_{002}\) is the peak for the crystalline fraction of biomass (i.e., cellulose) at about \(2\theta\) of 22.5°, while \(I_{am}\) is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about \(2\theta\) of 18° in most literatures [22, 23]. The structural features of samples were studied by Perkin Elmer-Spectrum 400 FT-IR/FIR spectrophotometer having resolution of 1 cm\(^{-1}\). KBr disc method was used to record the spectrogram in spectral region between 4000 and 400 cm\(^{-1}\).

Design of Experiment

The Box-Behnken design (BBD) was used in this study to optimize the pretreatment process for maximum cellulose production. Sulphuric acid content
(A), biomass loading (B), and retention duration (C) were all optimized (Table 1). The experiment design is suitable for a quadratic response surface and yields a second-degree polynomial equation.

The following equation (2, 3) was used to express the relationship between coded and actual values:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}$$

(2)

The coded value of the independent variable is represented by $x_i$.
The value of the independent variable is encoded as $X_i$.
The value of the independent variable at the central point is encoded as $X_0$.
The difference between $X_i$ and $X_0$ is $\Delta X_i$.

Using Design Expert v13, the response is determined from the following equation,

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

(3)

The response is $Y$, the independent variables are A, B, and C, the intercept is $\beta_0$, the linear coefficients are $\beta_1$, $\beta_2$, and $\beta_3$, the square coefficients are $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$, and the interaction coefficients are $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$.

Table 1 Coded and actual levels of the factors for three factor Box-Behnken Design

| Independent variables | Symbols | Coded and actual values |
|-----------------------|---------|-------------------------|
| $\text{H}_2\text{SO}_4$ (%) | $X_1$ | -1 | 0.1 | 0.55 | 1 |
| Temperature (°C) | $X_2$ | 60 | 90 | 120 |
| Time (h) | $X_3$ | 15 | 60 | 105 |

Lactic acid Fermentation

Wheat straw hydrolysate was adjusted to pH 7.0 and incubated at 50°C at 120rpm in Orbitex-LEXLH serial no: 2005 400 117 for fermentation after adding seed culture. Samples were collected periodically to measure lactic acid, residual glucose and xylose utilization.

Tolerance to inhibitors

The effect of inhibitors such as furfural and HMF on cell growth and lactic acid production of $B. \text{sonorenesis}$ DGS15 was evaluated by a modified method of Liu et al. [24]. Cells were grown in Bushnell Haas medium (10 g L$^{-1}$ xylose and 5g L$^{-1}$ yeast extract (pH 7.0) with different concentrations of furfural and HMF ranging between 0-2.4g L$^{-1}$ at 50°C for 48 h. Samples were collected at specific time interval and evaluated for cell growth. The value of optical density at 620 nm ($OD_{620}$) was estimated to describe the inhibition of cell growth [15]
Analytical method

Cell growth was analyzed by UV–Vis spectrophotometer (U-2900, HITACHI) at a wavelength of 600 nm. Glucose and xylose were estimated in supernatant by DNS method and orcinol method [25, 26] at 660nm. Furfural and HMF concentration were estimated using Tu et al. [27] at 414nm and 436nm respectively. Lactic acid was estimated using Borshchevskaya et al. [28] at 390nm. The overall productivity (g L\(^{-1}\) h) was expressed as concentration of LA (g L\(^{-1}\))/time of incubation (h) and the overall yield was expressed as grams L-lactic acid/grams sugar consumed [16].

Results and Discussion

Isolation and characterization of xylose utilizing, thermotolerant and lactic acid producing bacteria

Out of 25, fifteen bacterial isolates from compost, degraded wood, mango wood, bakery waste or brick kiln soil were identified as lactic acid producers at 50°C. Among these, isolate DGS15 was found to be best lactic acid producer yielding 9.24 g L\(^{-1}\) lactic acid at 50°C and pH 7 when grown in Bushnell Hass minimal media containing 10 g L\(^{-1}\) xylose as the carbon source and 5g L\(^{-1}\) yeast extract (Figure 1). DGS15 was thermo tolerant and efficiently converted lignocellulosic waste to lactic acid, which makes it an attractive candidate for industry.

**Figure 1:** L-lactic acid production from xylose by DGS15 in shake flask containing Bushnell Hass minimal media (xylose 10g L\(^{-1}\), yeast extract 5 g L\(^{-1}\)) at pH 6.0, 120 rpm, 50°C

The bacterial isolate DGS15, isolated from red soil of brick kiln (30° 20’ 19” 42.0’’ N, 76° 27’ 26.9” E) was Gram positive, facultatively anaerobic and positive for catalase, nitrate reductase, Voges Proskauer test (Table 2). It was capable of utilizing sucrose, glucose, and fructose as carbon source. The 1466 bp 16S rRNA gene sequence of *B. sonorenesis* DGS15 was assigned an accession number MT883287.1 after submission to the GenBank database and phylogenetic analysis showed 99.38% similarity with *Bacillus licheniformis* DSM 13 (Figure 2).

| Characteristics/Biochemical test | DGS15 |
|---------------------------------|-------|
| Carbohydrate fermentation       | Anaerogenic & fermentative |
| Urease                          | -     |
| Catalase                        | -     |
| Starch hydrolysis               | -     |
| Gram staining                   | +     |
| Capsule staining                | -     |
| Voges- proskauer                | +     |
| Nitrate reductase               | +     |
| Methyl red                      | -     |

\((+) = \text{positive; (-)} = \text{negative}\)
Figure 2: Neighbor-joining tree depicting the phylogenetic relationship between *Bacillus sonorenesis* DGS15 with other related bacterial species based on 16S rRNA gene sequences. The percentage of 1000 replicates were taken for representing bootstrap value. *Bacillus nakamurai* strain NRRL B-41091 was used as an outgroup. 0.01 scalebar represents the evolutionary distance of nucleotide position per substitution.

**Tolerance to presence of inhibitors in hydrolysates**

The over degradation of the biomass upon pretreatment can result in production of by-products such as furfural and hydroxymethylfurfural (HMF), respectively \[24\]. Specifically, the degradation of pentose can produce furfural and HMF can be formed from hexose degradation \[29\]. It has been reported that furan derivatives could induce the accumulation of reactive oxygen species and interfere with the activities of crucial fermentative enzymes \[30, 31\]. Moreover, negative effects on RNA and protein synthesis of various microorganisms by furfural stress were also reported \[32\]. Thus, inhibitory effects on cell growth and metabolism of microorganisms can be caused by furan derivatives.

**Table 3** HMF: 5-hydroxymethylfurfural; IC: inhibitor concentration. Inoculum of *B. sonorenesis* DGS15 was added and incubated for 24 h at 50°C and 120 rpm. ROD was defined as the percent OD620 of the experimental flask relative to the control flasks (no inhibitor added)

|          | Furfural | HMF |
|----------|----------|-----|
| IC (g L⁻¹) | ROD (%)  | IC (g L⁻¹) | ROD (%)  |
| 0.3      | 75 ± 0.03 | 0.6  | 85.7 ± 0.04 |
| 0.6      | 66.6 ± 0.02 | 1.2  | 42.8 ± 0.02 |
| 0.9      | 25 ± 0.08  | 1.8  | 14.3 ± 0.04 |
| 1.2      | 3.3 ± 0.04  | 2.4  | 4.2 ± 0.05  |

Susceptibility of *B. sonorenesis* DGS15 to the selected inhibitors such as furfural, 5-hydroxymethyl furfural that are produced after pretreatment of lignocellulosic biomass with sulphuric acid was tested (Table 3). *B. sonorenesis* was exposed to different concentrations of furfural and HMF in Bushnell Haas minimal media containing xylose and yeast extract to check bacterial growth. Furfural caused more severe inhibition in growth of *B. sonorenesis* DGS15 than HMF (Figure 3a). At their lowest tested concentrations, neither furfural (0.3 g L⁻¹) nor HMF (0.6 g L⁻¹) caused noticeable effects on *B. sonorenesis* fermentation and their cell growth profiles were similar to that of the control (0 g L⁻¹) (Figure 3a). HMF and furfural inhibited the growth of *B. sonorenesis* in dose dependent manner. With increase in furfural or HMF concentrations, the lag phase of cell growth was prolonged and the inhibitory effects of furfural and HMF caused the decline in growth of *B. sonorenesis*. At a concentration of 1.2 g L⁻¹, furfural was lethal to *B. sonorenesis* such that there was no cell growth. HMF at 1.2 g L⁻¹, showed no significant effects on the cell concentration after 48 h of incubation but the cell growth declined at 72 h of incubation. HMF was
lethal at 1.8 g L\(^{-1}\) and 2.4 g L\(^{-1}\) concentrations, when 2.4 g L\(^{-1}\) HMF was present in the fermentation medium, complete inhibition was observed in cell growth of \(B. \text{sonorenesis}\) at 72 h of incubation. (Figure 3b) Similar effect was observed due to furfural on growth of \(\text{Debaryomyces hansenii}\) which resulted in increased lag phase [33]. Also, butyric acid productivity was lowered as furfural or HMF concentrations increased. With increasing quantities of furfural and HMF, there were additional delays in cell growth and xylose consumption. At 1.2 g L\(^{-1}\) of furfural, \(\text{Clostridium tyrobutyricum}\) fermentation was completely inhibited, but HMF had less severe effects and \(C. \text{tyrobutyricum}\) could tolerate up to 2.4 g L\(^{-1}\) of HMF [27]. Below the tolerable concentrations, \(B \text{sonorenesis}\) was able to recover from the inhibition stress provoked by furfural and HMF.

**Figure 3: Effect of furfural (a) and HMF (b) on cell growth of \(B. \text{sonorenesis DGS15}\)**

Optimization of dilute sulphuric acid hydrolysis on reducing sugar concentration from wheat straw

The fitted data with coded values of independent variables and total reducing sugar concentration as a response variable has the following second order polynomial model equation:

\[
\text{where } A= \text{H}_2\text{SO}_4 \text{ concentration, } B= \text{Temperature, } C= \text{Time}
\]

**Table 4 Predicted value of reducing sugar as predicted by the model and the experimental values of reducing sugar**

| Run | Conc. (g/L) | Temp. (°C) | Time (h) | PRS (g/L) | ERS (g/L) |
|-----|-------------|------------|----------|------------|-----------|
| 1   | 0.99        | 100.8      | 69       | 11.29      | 11.13     |
| 2   | 0.99        | 97.19      | 95.14    | 11.09      | 10.9      |
| 3   | 0.99        | 100.54     | 102.3    | 11.03      | 10.08     |
| 4   | 0.98        | 106        | 79.5     | 11.36      | 11.21     |
| 5   | 0.99        | 111.97     | 60.8     | 11.25      | 11.18     |

PRS=Predicted RS, ERS=Experimental RS

The results of dilute acid hydrolysis of wheat straw for reducing sugar production are shown in Table 4. At an experimental run number of 4, temperature of 106°C, acid concentration of 0.98 v/v percent, and hydrolysis period of 45 minutes, a maximum concentration of 11.36 g L\(^{-1}\) was obtained. Also, at an experimental run number of 12, temperature of 60°C, acid concentration of 0.55% v/v, hydrolysis period of 15 minutes, the minimum reducing sugar of 3.4g L\(^{-1}\) was obtained. Also, the findings of the maximum and lowest concentrations of reducing sugars show that the total reducing sugar (TRS) extraction was lower at lower temperatures and acid concentrations. The increase in total reducing sugar (TRS) production at higher temperatures could be owing to sufficient temperature and acid concentration to hydrolyze wheat straw, but lower temperatures were insufficient to hydrolyze wheat straw.

Table 5 displays the results of an analysis of variance (ANOVA) for the fitted quadratic model. The model summary for regression coefficients (R\(^2\)
Table 5 ANOVA (Analysis of Variance) for the response surface quadratic model of the reducing sugar content

| Source          | Sum of Squares | df | Mean Square | F-value  | p-value |
|-----------------|----------------|----|-------------|----------|---------|
| Model           | 89.98          | 9  | 10          | 118.31   | < 0.0001 S |
| A-H$_2$SO$_4$ Conc | 7.37          | 1  | 7.37        | 87.26    | < 0.0001 |
| B-Temp         | 1.04           | 1  | 1.04        | 12.27    | 0.01    |
| C-Time         | 0.18           | 1  | 0.18        | 2.13     | 0.1878  |
| AB             | 30.36          | 1  | 30.36       | 359.3    | < 0.0001 |
| AC             | 8.7            | 1  | 8.7         | 102.99   | < 0.0001 |
| BC             | 1.82           | 1  | 1.82        | 21.57    | 0.0024  |
| A$^2$          | 17.98          | 1  | 17.98       | 212.8    | < 0.0001 |
| B$^2$          | 13.39          | 1  | 13.39       | 158.5    | < 0.0001 |
| C$^2$          | 10.83          | 1  | 10.83       | 128.12   | < 0.0001 |
| Residual       | 0.5915         | 7  | 0.0845      |          |         |
| Lack of Fit    | 0.4006         | 3  | 0.1335      | 2.8      | 0.1728  NS |
| Pure Error     | 0.1909         | 4  | 0.0477      |          |         |
| Cor Total      | 90.57          | 16 |             |          |         |
| Std. Dev.      | 0.2907         |    | R$^2$       | 0.9935   |         |
| Mean           | 6.94           |    | Adjusted R$^2$ | 0.9851 |         |
| C.V. %         | 4.19           |    | Predicted R$^2$ | 0.9259 |         |
|                |                |    | Adeq. precision | 35.4797 |         |

All the values were the means and standard deviations of three repeated experiments

S=Significant, NS=Not-Significant

99.35%, adjusted $R^2$ 98.51%, and anticipated $R^2$ 92.59%) reveals that the quadratic model fits the experimental data. The influence and relevance of factors in the regression equation were evaluated using ANOVA research for the quadratic model. The p values for all linear and interaction coefficients are 0.05 in the ANOVA results shown in Table 5, indicating that all variables and their interactions have a significant effect on dilute sulfuric acid hydrolysis of wheat straw i.e., Lack of Fit with F value and p value of 2.8 and 0.1728, respectively, indicates that Lack of Fit is not significant when compared to the pure error, indicating that the model was significant. The contour plots (shown in Figure 4) illustrate an infinite number of interactions of H$_2$SO$_4$ concentration with temperature and time of pretreatment. The interaction of independent variables was seen using the contours. The graph between the predicted and the actual experimental values (Figure 4a), the interaction of H$_2$SO$_4$ concentration with temperature (AB) (Figure 4b), dilute sulfuric acid concentration and hydrolysis time (AC) (Figure 4c), temperature and hydrolysis time (BC) (Figure 4d) on the total reducibility produces an elliptical and elliptical nature.

The following were the ideal settings for dilute sulfuric acid hydrolysis of wheat straw under this model for highest total reducing sugar output of 113.3 mg/g: concentration of H$_2$SO$_4$ 1 percent v/v, temperature 120°C, and hydrolysis period 60 minutes.

**Figure 4: Contour plots demonstrating (a) predicted vs actual reducing sugar concentration (b) temperature and dilute sulfuric acid concentration (AB), (c) dilute sulfuric acid concentration and hydrolysis time (AC), (d) temperature and hydrolysis time on total reducing sugar concentration (BC)**
**Validation of experiment:** To validate, the experiments of dilute sulfuric acid hydrolysis of wheat straw were conducted in triplicate under optimised conditions ($\text{H}_2\text{SO}_4$ concentration 1 percent v/v, temperature 120°C, hydrolysis time - 60 min) to ensure reproducibility of results predicted by BBD experiments and RSM analysis, the validation experiment results under ideal conditions were in good agreement with model predictions. The most abundant sugars found in the cellulosic biomass are glucose and xylose [34]. The composition analysis of native and pretreated wheat straw and was carried out to determine cellulose, hemicellulose and total lignin content. Pretreated wheat straw biomass had 40.4% cellulose, 18.4% hemicellulose, 12.4% lignin and 28.2 g L$^{-1}$ reducing sugar, while native wheat straw biomass had 36% cellulose, 25% hemicellulose, 20% lignin, and 0.94 g L$^{-1}$ reducing sugar (Table 6).

| Biomass     | C (%) | HC (%) | Lignin (%) | Red. sug. (g L$^{-1}$) | CrI (%) |
|-------------|-------|--------|------------|------------------------|---------|
| Native WS   | 36    | 25     | 20         | 0.94                   | 35.23   |
| Pretreated WS | 40.4  | 18.4   | 12.4       | 28.2                   | 44.9    |

C=Cellulose, HC=Hemicellulose

There was 30-36 fold increase in concentration of reducing sugars after pretreatment with acid. The dilute sulphuric acid pretreated hydrolysates and hot water treatment showed maximum amount of reducing sugars in wheat straw i.e., 28.20 g L$^{-1}$. Dilute (0.4 N) sodium hydroxide treatment was given to wheat straw for delignification of the structural component lignin. Delignified wheat straw hydrolysate after pretreatment had an increase in cellulose by 89% while decrease in hemicellulose and lignin content was observed by 73% and 62% respectively. Other researchers also concluded, rice straw that had not been treated included approximately 41.34% glucan and 28.46% xylan, the amount of glucan in the blood increased to 49.77% after dilute acid pretreatment [14]. The hemicellulose percentage of pretreated rice straw was 19%, while native rice straw had a 28 percent hemicellulose content. It could be because of its amorphous form, which allows it to hydrolyze quickly during dilute acid pretreatment. Due to the dissolving of amorphous components from the biomass, the cellulose content of acid pretreated rice straw increased by 47% [35].

**SEM**

SEM micrographs of native wheat straw (Figure 5a), pretreated wheat straw (Figure 5b) showed that upon pretreatment the structure of native wheat straw was distorted and the pretreatment led to structural changes in the biomass. SEM showed that the compact, ordered structure of the native biomass was destroyed in the pretreated biomass.

**Figure 5:** Scanning electron micrographs of a) native wheat straw, b) pretreated wheat straw (magnification: 1000X)
XRD

The XRD profile of native and pretreated wheat straw is presented in Figure 6. CrI in native and pretreated wheat straw was observed as 35.23% and 44.9% respectively (Table 6). An increase in CrI (9.71%) was observed in pretreated wheat straw in comparison with native biomass. Other researchers also found similar results in case of native rice straw (59.37%) CrI, dilute acid pretreatment of rice straw had a greater crystallinity degree (67.2%) [35]. An increase in CrI by 6% was also reported for Lantana camara biomass which was enzyme treated [36]. The fatty components of the biomass and the molecular mass of hydrocarbons define the crystallinity of the biomass [22]. The effect of acid pretreatment on crystallinity increased greater in the amorphous region than in the crystalline region [35]. The damage in crystallinity due to the acidic nature of sulphuric acid, delignification due to alkali NaOH and impulsive autoclaving, lead to enhanced saccharification of wheat straw.

Figure 6: XRD profile of native and pretreated wheat straw biomass

Fourier transform infrared (FTIR) spectroscopy analysis

Changes in the functional groups of native, pretreated wheat straw biomass are presented by FTIR spectra (Figure 7). In case of native wheat straw, the broad band at 3333.1 cm$^{-1}$ and 3334.2 cm$^{-1}$ is related to O-H stretching vibrations caused by the presence of alcoholic and phenolic hydroxyl group involved in the hydrogen bond. The change in higher wave number and broadening of the O-H stretching band was the result of pretreatment with sulphuric acid, which is an indication of weakened hydrogen bonding both intra and inter molecular and thus decrease in crystallinity [14]. The band position at 2915 and 2935 cm$^{-1}$ respectively for native and pretreated wheat straw were attributed to C-H stretching vibration in case of native and pretreated wheat straw respectively showed a small variation in peak intensity, suggesting that the methylene (-CH$_2$) and the methyl (-CH$_3$) portions of cellulose were distorted [37]. The highest reduction was observed in the 1640 cm$^{-1}$ band in case of native wheat, due to breakage in acetyl and uronic esters, ester linkage of the ferulic and p-coumaric acids and hemicellulose or lignin [38]. This glycosidic linkage of hemicellulose was absent in pretreated wheat which indicated that the pretreatment cleaved the glycosidic bond from the hemicellulose. The - CH deformation due to absorption within the methoxyl group of lignin and hemicellulose was represented by the absorption peak at 1400 cm$^{-1}$ in case of pretreated wheat straw [39, 40].

The release of acid-soluble lignin was determined due to adsorption in case of pretreated biomass. The structural changes in cellulose and hemicelluloses were determined by the bands in the peak range of 1000-1200 cm$^{-1}$ [38]. The change in the absorption peak at 1040 and 1066 cm$^{-1}$ in native and pretreated wheat straw respectively suggested a cellulose distortion in pretreated biomass [41].
Lactic acid production from wheat straw

Based on the efficient features of strain DGS15, a process was developed to produce lactic acid from wheat straw biomass. Wheat straw (10 g dry weight) were pretreated and the obtained hydrolysate was adjusted to pH 7.0, then fermentation was operated as described in methodology section. Upon dilute sulphuric acid pretreatment and hot water pretreatment on wheat straw, concentration of glucose and xylose was 19.06 g L$^{-1}$ and 9.14 g L$^{-1}$ and at 12 h, it declined to 10.5 g L$^{-1}$ and 5.02 g L$^{-1}$ at 48 h due to production 16.54 g L$^{-1}$ LA. At 72 h further decline in glucose and xylose concentration were observed to 2.87 g L$^{-1}$ and 4.32 g L$^{-1}$ respectively with increase in LA concentration to 27.5 g L$^{-1}$ (Figure 8). In case of wheat straw hydrolysate, the concentration of inhibitors furfural and HMF was 0.47 g L$^{-1}$ and 0.16 g L$^{-1}$ respectively where furfural was consumed after 72 h of fermentation and HMF got accumulated with 3.75-fold increase in concentration in the fermentation broth.

Figure 8: Lactic acid from wheat straw by Bacillus sonorenesis DGS15 in a shake flask containing (wheat straw hydrolysate, yeast extract 5g L$^{-1}$) at pH 7.0, 120 rpm, 50 °C

From above data it was inferred that hydrolysate of substrate wheat straw showed efficient LA production which may be due to release of more sugars upon pretreatment. Also, DGS15 can tolerate broad range of inhibitor concentration. Further, furfural gets utilized after 72 h of inoculation in wheat straw hydrolysate. Either furfural gets converted to HMF or is consumed by DGS15 to produce LA. By using Bacillus coagulans strain IPE22 produced LA from pretreated wheat straw with dilute sulphuric acid i.e., 2% (w/v) at 40°C of concentration of 38.73 g L$^{-1}$ at 60th h, [9] whereas using DGS15 and upon pretreatment with dilute (0.6 N) sulphuric acid in case of wheat straw hydrolysates, 25.41 g L$^{-1}$ lactic acid was produced after 60h at 50°C. Similar trend was observed with other researchers also. 0.59 g/g of LA was produced by Lactobacillus brevis whereas Lactobacillus pentosus produced 0.88 g/g of LA using wheat straw pretreated with sulphuric acid [34]. Wheat straw pretreated with lime produced 40.71 g L$^{-1}$ of LA by using Bacillus coagulans DSM 2314 after 60h of incubation at 50°C (pH 6.0) by using cellulose enzyme [42]. LA production from wheat straw with mesophilic Lactobacillus delbrueckii and Lactobacillus plantarum by SSF [43]. Treatment with alkali in case of wheat straw is an efficient process for production of LA as it removes lignin and fermentation of sugars to produce LA. Highly efficient L-lactate production (56.37 g L$^{-1}$ lactic acid) by a thermophilic strain Bacillus sp. NL01 was obtained from hydrolysate of lignocellulosic biomass which contained the solid residue produced upon enzymatic saccharification [44]. In wheat straw, the overall LA productivity was calculated to be 0.38 g L$^{-1}$/h. The yield of lactic acid after 72 h of fermentation was 0.97 g/g in case of wheat straw with
B. sonorenesis DGS15. Due to its inhibitor’s tolerance feature, this work confirmed that B. sonorenesis DGS15 could produce lactic acid from sulfuric acid pretreated wheat straw.

Conclusion

In this study, Bacillus sonorenesis DGS15, a thermotolerant lactic acid producer could efficiently utilize hemicellulosic xylose sugars from wheat straw hydrolysates and was even tolerant to inhibitors such as furfural and HMF present in lignocellulosic biomass hydrolysates. Pretreatment process consisting of biomass pretreatment by dilute sulfuric acid, dilute sodium hydroxide and hot water and fermentation for LA production from wheat straw was performed. Further, SEM, XRD, FTIR and composition analysis suggested the differences between native and pretreated wheat straw biomass. 27.5 g L\(^{-1}\) LA was produced from 10 g dry wheat. This work confirmed that B. sonorenesis DGS15 could produce LA from sulfuric acid pretreated wheat straw without detoxification due to its inhibitor tolerant characteristic. The process has the potential for industrial scale application where in, this strain can be effectively utilized for large scale bioconversion of lignocellulosic biomass to value added products.

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L-lactic acid production from xylose by DGS15 in shake flask containing Bushnell Hass minimal media (xylose 10g L$^{-1}$, yeast extract 5 g L$^{-1}$) at PH 6.0, 120 rpm, 50 °C
Figure 2

Neighbor-joining tree depicting the phylogenetic relationship between Bacillus sonorensis DGS15 with other related bacterial species based on 16S rRNA gene sequences. The percentage of 1000 replicates were taken for representing bootstrap value. Bacillus nakamurai strain NRRL B-41091 was used as an outgroup. 0.01 scalebar represents the evolutionary distance of nucleotide position per substitution.
Figure 3

Effect of furfural (a) and HMF (b) on cell growth of B. sonorenessis DGS15
Figure 4

Contour plots demonstrating (a) predicted vs actual reducing sugar concentration (b) temperature and dilute sulfuric acid concentration (AB), (c) dilute sulfuric acid concentration and hydrolysis time (AC), (d) temperature and hydrolysis time on total reducing sugar concentration (BC)
Figure 5

Scanning electron micrographs of a) native wheat straw, b) pretreated wheat straw (magnification: 1000X)
Figure 6

XRD profile of native and pretreated wheat straw biomass
Figure 7

FTIR spectra of native, pretreated wheat straw biomass
Lactic acid from wheat straw by Bacillus sonorenesis DGS15 in a shake flask containing (wheat straw hydrolysate, yeast extract 5g L\(^{-1}\)) at pH 7.0, 120 rpm, 50°C