Formulation of low-cost, lactose-free production medium for enhanced production of β-galactosidase using halotolerant A. tubingensis GR1.

Part of this chapter has been published as:

3.1 Introduction

The first step in achieving better production and characterization of an enzyme is the establishment of a suitable enzyme production technology. The majority of microbial enzymes in current industrial use are produced under conventional aerobic submerged fermentation (SmF), owing to its advantages such as purity of the product, greater control of growth conditions and scale-up processes (Wiseman 1985; Chandrasekaran 1997). SmF is the cultivation of organisms in liquid culture media. Although SmF process appears to be identical for the production of all enzymes, there is a need for optimization of process parameters such as nutrients, pH, temperature, incubation time etc. Development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium, and trace element sources. Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time while keeping the others at fixed level. However, statistical method offers several advantages over conventional method being rapid and reliable, short lists significant nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time, glassware, chemicals, and manpower (Srinivas et al. 1994). Statistical optimization by response surface methodology (RSM) is preferable because it is helpful in evaluating the interactions among the possible influencing parameters with limited number of experiments (Francis et al. 2003). It involves a specific design of experiments, which minimizes the error in determining the effect of parameters, and the results are achieved in an economical manner. However, 'one-variable-at-a-time' approach can be useful in determining the suitable operational intervals for the significant variables prior to response surface studies.

Commercially available β-galactosidase is obtained from microorganisms of different genera in particular from Kluyveromyces, Candida, Aspergillus, Bacillus spp. and E. coli (Panesar et al. 2006; Thigiel and Deak 1989; Pinheiro et al. 2003). Majority of the reports suggested the use of lactose or whey as the sole source of carbon and defined chemical medium for the production of β-galactosidase (Isobe et al. 2013; Raol et al. 2010; Gupte and Nair 2010). However, whey supplemented with other agricultural waste enhances the β-galactosidase production (Oberoi et al. 2008; Hatzinikolaou et
al. 2005). Response surface methodology has been used for efficient permeabilization of β-galactosidase using *Kluyveromyces lactis* (Teles de Faria et al. 2012). *Aspergillus* spp. also produces β-galactosidase by fermentation on different carbon sources (Maksimainena 2013; Awan et al. 2010; Gindy 2009) but a detailed statistical lactose-free medium optimization studies are not available for producing β-galactosidase using halotolerant *Aspergillus tubingensis*. Response surface methodology has also been applied for the production of various enzymes, such as cyclodextrin glucanotransferase (CGTase) (Mahat et al. 2004), chitinase (Gohel et al. 2005; Patel et al. 2007), gluco-amylase (Prajapati et al. 2013), pectinase (Nair and Panda 1997), and lipase (Murthy et al. 2000).

This chapter describes the formulation of low-cost, lactose-free medium by employing response surface methodology for the enhanced production of β-galactosidase using halotolerant *A. tubingensis* GR1 as well as its optimization of scale-up studies at laboratory scale bioreactor.

### 3.2 Materials and Methods

#### 3.2.1 Strain isolation and identification

Halotolerant fungus was isolated from the sediment soil samples collected from the man-made solar saltern located at Khambhat, Gulf of Cambay, Gujarat, India. Isolated fungus was screened for β-galactosidase activity as described in earlier in 2.2.8. Screened isolate was identified using FAST Microseq® D2 LSU rDNA fungal identification kit. (Applied Biosystems, Foster city, CA, USA). DNA extraction was carried out using Prepman™ ultra sample preparation reagent and D2 LSU rRNA gene was amplified and cycle sequencing was carried out as per the kit instructions. Amplification was carried out in a thermal cycler (9800, Applied Biosystems, Foster city, CA, USA) with reaction profile: initial denaturation at 95°C for 10 s followed by 35 cycles of denaturation 95°C for 30 s, annealing at 64°C for 15 s, extension at 72°C for 1 min and finally extension at 72°C for 5 min. The purified PCR product was sequenced and the phylogenetic relationship of the isolate was determined by comparing the sequence data with the existing sequences available through the gene bank database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).
3.2.2 Inoculum preparation and β-galactosidase production

The potato dextrose agar (PDA) plate containing 10 d old growth of the organism was used in inoculum. Spores were harvested from this plate by adding sterile distilled water containing 0.01% triton X-100 and 1x10^6 spores/mL inoculum were inoculated in 250 mL Erlenmeyer flask containing 100 mL Czapek Dox Lactose broth which consists of (in g/L) 30 Lactose, 3 NaNO₃, 0.5 NaCl, 1 K₂HPO₄, 0.5 MgSO₄.7H₂O, 0.01 FeSO₄ and pH 5.0. In order to examine the effect of various carbon sources on β-galactosidase production, lactose, maltose, polygalacturonic acid, deproteinized cheese whey, wheat bran and pectin were supplied individually at 1 g% (w/v) in the production medium. To examine the effect of various nitrogen sources on β-galactosidase production, peptone, casein hydrolysate, corn steep liquor (CSL), ammonium sulfate, sodium nitrate and urea were added individually at 0.5 g% (w/v) in the production medium. Effect of NaCl concentration on β-galactosidase production was studied using different concentration viz. 0.5, 1.5, 2.5, 3.5, 4.5 and 5.5 g% (w/v) of NaCl supplementation in the production medium having 1 g% (w/v) wheat bran as carbon and 0.5 g% (w/v) corn steep liquor as nitrogen source. To determine optimum pH for β-galactosidase production culture was inoculated in 100 mL sterile broth medium having 1 g% (w/v) wheat bran as carbon, 0.5 g% (w/v) CSL as nitrogen source, 4.5 g% (w/v) NaCl and keeping other variables as constant as mentioned above except pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0). pH of medium was adjusted with 1N HCl and 1N NaOH. All these flasks were incubated at 28±2ºC on a rotary shaker for enzyme production.

3.2.3 Optimization of the medium components for increased β-galactosidase production

The most important parameters, which affect the efficiency of β-galactosidase production, are found to be wheat bran, corn steep liquor and sodium chloride. In order to study the individual and combined effect of these parameters, statistically designed experiments were performed.

3.2.3.1 Central composite design (CCD)

The levels of the significant parameters as revealed from one factor at a time approach experiment and their interaction effects which influence the β-galactosidase production were analyzed and optimized by response surface central composite design
(CCD). RSM is useful for small number of variables (up to five) but is impractical for large number of variables, due to high number of experimental runs required. The level of the three major components wheat bran, CSL and NaCl were optimized, keeping other trace elements, pH, temperature and inoculum size as a constant.

According to the design, the total number of treatment combinations is $2^k + 2k + no$, where $k$ is the number of independent variables and $no$ is the number of repetition of experiments at the central point. Each factor in the design was studied at five different levels ($-\alpha, -1, 0, +1, +\alpha$) as shown in Table 3.1. This experimental design comprises a two level fractional factorial points (-1 and +1), central point (0) and axial or star points encoded as $-\alpha$ and $+\alpha$. All variables were set at a central coded value of zero. The minimum and maximum ranges of variables were determined on the basis of our previous experiments. The full experimental plan with respect to their values is listed in Table 3.2.

$\beta$-galactosidase (U/mL) was measured in triplicate in 20 different experimental runs. $\beta$-galactosidase production was analyzed using a second order polynomial equation and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given as:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

(1)

Where, $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ represent the constant, linear, quadratic effect of $X_i$ and interaction effect between $X_i$ and $X_j$, respectively for the production of $\beta$-galactosidase. Later, validation experiment was performed and maximum production of $\beta$-galactosidase was confirmed using the optimum values for variables predicted by response optimization.
Table 3.1 Experimental range and levels of the independent variables selected from one factor at time used for response surface central composite design.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Components</th>
<th>Range</th>
<th>Levels of variable studied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-α</td>
</tr>
<tr>
<td>X1</td>
<td>Wheat bran (g%)</td>
<td>0.50-5.00</td>
<td>-1.03</td>
</tr>
<tr>
<td>X2</td>
<td>CSL (g%)</td>
<td>0.10-1.00</td>
<td>-0.20</td>
</tr>
<tr>
<td>X3</td>
<td>NaCl (g%)</td>
<td>0.50-5.00</td>
<td>-1.03</td>
</tr>
</tbody>
</table>
Table 3.2 Full experimental central composite design with level of variables and the response functions.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>A: Wheat bran (g%)</th>
<th>B: CSL (g%)</th>
<th>C: NaCl (g%)</th>
<th>β-galactosidase production (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Predicted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.00</td>
<td>1.00</td>
<td>0.50</td>
<td>76.8</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>1.00</td>
<td>5.00</td>
<td>352.8</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>0.10</td>
<td>0.50</td>
<td>151.2</td>
</tr>
<tr>
<td>4</td>
<td>5.00</td>
<td>0.10</td>
<td>5.00</td>
<td>16.8</td>
</tr>
<tr>
<td>5</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>69.6</td>
</tr>
<tr>
<td>6</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>52.8</td>
</tr>
<tr>
<td>7</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>81.6</td>
</tr>
<tr>
<td>8</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>14.4</td>
</tr>
<tr>
<td>9</td>
<td>0.50</td>
<td>1.00</td>
<td>0.50</td>
<td>232.8</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
<td>0.10</td>
<td>5.00</td>
<td>259.2</td>
</tr>
<tr>
<td>11</td>
<td>5.00</td>
<td>0.10</td>
<td>0.50</td>
<td>35.2</td>
</tr>
<tr>
<td>12</td>
<td>5.00</td>
<td>1.00</td>
<td>5.00</td>
<td>26.4</td>
</tr>
<tr>
<td>13</td>
<td>2.75</td>
<td>-0.20</td>
<td>2.75</td>
<td>79.2</td>
</tr>
<tr>
<td>14</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>16.8</td>
</tr>
<tr>
<td>15</td>
<td>2.75</td>
<td>0.55</td>
<td>-1.03</td>
<td>72.0</td>
</tr>
<tr>
<td>16</td>
<td>2.75</td>
<td>0.55</td>
<td>2.75</td>
<td>28.8</td>
</tr>
<tr>
<td>17</td>
<td>2.75</td>
<td>1.30</td>
<td>2.75</td>
<td>84.0</td>
</tr>
<tr>
<td>18</td>
<td>2.75</td>
<td>0.55</td>
<td>6.53</td>
<td>84.0</td>
</tr>
<tr>
<td>19</td>
<td>-1.03</td>
<td>0.55</td>
<td>2.75</td>
<td>108.0</td>
</tr>
<tr>
<td>20</td>
<td>6.53</td>
<td>0.55</td>
<td>2.75</td>
<td>12.0</td>
</tr>
</tbody>
</table>

3.2.4 Enzyme extraction and assay

The experiments were performed according to the design matrix (Table 3.2) in 250 mL Erlenmeyer flasks containing 100 mL of production medium having pH 4.0. The production flasks were kept at 28°C under shaking condition at 150 rpm. The enzyme source from experimental flasks was harvested after 96 h of incubation by centrifuging the content at 10,000 rpm at 4°C after discarding the fungal biomass. Clear supernatent after centrifugation was used as an enzyme source for determining
\( \beta \)-galactosidase activity. Enzyme activity was assayed in the reaction mixture containing 0.9 mL of sodium-citrate buffer (75 mM, pH 5.0) and 0.1 mL of enzyme source. 1.0 mL of 2 mM O-nitrophenyl-\( \beta \)-D-galactopyranoside (ONPG) was added to this mixture and reaction mixture was incubated at 50\(^\circ\)C for 5 min. After incubation reaction was stopped by addition of 3.0 mL 0.1 N NaOH. Released O-nitrophenol was determined spectrophotometrically at 420 nm using calibration curve prepared with O-nitrophenol under the same conditions (Cruz et al. 1999). One unit (U) of \( \beta \)-galactosidase activity was defined as the enzyme liberating one \( \mu \)M O-nitrophenol (molar extinction coefficient of ONP is = 4166 Lmol\(^{-1}\)cm\(^{-1}\)) in one minute under the standard assay conditions.

3.2.5 Software and data analysis
The results of the experimental design were analyzed and interpreted using Design Expert Version 8.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software.

3.2.6 Bioreactor and accessories
The studies were carried out in a 7.0 L bench-top fermenter (BIOCARE 7L, Shri Biocare India Ltd., Ahmedabad, India) having a working volume of 5L. The vessel has a flanged glass tube body detachable from stainless steel head plate and stainless steel bottom dished head. The bottom is jacketed for circulation of temperature controlled water. Ports are present in the head plate for inoculation and acid-base addition. The head plate and vessel also have arrangements for a foam probe, sparger, harvesting tube, exhaust condenser, pH and dissolved oxygen electrodes and a thermo-well for temperature sensor. Peristaltic pumps were attached to control the foam and pH by automatic addition of an antifoam silicon agent or an acid/base respectively. The agitation system consists of a removable motor located on top of the vessel and connected to the agitation shaft with a multi jaw coupling. The agitation shaft made of stainless steel and has three six-blade stainless steel impellers. The temperature was controlled by circulating chilled water from a chilling water bath.

3.2.7 Production of \( \beta \)-galactosidase in bench-top bioreactor
The bioreactor was filled with 5L of optimized culture medium and then sterilized by autoclaving at 121\(^\circ\)C for 20 min. A regulation system was used to control the temperature at 28\(^\circ\)C and pH 4.0 throughout of the experiment after sterilization.
Foaming was controlled with the addition of silicon oil antifoaming agent (Himedia, Mumbai, India). Periodically samples were withdrawn to perform enzyme assay as mentioned above hence all the experiments were performed in duplicate to ensure data and mean values were represented. Following parameters were optimized to achieve enhanced enzyme production.

3.2.7.1 Effect of inoculum age and size on β-galactosidase enzyme production

Effect of inoculum age on production of β-galactosidase enzyme was studied by taking two different inoculum viz. spore form of inoculum and vegetative mycelium inoculum. Spore inoculum was prepared by growing the culture on PDA plate for 10 days at 28°C temperature and spores were harvested from this plate by adding sterile distilled water containing 0.01% triton X-100. The inoculum had a spore concentration of 1x10^6 spores/mL. Vegetative mycelium inoculum was prepared by growing the 1x10^6 spores in 100 mL potato dextrose broth at 28°C temperature. After 48 h of incubation the grown vegetative mycelia were used as an inoculum for mycelial process. Each of the inoculum was added in to production media at the rate of 10% (v/v) and the production of enzyme was carried out at constant pH, temperature, agitation and aeration rates i.e. 4.0, 28°C, 150 rpm and 2.0 vvm, respectively.

3.2.7.2 Effect of agitation speeds and aeration rates on β-galactosidase production

The air flow rates studied were 2.0, 3.0, 4.0 and 5.0 vvm at agitation speeds of 150 and 250 rpm corresponding to the following peripheral speeds of 0.393 and 0.655 m/s, respectively. Medium was inoculated with 8% (v/v) inoculum and production carried out at constant pH and temperature i.e. 4.0 and 28°C, respectively.
3.3 Results and discussion

3.3.1 Identification of the fungal isolate

A 618 bp sized 28S rDNA sequence of the isolate was obtained through PCR amplification (Fig 3.1). The sequence had 97% homology with *A. tubingensis* showing 928 maximum score having 0.0 e-value. The sequence was deposited in the NCBI gene bank bearing the Accession no. **KC968906**. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The analysis involved 8 nucleotide sequences and the ambiguous positions were removed for each sequence pair. There were a total of 1814 positions in the final dataset. The phylogenetic tree was drawn using bioinformatics software MEGA 5.05 (Fig 3.2).

![Figure 3.1 618 bp PCR product with DNA marker.](image)
Figure 3.2 Phylogenetic relationships on the basis of homology index for a halotolerant fungal isolate *A. tubingensis* GR1.

3.3.2 Production of β-galactosidase enzyme

Amongst the different carbon sources tested, wheat bran showed maximum enzyme activity (159±2.1 U/mL) whereas; β-galactosidase production (75±2.05 U/mL) was low in the lactose amended medium (*Fig. 3.3*). When lactose was used as carbon source then β-galactosidase releases glucose, which leads to formation of catabolite repressor and reduces the β-galactosidase production whereas, on a polysaccharide like wheat bran, polygalacturonic acid and pectin, β-galactosidase releases terminal galactose residues from the galactan and xyloglucan side chain resulting release of galactose which not affect the β-galactosidase production. Similar results were also reported by McKay (1991); de Vries and Visser (2001); Fekete et al. (2008).

The effects of organic and inorganic nitrogen sources were further studied keeping optimized concentration (1 g% w/v) carbon source and results were illustrated in *Fig. 3.4*. 
**Figure 3.3** Effect of different carbon sources on production of β-galactosidase.

**Figure 3.4** Effect of different nitrogen sources on production of β-galactosidase.
β-galactosidase production varied from 77~169 U/mL. It was found that corn steep liquor (CSL) was most effective for β-galactosidase production (169 U/mL). Fekete et al. (2008) reported that metabolic fate of galactose in A. nidulans proceeds through reductive pathway and it prefers ammonium salts rather than nitrate as nitrogen source. In our study, we found that after degradation of wheat bran, resulted galactose molecules served as carbon source and corn steep liquor supply enough ammonium ions and other vitamins for reductive metabolic fate of galactose.

Apart from carbon and nitrogen source, another significant parameter which affects the production of β-galactosidase was sodium chloride (NaCl). The effect of different concentration of NaCl on β-galactosidase production using halotolerant A. tubingensis GR1 was shown in Fig. 3.5. As the concentration of NaCl increases, β-galactosidase production and fungal biomass increased significantly. Sheridane and Brenchley (2000) reported β-galactosidase production using salt-tolerant psychrophilic isolate Planococcus sp. but to the best of our knowledge this is the first report which shows the increased production of β-galactosidase at 4.5 g NaCl concentration.

The hydrogen ion concentration of environment has the maximum influence on the microbial growth and enzyme production. The most favorable pH value is the point where the enzyme production is highest while increase or decrease in pH from that point leads to the decrease in enzyme production. As shown in Fig. 3.6 different pH levels were selected to study the effect of pH on enzyme production using isolate A. tubingensis GR1 and optimum enzyme production was obtained at pH 4.0. Nizamuddin et al. (2008); Panesar (2008) and Pavani et al. (2011) have reported the β-galactosidase production at pH 5.0 while in our experiment we observed that the pH 4.0 was found to be most suitable for the maximum enzyme production by A. tubingensis GR1.
Figure 3.5 Effect of sodium chloride concentration on production of β-galactosidase and biomass production.

Figure 3.6 Effect of pH on β-galactosidase production.
3.3.3 Response surface methodology (RSM)

The central composite design was employed to evaluate the interaction among the significant factors and also to determine their optimal levels. In the present work, experiments were planned to obtain a quadratic model consisting of $2^3$ trials. The plan included twenty experiments and two levels of concentration for each factor. In order to study the combined effect of these variables, experiments were performed at different combinations. The central composite experimental plan along with the predicted and observed response for each individual experiment is summarized in materials and methods section (3.2.3.1) as Table 3.2. It also shows the production of β-galactosidase (U/mL) corresponding to combined effect of all three components in the specified ranges.

The optimum levels of selected variables were obtained by solving the regression equation and by analyzing the response surface contour and surface plots. The larger the magnitude of the $t$-value and smaller the $p$-value, more significant is the corresponding coefficient (Myers and Montgomery 2002). The regression equation obtained after the analysis of variance (ANOVA) provides an estimate of the level of β-galactosidase production as a function of wheat bran, corn steep liquor and NaCl concentration.

The production of β-galactosidase may be best predicted by the following model:

\[
Y = 43.71 - (75.96*A) + (19.74*B) + (15.71*C) - (11.1*A*B) - (32.7*A*C) - (6.9*B*C) + (20.13*A^2) + (27.77*B^2) + 26.50*C^2
\]  

(2)

Where $Y = \beta$-galactosidase production (U/mL),

$A =$ Wheat bran (g%), $B =$ Corn steep liquor (g%) and $C =$ NaCl (g%).

The statistical significance of the second order model equation was evaluated by $F$-test analysis of variance as shown in Table 3.3 which revealed that this regression is statistically highly significant for β-galactosidase production.
Table 3.3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for level optimization of β-galactosidase production using *A. tubingensis* GR1.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>P-value</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>13219.008</td>
<td>2</td>
<td>6609.504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>120116.7363</td>
<td>9</td>
<td>13346.3</td>
<td>3.58732</td>
<td>0.0430</td>
<td>Significant</td>
</tr>
<tr>
<td>A-Wheat Bran</td>
<td>78810.74693</td>
<td>1</td>
<td>78810.75</td>
<td>21.18336</td>
<td>0.0017</td>
<td>Significant</td>
</tr>
<tr>
<td>B-Corn Streep</td>
<td>5325.041395</td>
<td>1</td>
<td>5325.041</td>
<td>1.431306</td>
<td>0.2658</td>
<td></td>
</tr>
<tr>
<td>C-NaCl</td>
<td>3371.583623</td>
<td>1</td>
<td>3371.584</td>
<td>21.18336</td>
<td>0.0017</td>
<td>Significant</td>
</tr>
<tr>
<td>AB</td>
<td>985.68</td>
<td>1</td>
<td>985.68</td>
<td>0.264939</td>
<td>0.6207</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>8554.32</td>
<td>1</td>
<td>8554.32</td>
<td>2.299296</td>
<td>0.1679</td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>380.88</td>
<td>1</td>
<td>380.88</td>
<td>0.102376</td>
<td>0.7572</td>
<td></td>
</tr>
<tr>
<td>A²</td>
<td>5840.608373</td>
<td>1</td>
<td>5840.608</td>
<td>1.569884</td>
<td>0.2456</td>
<td></td>
</tr>
<tr>
<td>B²</td>
<td>11109.85861</td>
<td>1</td>
<td>11109.86</td>
<td>2.986194</td>
<td>0.1222</td>
<td></td>
</tr>
<tr>
<td>C²</td>
<td>10115.01024</td>
<td>1</td>
<td>10115.01</td>
<td>2.718791</td>
<td>0.1378</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>29763.2637</td>
<td>8</td>
<td>3720.408</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>27292.2237</td>
<td>5</td>
<td>5458.445</td>
<td>6.6269</td>
<td>0.0752</td>
<td>Not significant</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2471.04</td>
<td>3</td>
<td>823.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>163099.008</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ R^2 = 0.80; \text{ Adeq Pre} = 7.533 \]

The model F-value 3.59 implies that the model is significant. There is only a 4.30% chance that a large ‘model F-Value’ could occur due to noise. P-value less than 0.050 indicate that the model terms are significant. Thus, in this study only A is a significant model terms (Table 3.3). The Lack of fit F-value of 0.0752 implies the lack of fit is not significant relative to the pure error. Non-significant lack of fit is good for the model to fit. The \[ R^2 \] value (multiple correlation coefficient) closer to 1 denotes better correlation between observed and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the experiment is compared. The lower
reliability of the experiment is usually indicated by high value of CV. In the present case low CV (67.05) denotes that the experiment performed is reliable. Adequate precision measures the signal to noise ratio. A ratio greater than 4.00 is desirable. In present study, the ratio is 7.533 which indicate an adequate signal.

The effect of interaction of variables β-galactosidase yield was studied by changing levels of any two independent variables while keeping the third independent variable at its constant level. The response surface plots or contour plots can be used to predict the optimal values for different test variables. Therefore, three response surface plots were obtained by considering all the possible combinations. The effect of interaction between wheat bran and corn steep liquor on β-galactosidase, revealed that both the components at their higher level had no significant effect on the β-galactosidase production but increase in corn steep liquor concentration lead to gradual increase in the β-galactosidase production. But increase in wheat bran concentration lead to decrease in the β-galactosidase production. Wheat bran and corn steep liquor concentration at higher level showed negative effect on production but corn steep liquor at higher level and wheat bran at lower level showed positive effect on the β-galactosidase production (Fig 3.7). Both wheat bran and NaCl at higher concentration lead to decrease in β-galactosidase production while higher NaCl concentration and lower wheat bran showed positive effect on β-galactosidase production (Fig 3.8). Thus, NaCl at higher and wheat bran at lower level were found to be significant for β-galactosidase production. The interaction between corn steep liquor and NaCl also play an important role in β-galactosidase production; both higher levels were found to be significant for β-galactosidase production. It was noticed that the concentration of corn steep liquor was directly proportional in the production of β-galactosidase (Fig. 3.9). According to the response surface point prediction analysis, wheat bran concentration of 5 g/L, corn steep liquor (10 g/L) and NaCl (50 g/L) maximized the yield of β-galactosidase up to 352.8 U/mL.
Figure 3.7 Response surface graph showing interaction effect of wheat bran and corn steep liquor (CSL) on β-galactosidase production.
Figure 3.8 Response surface graph showing interaction effect of wheat bran and NaCl on β-galactosidase production.
Figure 3.9 Response surface graph showing interaction effect of corn steep liquor and NaCl on β-galactosidase production.
3.3.4 Validation of the quadratic model

In order to confirm the above mentioned optimized medium constitution and condition an experiment for β-galactosidase production was performed in duplicate. Under these suggested condition the mean value of the β-galactosidase yield was 352.8 U/mL, which was slightly higher than the predicted value of 297.54 U/mL. Thus, the model developed was accurate and reliable for predicting the production of β-galactosidase by halotolerant A. tubingensis GR1.

3.3.5 Production of β-galactosidase in bench-top bioreactor

As shown in Fig. 3.10 bioreactor was assembled, filled with production medium and sterilized at 121°C for 20 min.

![Set up of bench-top bioreactor for production of β-galactosidase from A. tubingensis GR1.](image)

**Figure 3.10** Set up of bench-top bioreactor for production of β-galactosidase from A. tubingensis GR1.

Inoculum age and size significantly affects the β-galactosidase production. As shown in Fig. 3.11 spore inoculum shows good production as compared to mycelium inoculum. At prolonged incubation time, vegetative mycelium culture may get degenerated and lysis occur which leads to decreased enzyme production whereas, in case of spore inoculum, germination of spore and vegetative mycelium life get prolonged and after 120 h of incubation also gives better enzyme production.
**Figure 3.11** Effect of inoculum age on production of β-galactosidase in bench-top bioreactor.

**Fig. 3.12** shows the effect of inoculum size on the production of β-galactosidase enzyme. The enzyme production increased with the concomitant increased in inoculum size up to 8% (v/v), thereafter no improvement in enzyme production was observed. The maximum enzyme production 290.58 U/mL was observed with 8% (v/v) inoculum. The low enzyme production may be attributed to the low density of the starter culture (2% (v/v) inoculum level). Our results are also in tune with Panesar (2008). Therefore, an inoculum of 8% (v/v) can be considered optimal for achieving maximum enzyme production.
3.3.6 Effect of agitation speeds and aeration rates on β-galactosidase production

The results obtained after 120 h of culture incubation at different agitation speeds for aeration rates of 2.0, 3.0, 4.0 and 5.0 vvm are presented in Fig. 3.13.

Figure 3.13 Production of β-galactosidase enzyme at different peripheral speeds and aeration rates in bench-top bioreactor.
At all the peripheral speeds tested, improvement in β-galactosidase production was observed when aeration rates increased from 3.0 to 4.0 vvm. Increases in the aeration rate more than 4.0 vvm resulted in reduced enzymatic activity. The optimum β-galactosidase production of 355.36 (U/mL) was obtained in 4.0 vvm aeration rate at 0.393 m/s peripheral speed. At high peripheral speed (0.655 m/s), the decrease in β-galactosidase production has been attributed to the effect of hydrodynamic stress, which may cause hyphal disruption and leakage of intracellular compounds (Chipeta et al. 2008). The harmful effect of the shear forces as a result of agitation intensity has been reported to cause decreased enzyme production in some filamentous fungi (Palma et al. 1996; Lenartovicz et al. 2003; Techapun et al. 2003).

β-galactosidase production in 4.0 vvm was higher than 2.0 vvm aeration rate. This indicated that low aeration rate had more negative effect on β-galactosidase production than high aeration rate. However, it has been reported that improving aeration rate has a positive effect on enzyme activity from aerobic microorganisms (Bakri et al. 2002; Jafari et al. 2007; El-Enshasy et al. 2008). Similar results were obtained with Thermomyces lanuginosus RT9 by Hoq et al. (1994) who observed an increase in β-xylanase production with increasing aeration to 1.0 vvm. However, when aeration was increased to 1.5 vvm, β-xylanase production decreased 1.5-fold. Hence, when the aeration rate was increased to more than 4.0 vvm, β-galactosidase production gradually decreased. The higher enzyme inactivation in highly aerated culture may be due to irreversible oxidation of amino acid residues of the enzyme structure (Cabiscol et al. 2000).

### 3.4 Conclusion

In this study, the improvement of β-galactosidase production by halotolerant A. tubingensis GR1 has been reported. The individual and interactive role of carbon and nitrogen sources and sodium chloride concentration on β-galactosidase was investigated by Response Surface Central Composite Design. To the best of our knowledge, it is for the first time that statistical approach has been employed and showed significant results for optimizing the medium components for maximal β-galactosidase production under submerged fermentation using a halotolerant A. tubingensis. The enzyme yield and the production were found to be significantly influenced by wheat bran, corn steep liquor and sodium chloride but not by the
lactose. The data obtained after optimization has resulted in 352.8 U/mL compared to unoptimized medium (75 U/mL) for enzyme production. In the scale-up study, it was found that β-galactosidase production was significantly affected by inoculum size as well as aeration and agitation rate. Under optimized aeration rate (4.0 vvm) and agitation speed (0.393 m/s peripheral speed) enhanced β-galactosidase production from *A. tubingensis* GR1 in 5 L bench-top bioreactor was achieved that is 355.36 U/mL.
3.5 References


