An attempt was made to isolate amylolytic yeasts from curd, buttermilk and lassi like dairy products from various domestic and local dairy vendors. Among yeasts species screened for amylolytic activity, yeast with maximum glucoamylase activity was used for further study. On basis of 18s rDNA partial genome sequencing, the yeast was identified as *Kluyveromyces marxianus*. The effect of various carbon sources, nitrogen sources and growth conditions on production of glucoamylase was investigated. Medium optimization was carried out using...
one variable at a time approach as well as statistical method involving Plackett-Burman design. Maximum amount of glucoamylase (937 ± 45 U/L) was produced after 96 hours of incubation at 30°C on using 7% inoculum applying one variable at a time strategy. Application of Plackett-Burman design for medium optimization resulted in about 25% increase in production of glucoamylase. Statistical optimization included utilization of inexpensive substrates like corn steep liquor, starch, rice flour, corn flour and soya flour in various prescribed combinations. Major medium components optimized for production of glucoamylase by statistical method are starch (2%), corn steep liquor (0.5%) and rice flour (0.1%). The result indicates economically feasible production of glucoamylase from *Kluyveromyces sp.* by using low priced substrates. An attempt was made to isolate amylolytic yeasts from curd, buttermilk and lassi like dairy products from various domestic and local dairy vendors. Among yeasts species screened for amylolytic activity, yeast with maximum glucoamylase activity was used for further study. On basis of 18s rDNA partial genome sequencing, the yeast was identified as *Kluyveromyces marxianus*. The effect of various carbon sources, nitrogen sources and growth conditions on production of glucoamylase was investigated. Medium optimization was carried out using one variable at a time approach as well as statistical method involving Plackett-Burman design. Maximum amount of glucoamylase (937 ± 45 U/L) was produced after 96 hours of incubation at 30°C on using 7% inoculum applying one variable at a time strategy. Application of Plackett-Burman design for medium optimization resulted in about 25% increase in production of glucoamylase. Statistical optimization included utilization of inexpensive substrates like corn steep liquor, starch, rice flour, corn flour and soya flour in various prescribed combinations. Major medium components optimized for production of glucoamylase by statistical method are starch (2%), corn steep liquor (0.5%) and rice flour (0.1%). The result indicates economically feasible production of glucoamylase from *Kluyveromyces sp.* by using low priced substrates.

**Keywords:** Glucoamylase, Yeast, *Kluyveromyces*, Amylolytic yeast.
Glucoamylase (EC 3.2.1.3) is an exo-acting enzyme. It is known for its ability to act on non-reducing chain ends of amylopectin, amylose and glycogen to yield β-D-glucose. To liberate β-D-glucose from these substrates, it cleaves α-1,4 linkages from non-reducing end (Marín-Navarro and Polaina, 2011). Glucoamylases is considered important in degradation of starchy substrates and represents about 25-33% in the worldwide enzyme market and comes to second rank after proteases (Gupta et al., 2003). This enzyme plays an important role in various industrial applications especially in saccharification where starch is hydrolyzed to glucose to produce high glucose syrups which is used as substrate for various industrial fermentations. Glucoamylase alone is efficient for starch hydrolysis and can be used for saccharification of starch obtained from seeds for fermentative production of ethanol (Uchida et al., 2014). The high glucose syrups can also be used for making other products such as ice-creams, soft drinks, sauces, bread, canned foods, tinned fruits etc. (Nigam and Singh, 1995 and Nguyen et al., 2002). It is also possible to carry out saccharification and fermentation together where source of enzyme is added to fermentation medium containing starch and the medium is inoculated with microorganism producing desired product (Izmirlioglu and Demirci, 2016; Szymanowska-Powałowska et al., 2014). Glucoamylase co-immobilized with glucose oxidase had been used in construction of electrochemical sequential biosensor capable of determining starch (Lang et al., 2014).

Glucoamylase is produced through various botanical, animal and microbial sources among which microbial sources are significantly important and includes filamentous molds, yeast and bacteria (Souza, 2010). Production of glucoamylase through microbial sources is more advantageous than the other sources since the life span of microbes is shorter and the isolation of enzyme is comparatively easy and comfortable (Selvakumar et al., 1996). For different kind of microbial species, various production media have been optimized starting from laboratory scale to industrial fermenters. Glucose syrup, maltose syrup and α-lactose are major substrates for industrial production of glucoamylase which conquers more than 60% of total production cost due to which, the economics and future developments could become limited (Pedersen et al., 2000). So it is a matter of commercial interest to go for production of glucoamylase using cheaper substrates. To increase production of microbial metabolites various conventional approaches are applied which includes manipulation in
physical parameters and nutritional requirements as well as alteration in genetic makeup of the microbe (Greasham, 1983). Wise selection of different carbon, nitrogen and other sources may result in development of economical medium. In addition to the conventional methods, different statistical methods can also be used for manipulation of nutritional requirements. In conventional method only one variable is studied at a time keeping the other variables constant. Statistical methods offer several advantages over the previous method especially in terms of reduction in total number of experiments required to shortlist the significant nutrients rapidly with reliability (Rajendran et al., 2008).

The objective of our study was based on the same economical interest. In present study, attempts were made to optimize the cultural conditions, carbon sources and nitrogen sources for glucoamylase production using newly isolated yeast. In addition, statistical method was also applied to formulate production medium composed of cheaper substrates.

**MATERIAL AND METHODS**

**Isolation and identification of yeast:** From the total collected samples, 63 yeasts were isolated and screened for the production of glucoamylase (Shah and Parikh, 2016). One of these yeast strains that gave maximum yield of glucoamylase was selected for production of glucoamylase. The yeast was grown on YPD medium composed of w/v each of 1 g% yeast extract, 2 g% peptone and 2 g% d-glucose. For the purpose of maintenance, YPD agar containing 2% w/v starch (YPDS) was used. Yeast DNA was isolated and purified using Chromous Genomic DNA Spin-50 kit and used for downstream processes (ITS1: TCCGTRSGNGAACYTGHGG; ITS4: TCCTCCGCTTATTKATDTGC). PCR amplification was carried out using universal 18s rDNA ITS primers. Amplification was carried out using reaction profile: Stage-1 included initial denaturation at 94°C for 5 minutes; Stage-2 included 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and ultimately extension at 72°C for 1 minute; finally, Stage-3 included final extension at 72°C for 5 minutes. DNA from electrophoresed gel was extracted using Chromous Gel Extraction Spin-50. The extracted purified PCR product was used for
sequencing using ABI 3500 XL Genetic Analyzer (Applied Biosystems, Fostercity, CA, USA) followed by determination of phylogenetic tree of the yeast isolate by comparing the sequencing data with the existing sequence data available at GenBank, National Center for Biotechnology Information.

**Glucoamylase assay:** Assay of glucoamylase was performed by the method of Bernfeld (1955). Biomass was removed from the production medium by centrifugation (10000 rpm, 4°C) and the cell free culture supernatant was used as crude enzyme for assay purpose. Glucoamylase assay was performed using 1% starch as substrate prepared in 50mM sodium acetate buffer (pH 6.0). Same buffer was used for dilution of crude enzyme wherever required. The reaction mixture for enzyme assay was prepared by mixing 1.0 mL of 1 g% (w/v) starch and 1.0 mL of crude enzyme (diluted as required). The amount of glucose released after utilization of substrate starch by glucoamylase was measured using DNS (3,5-Dinitrosalicylic acid) method (Miller, 1959). As a standard, glucose was used. One glucoamylase unit (U) was defined as the amount of enzyme liberating one μmole of glucose equivalent per minute from starch under the specified assay conditions. The reaction products produced during enzyme assay were studied by thin layer chromatography. The amylolytic enzyme produced by the yeast was identified as glucoamylase since the major end product produced by action of amylolytic enzyme on starch was glucose only.

**Preparation of inoculum for production of glucoamylase:** Initially, yeast was allowed to grow in YPD broth at 30°C for 24 hours in shaking incubator at 150 revolutions per minute. Yeast biomass was separated by centrifugation and resuspended in YPS broth containing w/v of each 1 g% yeast extract, 1 g% peptone and 0.5 g% starch to original volume and incubated at 30°C for 24 hours in shaking incubator at 150 rpm to induce production of glucoamylase. After allowing yeast culture for induction of glucoamylase as in previous step, yeast biomass was separated by centrifugation and resuspended in YPS production
medium (containing same concentrations of yeast extract and peptone as explained earlier for YPS broth but with 2 g% w/v starch) till its optical density reaches 1.0 at 600 nm. Freshly prepared such yeast suspension was used as inoculum during further studies.

**Optimization of cultural conditions for production of glucoamylase:** Erlenmeyer flasks (250 ml capacity) contained 100ml YPS production medium, sterilized at 121°C for 15 minutes and were used for optimization of cultural conditions for glucoamylase production from the yeast isolate. The cultural conditions that were optimized includes study of incubation temperature, initial medium pH, amount of inoculum and time of incubation used for production of glucoamylase. The study of glucoamylase production using different concentrations of inoculum and time of incubation were optimized together where the values of inoculum volume studied were 1, 3, 5, 7 and 9 % (v/v) while the incubation time was considered in multiple of 24 hours. Production of glucoamylase was studied at different temperatures like 25, 30, 37, 40, 45°C etc. at constant pH 6.0 in all flasks. Effect of initial pH of medium on glucoamylase production included different pH values viz. 4, 4.5, 5, 5.5, 6, 6.5, 7 and 8 at constant 30°C incubation temperature. The presence of starch in the fermentation medium throughout incubation was detected using iodine reagent (0.2% Iodine in 0.4% KI solution).

**Effects of various carbon and nitrogen sources on glucoamylase production:** Production of glucoamylase was studied using various carbon and nitrogen sources to optimize production medium. To examine the effect of carbon sources on production of glucoamylase, glucose, maltose, lactose, sucrose, starch and molasses were individually supplied at 2 g% (w/v) concentration in the production medium containing 2 g% (w/v) peptone as nitrogen source. In order to study the effect of various nitrogen sources on glucoamylase production, peptone, malt extract, yeast extract, urea and ammonium sulfate were supplied in the production medium containing 2 g% (w/v) starch as carbon source. The concentration of
urea and ammonium sulfate were 1 g% (w/v) while for other nitrogen sources it was 2 g% (w/v). A study on feasibility of nontraditional carbon sources like rice flour and corn flour as well as nitrogen sources like soya flour and corn steep liquor for glucoamylase production was also carried out within the present study. The pH of medium was adjusted with 1 N HCl and 1 N NaOH. Sugars in these media were filter sterilized separately of the medium and added aseptically to sterile medium base.

**Screening of critical nutrient components using Plackett-Burman design:** In the present study, Plackett-Burman design was used to study and screen important medium ingredients as well as for evaluation of their effect on the production of glucoamylase. Total 7 medium components were screened which includes starch, corn steep liquor, rice flour, corn flour, soya flour, peptone and urea. Experimental design for screening of variables was prepared as presented in Table 1.

**Table 1. Variables and their values used to design experiment using Placket-Burman method for glucoamylase production by yeast**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Medium components</th>
<th>- Value</th>
<th>+ Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>Corn flour</td>
<td>1</td>
<td>0.1</td>
<td>Gram</td>
</tr>
<tr>
<td>X2</td>
<td>Starch</td>
<td>2</td>
<td>0.2</td>
<td>Gram</td>
</tr>
<tr>
<td>X3</td>
<td>Corn steep liquor</td>
<td>2.5</td>
<td>0.5</td>
<td>mL</td>
</tr>
<tr>
<td>X4</td>
<td>Soya flour</td>
<td>1.5</td>
<td>0.5</td>
<td>Gram</td>
</tr>
<tr>
<td>X5</td>
<td>Peptone</td>
<td>2</td>
<td>0.2</td>
<td>Gram</td>
</tr>
<tr>
<td>X6</td>
<td>Urea</td>
<td>0.5</td>
<td>0.05</td>
<td>Gram</td>
</tr>
<tr>
<td>X7</td>
<td>Rice flour</td>
<td>0.1</td>
<td>1</td>
<td>Gram</td>
</tr>
</tbody>
</table>

Experimental design used to identify the critical medium components required for prominent glucoamylase production was based on 2 factorials. The screening of \( n \) variables by such
design requires \( n+1 \) experiments to be performed (Plackett and Burman, 1946). The design is based on assumption that there are no interactions between all the constituents of production medium. The concentrations of all the variables (medium components) were studied at two widely spaced intervals designated as -1 and +1 which indicates low and high level respectively (Table 2). Following equation illustrates the calculation of the individual effect of variables on glucoamylase production.

\[
E(X_i) = \frac{2(A^+ - A^-)}{N}
\]

Where, \( E(X_i) = \) Effect of variable \( X \) under study; \( A^+ \) and \( A^- \) = Response (activity of glucoamylase) obtained on performing trial at higher (+1) and lower (-1) level respectively; \( N = \) total number of trials.

The formula used to estimate the experimental error is given below and includes calculation of the variance for dummy variables.

\[
V_{\text{eff}} = \frac{\Sigma(Ed)^2}{n}
\]

Where, \( V_{\text{eff}} = \) Variance of the effect of respective level; \( Ed = \) the effect of level for the dummy variables; and \( n = \) Number of dummy variables used in the experiment.

Student’s t-test was used to determine the significance level (p-value) for effect of each concentration where Standard error (SE) was the square root of variance of an effect.

\[
T(X_i) = \frac{E(X_i)}{SE}
\]

**Software and data analysis:** Plackett-Burman experimental design was made using statistical software Design-Expert 8.0 (Stat Ease Inc., USA). Effect of individual variables on glucoamylase production was determined during analysis of the results in the same software.

**Table 2.** Experimental design by Plackett-Burman method, \( X_1 - X_7 \) are independent variables and \( D_1 - D_4 \) are dummy variables
RESULTS AND DISCUSSION

Identification of yeast: On PCR amplification of 18S rDNA from yeast genomic DNA, 680 bp long fragment was obtained (Fig. 1) followed by sequencing. The sequence was found to be having 99% homology with *Kluyveromyces marxianus* showing maximum score 1197 with 0.0 e-value. The sequence was submitted to GenBank, NCBI (Accession no. KY073322). The evolutionary history (Fig. 2) was extrapolated using the Neighbor-Joining method (Saitou and Nei, 1987). To represent the evolutionary history of the analyzed taxa, bootstrap consensus tree derived from one thousand replicate was taken (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Next to the branches, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (Felsenstein, 1985). Maximum Composite Likelihood method (Tamura *et al.*, 2004) was used to compute the evolutionary distances which was represented in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were

<table>
<thead>
<tr>
<th>Run</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>X4</th>
<th>X5</th>
<th>X6</th>
<th>X7</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>Glucoamylase Produced (U/L)</th>
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<td>-1</td>
<td>+1</td>
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<td>+1</td>
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1\textsuperscript{st}+2\textsuperscript{nd}+3\textsuperscript{rd}. All positions containing gaps and missing data were eliminated. There were a total of 528 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0 (Tamura \textit{et al}., 2013).

**Figure 1.** 656bp PCR product (lane 1) with DNA marker (lane 2)

**Figure 2.** Evolutionary relationships of taxa

**Optimization of incubation time and proportion of inoculum on glucoamylase production:** Inoculum was prepared as explained earlier. The effect of proportion of inoculum was studied on the basis of amount (% in ml, v/v) of inoculum used to inoculate fermentation medium. On increasing concentration of inoculum, glucoamylase production was found to be enhanced till 7% inoculum was used (Fig. 3). Further increase in inoculum resulted in decline in glucoamylase production. Time of incubation is one more essential factor required to be considered. Presence of starch as carbon source induced production of
glucoamylase and it was increased as time of incubation was increased. Glucoamylase activity was found to be declined after complete utilization of starch as detected on mixing iodine reagent with sample from fermentation medium which indicates that glucoamylase production was ceased after complete utilization of starch. In present study, maximum yield of glucoamylase (839 ± 21 Unit/L) was obtained after 96 hours of incubation. Li et al. (2007) reported that glucoamylase production by yeast Aureobasidium pullulans required 56 hours of incubation. In our case, glucoamylase production reached maximum after 96 hours incubation followed by decline.

![Figure 3](image_url)

**Figure 3.** Optimization of incubation time and proportion of inoculum (%) used for the production of glucoamylase

**Production of glucoamylase using different substrates:** On evaluating production of glucoamylase using different substrates as carbon sources (Fig. 4), starch and glucose were found to be producing maximum and minimum yield of glucoamylase as 928 ± 25 and 13 ± 3 Unit/L respectively. Starch as well as maltose can be used for glucoamylase production since both are noted as substrates for production. Clementi et al. (1986) utilized various carbohydrates for glucoamylase production using yeast Schwanniomyces castellii found starch as better substrate in comparison to maltose. Results obtained in our experiments...
agrees with the same, glucoamylase production achieved using maltose was 47% lesser as compared to glucoamylase production using starch. In addition, cheaper substrates like rice flour (549 ± 20 Unit/L) and corn flour (467 ± 11 Unit/L) were also able to yield remarkable amount of glucoamylase. As a substrate for glucoamylase production, glucose was found to be poor as compared to all others substrates.

![Figure 4](image.png)

**Figure 4.** Production of glucoamylase using various carbon sources

**Influence of organic and inorganic nitrogen sources on glucoamylase production:**

Glucoamylase production was studied using inorganic as well as organic nitrogen sources (Fig. 5) where starch (2 g% w/v) was used as carbon source. Amongst all the organic as well as inorganic nitrogen sources, utilization of peptone led to maximum yield of glucoamylase (918 ± 52 Unit/L). Use of corn steep liquor also yielded notable amount of glucoamylase (842 ± 58 Unit/L) as a cheapest nitrogen source amongst the nitrogen sources studied in the experiment. Mohamed *et al.* (2007) observed that inorganic nitrogen source are better nitrogen sources for glucoamylase production from *Candida famata*, but our results are similar to Kumar *et al.* (2001) where organic nitrogen sources were found to give higher glucoamylase production as compared to inorganic nitrogen sources.
Effect of medium pH and incubation temperature on production of glucoamylase: In case of submerged fermentation where the microbes get direct exposure to hydrogen ion concentrations, microbial growth as well as enzyme production are influenced. Maximum enzyme production is achieved at optimum medium pH and any increase and decrease in pH results in reduction of enzyme production simultaneously. As shown in Fig. 6, maximum glucoamylase production was obtained at pH 6.0 while further increase or decrease in medium pH declined the production of glucoamylase. Kumar et al. (2001) reported production of glucoamylase by *Pichia subpelliculosa* within mesophilic range i.e. 30°C which is commonly used incubation temperature for growth of yeasts. Similarly as seen in Fig. 7, maximum glucoamylase production occurred at 30°C. It indicates that glucoamylase production could be proportional to the growth of yeasts. Since temperatures higher or lower than 30°C doesn’t support growth of majority of yeasts, glucoamylase production was found to be declined on increasing or decreasing the incubation temperature.
**Figure 6.** Effect of initial medium pH on glucoamylase production

**Figure 7.** Effect of incubation temperature on glucoamylase production

**Screening of medium components using Plackett-Burman design:** Statistical methodology is employed especially due to its ease of application as compared to traditional methods. Mosbah et al. (2015) successfully utilized full factorial design for optimization of glucoamylase production by *Candida famata*. Plackett-Burman designs were found to be useful for determination of screening of medium components for glucoamylase production by *Colletotrichum* sp. (Prajapati et al., 2013). Medium components significantly required for production of glucoamylase were screened using statistical methodology which also
included selection of concentration of medium components necessary to achieve maximum production. Most common method includes initial screening of ingredients to understand their individual effect on the enzyme production followed by selection of certain better ingredients for further optimization. Total seven different medium components as carbon and nitrogen sources were screened using Plackett-Burman design in 12 run experiment with two levels of concentration for each of the variables (Table 2). The results obtained in form of glucoamylase activity from each of the run was considered as effect of the experimental design. Screening of the components was carried out at 95% confidence level on the basis of the effects obtained.

**Table 3. Statistical analysis of results obtained by medium components**

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Effect</th>
<th>Standard error</th>
<th>t-value</th>
<th>P-value</th>
<th>Confidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corn flour</td>
<td>-28.1</td>
<td>78.18</td>
<td>-0.36</td>
<td>0.74</td>
<td>26.30</td>
</tr>
<tr>
<td>2</td>
<td>Starch</td>
<td>296.33</td>
<td>78.18</td>
<td>3.79</td>
<td>0.02</td>
<td>98.07</td>
</tr>
<tr>
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<td>Corn steep liquor</td>
<td>-319.23</td>
<td>78.18</td>
<td>-4.08</td>
<td>0.02</td>
<td>98.49</td>
</tr>
<tr>
<td>4</td>
<td>Soya flour</td>
<td>-62.2</td>
<td>78.18</td>
<td>-0.80</td>
<td>0.47</td>
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<td>Peptone</td>
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<td>78.18</td>
<td>-1.01</td>
<td>0.37</td>
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</tr>
<tr>
<td>6</td>
<td>Urea</td>
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<td>78.18</td>
<td>-0.33</td>
<td>0.76</td>
<td>24.20</td>
</tr>
<tr>
<td>7</td>
<td>Rice Flour</td>
<td>-331.73</td>
<td>78.18</td>
<td>-4.24</td>
<td>0.01</td>
<td>98.67</td>
</tr>
</tbody>
</table>

Statistical analysis of result for the experiment performed on the basis of Plackett-Burman design is presented in Table 3. Component can be considered as effective which shows significance at or above 95% confidence level as well as the component with negative effect but the required amount of that variable could be lower than the indicated as low (-1) concentration in the experimental design. Present study indicated that starch, rice flour and corn steep liquor are significant variables since the confidence level for them was higher than 95% while remaining variables with confidence level lesser than 95% were considered as insignificant. So the medium for production of glucoamylase can be composed using +1 values of significant variables with positive t-value and -1 values of significant variables with negative t-value. Since substrates containing higher starch content were found to be
increasing glucoamylase production significantly giving inducing effect, they can be used efficiently as a carbon source.

**CONCLUSION**

The yeast was found to be producer of glucoamylase. The optimization revealed the mesophilic nature of fermentative production and acidic conditions required for production medium. Glucoamylase production was influenced by different carbon and nitrogen sources. Starch acted as better carbon source for production of glucoamylase than maltose. About 50-60% costs of enzyme production are the expenses made on carbon sources, so utilization of cheaper starchy substrates like rice flour and nitrogen source like corn steep liquor in the production medium can reduce the cost of glucoamylase production. One variable at a time approach for medium optimization is time consuming and laborious approach so the time and labor can be reduced on application of statistical methods for medium optimization. Present study achieved about 25% increase in glucoamylase production on utilizing Plackett-Burman design for statistical optimization of medium ingredients and included cheaper substrates which demonstrates it cost-effectiveness. Statistically optimized medium for glucoamylase production by *Kluyveromyces sp.* studied here may include w/v of each of 2 g% starch, 0.5 g% corn steep liquor and 0.1 g% rice flour.

**REFERENCES**


