β-Galactosidase production and ethanol fermentation from whey using

*Kluyveromyces marxianus* NCIM 3551

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β-Galactosidase production and ethanol fermentation from whey were studied using *Kluyveromyces marxianus* NCIM 3551 at laboratory scale. Optimum β-galactosidase production and ethanol fermentation was obtained with 16 h old culture at an inoculum size of 10% over an incubation period of 20 h at pH 5.0 and at 25°C. Nitrogen supplementation was found to have not much effect on β-galactosidase production and ethanol fermentation.

*Keywords*: Alcohol fermentation, β-Galactosidase, *Kluyveromyces marxianus* NCIM 3551, Whey, Yeast

**Experimental Section**

**Materials**

Whey was prepared from cow’s whole milk purchased from local market. Milk (200 ml) pH (3.5) was adjusted with 1 N HCl. It was then boiled for 20 min and cooled. Clear filtrate was obtained by filtering it through muslin cloth followed by Whatman filter paper No. 1. Whey (150 ml) thus obtained was sterilized by autoclaving after adjusting to desired pH using 0.1N NaOH or 0.1 N HCl. *K. marxianus* NCIM 3551 was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune (India).

**Maintenance and Preparation of Culture**

Yeast culture was revived by incubating at 30°C for 48 h on maintenance medium (MM) containing: glucose, 1; peptone, 0.5; malt extract, 0.3; and yeast extract, 0.3% w/v. Culture was maintained and preserved at 4°C by serial subculture at 2 weeks intervals on MM agar slants. MM (50 ml) was inoculated with a loopful of culture from agar slants and incubated at 30°C for 18 h on a rotary shaker at 100 rpm.

**Production Medium**

Clarified whey was used as production medium for β-galactosidase and ethanol. Whey medium (50 ml) was inoculated with 10% (v/v) of starter culture and incubated on a rotary shaker at 100 rpm. At various time
intervals, samples were drawn from flask and assayed for β-galactosidase activity and ethanol concentration.

Extraction Methods

**SDS-Chloroform Method**

Cell suspension (0.1 ml) was mixed with 0.9 ml Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl and 0.001 M MgSO₄). To this, chloroform (100 µl) and 0.1% SDS solution (50 µl) was added. Suspension was centrifuged at 1398 x g to obtain cell free extract.

**Isoamyl Alcohol Method**

A volume of re-suspended cells in 0.2 M phosphate buffer (pH 6.5) containing dry cell mass (10 - 20 mg) was mixed with isoamyl alcohol (5 ml) and diluted up to 25 ml with 0.2 M phosphate buffer (pH 6.5). Mixture was shaken for 15 min at room temperature (RT) to make cell envelope permeable and used for enzyme assay.

**Liquid Nitrogen Method**

Biomass was ground with liquid nitrogen using mortar-pestle and later centrifuged at 4000 x g for 20 min. Supernatant was used for enzyme assay.

**Toluene Method**

Cell suspension (1 ml) was mixed with equal quantity of cold toluene for 15 min with intermittent stirring. Mixture was vortexed for 5 min and then centrifuged at 4000 x g at 5°C for 20 min. Supernatant obtained was used for enzyme assay.

**Toluene-Acetone Method**

Production culture (10 ml) was centrifuged at 4000 x g at 5°C for 20 min. Pellet obtained was dissolved in 2 ml of 0.2 M phosphate buffer (pH 7.0), ground with alumina(2 g) and toluene: acetone (9:1) solution (0.1 ml). Suspension was redissolved in buffer (8 ml) and centrifuged at 4000 x g at 5°C for 10 min. Supernatant obtained was used for enzyme assay.

β-Galactosidase Enzyme Assay

Biomass was washed twice with 0.1 M phosphate buffer (pH 7.0) and re-suspended in same buffer. Appropriately diluted cell suspension (0.1 ml) was spectrophotometrically assayed for enzyme activity using O-nitrophenol-β-D-galactopyranoside (ONPG) as a substrate. One unit of enzyme activity is the enzyme quantity that liberated 1 µmole of O-nitrophenol per min under assay conditions. Values expressed are mean ± standard error of three independent experiments.

Ethanol Estimation

Ethanol was measured using potassium dichromate (K₂Cr₂O₇) method. Potassium dichromate reagent (10 ml) was added to 1 ml of whey (1:10 diluted) and was incubated for 30 min at RT. After incubation, distilled water (100 ml) and 4 ml of potassium iodide (25%) was added in each flask. Solution was titrated against 0.1 N sodium thiosulfate with starch (1%) as an indicator. Values expressed are mean ± standard error of three independent experiments.

Results and Discussion

**Effect of Extraction Methods on β-Galactosidase Activity**

Chloroform-SDS method was found best among methods tried for enzyme extraction (Fig. 1). Cell disintegration, applied in production of intracellular β-galactosidase from *Kluyveromyces* sp., can be achieved by application of shear forces (mechanical methods) and digestion or permeabilization of cell envelopes (chemical methods). For enzyme extraction using permeabilization of cells by organic solvents, performance of organic solvents is dependent on incubation time, incubation temperature and concentration of both cells and solvents. Chloroform-SDS method is reported ideal for extraction of β-galactosidase from yeast cells.

**Effect on β-Galactosidase Activity and Ethanol Production of Following Parameters**

**Incubation Period**

Maximum β-galactosidase activity was observed after 20 h of incubation (Fig. 2a) and also was found to be optimal for ethanol production (Fig. 2b). A decrease in enzyme activity was observed with further increase in incubation time (24 - 32 h), may be attributed to culture reaching stationary phase. Earlier studies have also reported optimal incubation period (18 - 24 h).
However, incubation period extending up to 30 h is also reported\(^\text{11}\).

**Inoculum Size and Age**

Different inoculum levels (4 - 16\%) were used as starter culture for \(\beta\)-galactosidase activity and ethanol production. Enzyme activity increased with inoculum size; maximum activity was observed at 16\% (Fig. 3a). However, in case of alcohol production, concentration of alcohol produced gradually increased with inoculum size up to 10\% and thereafter was found to decrease (Fig. 3b). Inoculum age was also found to have an effect on \(\beta\)-galactosidase production (Fig. 4a). Activity was maximum with a 16 h culture; increasing from 12 - 20\% and thereafter decreasing. Alcohol produced remained almost constant with inoculum age of up to 20 h and decreased thereafter (Fig. 4b). Therefore, a 16 h old culture and a 10\% inoculum size was used in further experiments to maintain optimal production of \(\beta\)-galactosidase and alcohol. Earlier studies used cultures varying in inoculum age from 20 h\(^\text{7,22}\) to 24 h\(^\text{21}\).

**pH**

Enzyme activity was constant till pH 4.5, peaked at pH 5.0, and decreased between pH 5.5 and 6.0 (Fig. 5a).
Temperature

β-Galactosidase production was better under shake flask conditions (SFC) at RT of 25°C (Fig. 6a). Similarly, alcohol production was also better at RT (25°C) under SFC (Fig. 6b). Temperatures (28 - 31°C) have been used in earlier studies for optimal β-galactosidase production. Earlier studies have also supported SFC for β-galactosidase production.

Nitrogen Source

Effect of supplementing whey with different nitrogen sources on β-galactosidase (Fig. 7a) and ethanol production (Fig. 7b) was studied. Nitrogen supplementation increases production of β-galactosidase and ethanol by reducing lag period and increasing alcohol production increased with increasing pH up to 4.5 and was constant till pH 5.5 and dropped at pH 6.0 (Fig. 5b). Earlier studies for optimal β-galactosidase production used pH 4.68 with *K. lactis* 20, pH 5.0 with *K. marxianus* 23, and pH 5.5 with *K. marxianus* 24.

Alcohol production increased with increasing pH up to 4.5 and was constant till pH 5.5 and dropped at pH 6.0 (Fig. 5b). Earlier studies for optimal β-galactosidase production used pH 4.68 with *K. lactis* 20, pH 5.0 with *K. marxianus* 23, and pH 5.5 with *K. marxianus* 24.

**Fig. 5**—Effect of pH on: a) β-D-Galactosidase activity; b) Ethanol production

**Fig. 6**—Effect of temperature on: a) β-D-Galactosidase activity; b) Ethanol production

**Fig. 7**—Effect of nitrogen supplementation on: A) β-D-Galactosidase activity; B) Ethanol production (a, whey; b, whey + ammonium sulphate; c, whey + ammonium nitrate; d, whey + urea; and e, whey + yeast extract)
bioconversion efficiency of ethanol production.\(^{27,28}\) Ethanol production studied earlier using Kluyveromyces\(^{29,30}\)(conc. 13 - 22 g/l) is in agreement with present study.

**Conclusions**

Optimal production of \(\beta\)-galactosidase and ethanol fermentation from whey using \(K.\) marxianus NCIM 3551 was found at following process conditions: pH, 5.0; temp., 25\(^\circ\)C (RT); incubation period, 20 h; and inoculums size, 10%.

**References**