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Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23

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ABSTRACT

Mutants of *Candida magnoliae* NCIM 3470 were generated by ultra-violet and chemical mutagenesis to enhance erythritol production. The mutants were screened for higher reductase activity on agar plates containing high concentration of glucose and 2,3,5-triphenyl tetrazolium chloride (TTC). One of the mutants named as R23 gave maximum erythritol production, 60.3 g L^{-1}, compared to 14 g L^{-1} of the parent strain. Glucose and yeast extract were identified as critical medium components which decide the ratio of polyols produced, mainly erythritol, mannitol and glycerol. In order to enhance the production of erythritol and to minimize the production of mannitol and glycerol, a four component-five level-three response central-composite-rotatable-design (CCRD) of response surface methodology (RSM) model was used. The optimum medium composition for erythritol production was found to contain (g L^{-1}) glucose 238, yeast extract 9.2, KH_2PO_4, 5.16 and $MgSO_4$ 0.23. Moreover, erythritol production was studied in a 10 L fermentor in batch and fed-batch mode using RSM optimized medium. In fed-batch fermentation, 87.8 g L^{-1} erythritol was produced with 31.1% yield, without formation of any other polyols. Thus present study involving strain improvement followed by media and process optimization resulted in 6.2-fold increase in erythritol production and 3.4-fold increase in the yield.

1. Introduction

Erythritol is a naturally occurring four-carbon sugar alcohol which is used as food grade bulk sweetener. It is present in small quantities in fruits and fermented foods. It is 60–70% as sweet as sucrose with a very low caloric value of 0.2 calories per gram [1,2]. Erythritol has been permitted to be used as a flavour enhancer, formulation aid, humectant, non-nutritive sweetener, stabilizer, thickener, sequestrant and texturizer at maximum levels of 100% in sugar substitutes [3].

Industrial-scale production of erythritol by chemical means involves catalytic hydrogenation of starch at high temperature and pressure using nickel as a catalyst but this process has not been industrialized because of its low efficiency [4]. On the contrary, biosynthetic routes offer the potential for safer and environment friendly erythritol production. Erythritol can be produced using osmophilic yeasts belonging to the genus *Aureobasidium*, *Candida*, *Moniliella*, *Pichia*, *Pseudozyma*, *Trigonopsis*, *Trichosporon*, *Trichosporonoides* and *Yarrowia* [2,5–7].

Candida magnoliae has been reported to produce several polyols and organic acids such as erythritol, glycerol, mannitol, xylitol, citric acid, gluconic acid, butyric acid and ethanol [8–11]. Interestingly, the composition of polyols produced by *C. magnolia* depends on the nature, composition and concentration of media constituents.

Development of a commercially viable process requires: (i) strain with high yield and productivity with minimal amount of interfering by-products, (ii) optimized fermentation medium composition having low-cost nutrients, (iii) optimized process parameters and (iv) ease of downstream processing for product recovery [12]. Conventional method of medium optimization is laborious and time consuming. Moreover, being linear, this method does not account for the synergistic effect between operational variables during fermentation process. These limitations can be overcome by application of statistical based approach [13,14].

Among different statistical methods, response surface methodology (RSM) has been extensively used for media optimization. Using RSM, it is possible to enhance the production of desired product selectively, by keeping the by-products at minimal possible levels. Since the separation of erythritol from the mixture of polyols is expensive and tedious, RSM appears to be an attractive method of media optimization.

The present study was initiated to improve erythritol production keeping the by-products at minimal possible levels.

The objective of the present study was to improve erythritol production from *C. magnoliae* by mutagenesis followed by media and process optimization to minimize formation of mannitol and glycerol.

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2. Materials and methods

2.1. Microorganisms and media

C. magnoliae NCIM 3470 was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The culture was maintained on malt extract glucose yeast extract peptone (MGYP) agar slants. All media ingredients were purchased from HiMedia, Mumbai, India. *N*-Methyl *N*-nitro *N*-nitroso guanidine (NTG), Ethyl methyl sulphonate (EMS), sugars and sugar-alcohols were purchased from Sigma–Aldrich, USA.

2.2. Culture conditions

A loop-full of respective culture from freshly prepared slant was inoculated into 5 mL liquid fermentation medium (LFM) in 150 mm × 25 mm test tubes and incubated at 28 °C, 210 rpm, for 48 h. Two and a half millilitre of these seed cultures were aseptically transferred to 250 mL Erlenmeyer flasks containing 22.5 mL liquid medium. The LFM was composed of ($g\,L^{-1}$) glucose 250, yeast extract 10, KH_2PO_4 5 and $MgSO_4{\cdot}7H_2O$ 0.25. Initial pH of the medium was adjusted to 6.0. Glucose was autoclaved separately for all the experiments. All experiments were performed in triplicate, using 250 mL Erlenmeyer flasks containing 25 mL fermentation broth incubated at 28 °C and agitated at 210 rpm for 168 h unless otherwise mentioned. At the end of the incubation, final volume of the culture broth was adjusted to 25 mL by sterile distilled water. Samples were withdrawn at fixed interval and analyzed for optical density at 600 nm, pH, residual glucose and polyol concentration.

2.3. Mutagenesis

With initial set of culture conditions, *C. magnoliae* NCIM 3470 produced 14 $g\,L^{-1}$ erythritol with a yield of 9.5% in LFM. The parent strain was grown in LFM for 48 h. The culture broth was centrifuged at 10,000 × *g* and the cells were washed twice with sterile physiological saline by suspending them followed by centrifugation. The cell count was adjusted to 1×10^5 cells mL^{-1} by counting cells under a microscope using Neubauer improved cell counting chamber (Marienfeld, Germany). Two millilitre of the above cell suspension was UV irradiated using germicidal lamp (Sankyo Denki Co. Ltd., Japan) for 0–3 min at a distance of 20 cm. For chemical mutagens (EMS and NTG) 5 mL of cell suspension with 1×10^5 cells mL^{-1} was treated with 20 μL EMS or 50 μL NTG solution (2 mg mL^{-1} in 50 mM acetate buffer pH 5.5), for 0–60 min. At fixed time interval, 0.5 mL filter-sterilized 5% sodium thiosulfate was mixed with 0.5 mL treated cell suspension to inactivate EMS and NTG. A combined UV and EMS/NTG treatment was carried out by irradiation of the cell suspension with UV for 30 s followed by EMS/NTG treatment for 20 min. After mutagenesis treatment, 20 μL mutagenized cell suspension containing approximately 2000 treated cells was spread plated on LFM agar plates with glucose/sucrose concentration between 400 and 600 $g\,L^{-1}$. To exert constant high selection pressure for selecting mutants in some of mutation series, plates were incorporated with 200 $g\,L^{-1}$ KCl with 20 $g\,L^{-1}$ sugar. Plates were incorporated with 0.1 $g\,L^{-1}$ TTC to screen the mutants with high reductase activity. A survival curve was drawn and used to get 90–95% kill.

2.4. Mutant selection

Plates with treated cells were incubated at 28 °C for six to seven days; colonies were selected from the plates, which showed 5–10% of survival. The colonies which appeared dark red in colour, bigger in size, smooth, non-pigmented and non-sticky on high osmotic pressure agar plates incorporated with TTC were selected. The selected colonies were inoculated in tubes with 5 mL LFM medium in 150 mm × 25 mm test tubes and incubated at 210 rpm, at 28 °C for 3 days and residual glucose was analyzed by enzyme kit. Mutants which showed higher glucose utilization compared to parent were selected and further evaluated in Erlenmeyer flasks with 25 mL medium. The mutants with enhanced erythritol production and minimal by-products formation based on HPLC analysis were selected and maintained on LFM agar slants.

2.5. Screening of media components for erythritol production by *C. magnoliae* mutant R23

2.5.1. Choice of carbon source

Glucose from the LFM was substituted with different carbon sources viz sucrose, fructose, glycerol and glucose–fructose mixture to study the effect of carbon sources on erythritol production by *C. magnoliae* mutant R23. The carbon sources were used at 250 $g\,L^{-1}$. Respective media were inoculated with 48 h seed culture and incubated at 28 °C, 210 rpm on rotary shaker. Samples were analyzed at 168 h for OD at 600 nm, pH, residual glucose and polyol concentration by HPLC. The effect of glucose concentration on erythritol production was further studied by varying the glucose concentration from 150 to 400 $g\,L^{-1}$.

2.5.2. Effect of nitrogen source

The effect of nitrogen source on erythritol production by *C. magnoliae* mutant R23 was evaluated in shake flasks. Two and half millilitre of seed cultures were inoculated in 250 mL Erlenmeyer flasks with 25 mL medium with varying yeast extract concentration from 2 to 16 $g\,L^{-1}$ and incubated at 28 °C, 210 rpm for seven days. Samples were analyzed as mentioned earlier.

2.5.3. Effect of metal ions on polyol production by *C. magnoliae*

Effect of metals ions namely Ca^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Mo^{+2}, Zn^{+2} and B^{+2} on polyol production were studied in shake flasks with 25 mL LFM. Three different concentrations of trace metals predominantly 10, 50 and 100 mg L^{-1} were added to individual flasks containing LFM. The flasks were incubated for 168 h and analyzed for growth and polyol production.

2.6. Comparison of erythritol production by *C. magnoliae* NCIM 3470 and mutant R23

To compare the erythritol producing mutant R23 with parent *C. magnoliae*, respective seed cultures were grown in 25 mL LFM in 250 mL Erlenmeyer flasks as described above and samples were analyzed at regular intervals of 24 h for OD, pH, concentration of residual substrate and polyol by HPLC. Glucose uptake rate, productivity and erythritol yield for the mutant generated were compared at flask level.

2.7. Optimization of media components: experimental design and statistical analysis

Four media components viz. glucose, yeast extract, KH_2PO_4 and $MgSO_4$ were chosen for this study. A central composite factorial design of $2^4 = 16$ plus 6 *centre points* plus 8 (i.e. 2 × 4) *star points* leading to a total of 30 experiments were performed in duplicate. Each flask was inoculated with 2.5 mL inoculum and incubated for seven days and analysis of polyols was done by HPLC as described earlier.

The coded values of independent variables are given in Table 1. The values of the three dependent responses (i.e. concentration of