Renewable Energy 109 (2017) 406–421

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

Augmentation of ethanol production through statistically designed growth and fermentation medium using novel thermotolerant yeast isolates

Richa Arora [a, b, 2], Shuvashish Behera [a, 2], Nilesh Kumar Sharma [a, b], Sachin Kumar [a, *, 1]

[a] Biochemical Conversion Division, Sardar Swaran Singh National Institute of Bio-Energy, Kapurthala, 144601, India
[b] I.K Gujral Punjab Technical University, Kapurthala, 144601, India

ARTICLE INFO

Article history:
Received 3 June 2016
Received in revised form
14 March 2017
Accepted 18 March 2017
Available online 21 March 2017

Keywords:
Thermotolerant yeast
Kluyveromyces marxianus
Ethanologen
Optimization
Face-centered central composite design

ABSTRACT

Overproduction of metabolites, high product yield and process economics are greatly influenced by the media composition used for growth and fermentation. The main purpose of this study is to enhance the ethanol production through statistical tool of response surface methodology (RSM) by optimizing media components for the growth and fermentation of thermotolerant isolates *Kluyveromyces marxianus* NIRE-K1 and NIRE-K3. Five different salts were used in the Face-centered Central Composite Design (FCCD), with the responses of biomass formation and ethanol production for growth and fermentation, respectively. Yeast extract and K_2HPO_4 were found to be the key media components for the growth and fermentation which is revealed from their interaction in both the yeast isolates. Further studies on batch fermentation kinetics using the optimized values of the medium composition for *K. marxianus* NIRE-K1 and NIRE-K3 resulted in final ethanol concentration of 17.73 (86.27% of theoretical ethanol yield) and 19.01 g l^{-1} (94.12% of theoretical ethanol yield), respectively. An increase in the ethanol yield and productivity by 11.36, 10.42% and 2.0, 2.7% was revealed in NIRE-K1 and NIRE-K3, respectively, as compared to our previous study.

1. Introduction

The drastic increase in the energy crisis, green house gas emissions and exhaustion of fossil fuel reserves have led to the development of renewable energy technologies [1–3]. However, one of the major challenges in the fuel production from renewable resources lies in the development of improved strains with efficient ethanol production [4,5]. Current research primarily focuses on the utilization of thermotolerant yeasts for efficient bioconversion of biomass to ethanol [6–9]. Thermotolerant ethanologenic fermentations are reported to be superior rather than the conventional mesophilic ones with higher bioconversion rates, continuous product recovery, economically viable processes due to lesser requirement of cooling and reduced risk of contamination [4,10–12].

Apart from the use of thermotolerant ethanologens, the commercialization of a bioprocess and its economics depends upon the cost for the cultivation of the culture and its subsequent ethanol yield. The biochemical and nutritional requirements of the bioprocessing strains is highly influenced by carbon, nitrogen sources along with supplements like amino acids, vitamins, antibiotics, etc., which further aids in the cost [13]. Also, mineral salts are generally used in ethanol producing industries to supplement the fermentation media and provide acceptable yields [14]. Moreover, various medium components have strong interactions which may affect the competence of the process, both positively and negatively [15]. Thus, there is a need to develop a medium formulation for convenient, cost-effective and efficient bioprocess technology for bioethanol production.

There are two methods for evaluating the optimal level, empirical method and statistical method. The former has several limitations because it involves substantial amount of time and labour taking OFAT (one-factor-at-a-time) approach into account. Moreover, it does not account for the interaction among the variables which strongly influences the bioprocess [16]. On the other hand, the latter involves the statistical tools like response surface

* Corresponding author. Biochemical Conversion Division, Sardar Swaran Singh National Institute of Bio-Energy, Jalandhar-Kapurthala Road, Wadala Kalan, Kapurthala, 144601, Punjab, India.
E-mail address: sachin.biotech@gmail.com (S. Kumar).
[1] Present Address: Department of Chemical & Biological Engineering, South Dakota School of Mines and Technology, Rapid City, SD, 57701, USA.
[2] Equal contribution.

http://dx.doi.org/10.1016/j.renene.2017.03.059
0960-1481/

methodology (RSM) for designing experiments, constructing models suitably fitting with the data and evaluation of positive and negative interactions among various variables and finally providing the optimal solutions with reduced number of experimental runs [17,18]. The main goal of RSM is to chase the optimum values of the parameters in such a manner that the maximum response can be obtained. Optimization of different medium composition using RSM has been done by several researchers for alcoholic fermentations [14,19–23].

Several researchers have reported the optimization of growth and fermentation conditions of thermotolerant *Kluyveromyces marxianus* for the production of inulinase [24]; β-galactosidase [25]; ethyl acetate [26]; hydrogen [27]. However, to the best of our knowledge, the statistical optimization of medium composition for both growth and ethanol fermentation of thermotolerant *K. marxianus* has not been reported yet. Therefore, the present study was carried out to optimize medium components for novel thermotolerant ethanologenic yeast *K. marxianus* NIRE-K1 and NIRE-K3 for biomass and ethanol production using RSM, thereby, formulating a new medium for cost-effective bioethanol production with enhanced ethanol yield. In addition to this, the comparison of optimum medium components for growth and fermentation would aid in better understanding of the physiology of this yeast.

2. Materials and methods

2.1. Microorganism and culture conditions

Two thermotolerant yeast *K. marxianus* NIRE-K1 (MTCC 5933) and NIRE-K3 (MTCC 5934) used in this study were isolated and reported in our earlier study [28]. The cultures were maintained on yeast extract-peptone (YEP) medium [(g l^{-1}): yeast extract (Himedia, Mumbai, India), 10; peptone (Himedia, Mumbai, India), 20; glucose (Himedia, Mumbai, India), 20; phytagel (Sigma-Aldrich, USA), 15; pH, 5.5]. Both the cultures were stored in refrigerator at 4 ± 0.5 °C for future use and the stock cultures were maintained in 30% glycerol at −80 °C.

2.2. Inoculum preparation

Inoculum was prepared in 500 mL cotton plugged erlenmeyer flask containing 100 ml salt medium (as mentioned above but without phytagel) having 1% glucose as the carbon source. A loopful pure culture of both the isolates *K. marxianus* NIRE-K1 and NIRE-K3 were inoculated separately in the flasks as mentioned in Arora et al. [28] through the incubation of 24 h at 45 °C in shaking condition and were used for the inoculation in the subsequent runs for optimization.

2.3. Growth and fermentation medium

Growth and fermentation medium for both the isolates were prepared separately in 100 mL cotton plugged and capped erlenmeyer flask containing 25 ml of salt medium (as mentioned above but without phytagel), respectively. The initial glucose concentrations in growth and fermentation optimization were 10 g l^{-1} and 40 g l^{-1}, respectively. Optimization of growth media for both the isolates was carried out in aerobic condition through the inoculation of 25 μl inoculums in each flask. However, optimization of fermentation media for both the isolates was carried out in anaerobic conditions through the inoculation of 2 g l^{-1} harvested cells. All the optimization experiments were run for 16 h at pH 5.5, temperature 45 °C and shaking at 150 rpm in an orbital shaker incubator (New Brunswick Innova 43/43R Shaker, Germany).

2.4. Optimization of growth and fermentation medium components using FCCD

Medium components playing an indispensible role for the growth and fermentation of *K. marxianus* NIRE-K1 and NIRE-K3 were optimized according to (RSM) using Design Expert software version 8.0 (STAT-EASE Inc., Minneapolis, USA). FCCD (Face-centered Central Composite Design) was employed to study the combined effect of the components like yeast extract, di-potassium hydrogen phosphate (K_2HPO_4), sodium di-hydrogen phosphate (NaH_2PO_4), magnesium sulphate ($MgSO_4$) and ammonium sulphate [$(NH_4)_2SO_4$] on the biomass (g l^{-1}) and ethanol concentration (g l^{-1}) as the responses. All the variables were studied at three levels viz. low (−1), middle (0) and high (+1), with alpha value of 1. The real and coded values of these variables have been presented in Table 1. The software displayed 50 experimental runs, with 8 runs at the middle points. Similar runs at the central values ensure the accuracy of the data along with reproducibility of the model. Further, to enhance the accuracy of the model, the experiments were performed in duplicate and the values of the responses were the means of two replications.

The statistical significance of the model was estimated by analysis of variance (ANOVA) with p-value < 0.05 i.e. above 95% confidence level and insignificance of lack of fit test. The responses of the dependent variables were analyzed using the polynomial Equation (1) of second order. The variance for each variable was divided into linear, quadratic and interactive parts mentioned below:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{55}x_5^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{34}x_3x_4 + b_{35}x_3x_5 + b_{45}x_4x_5 \quad (1)$$

where, Y is predicted response, x_1, x_2, x_3, x_4 and x_5 are the coded levels of independent parameters, b_0 is the offset term, b_1, b_2, b_3, b_4 and b_5 are the linear effects, b_{11}, b_{22}, b_{33}, b_{44} and b_{55} are the quadratic effects and b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, b_{45} are the interaction effects.

The quality of the models developed were evaluated by three types of R-squared values i.e. coefficient of determination, adjusted R^2 and predicted R^2. The fitted polynomial equations were then expressed in the form of contour and three dimensional plots, to illustrate the relationship between the responses and any two variables to be optimized, keeping the other variables at central positions. The interaction of any two variables under the study can be examined from the prototype of the contour plots. Further, numerical optimization method was used for obtaining the optimal solutions.

2.5. Validation through growth and fermentation

Confirmatory experiments under optimized conditions for both

Table 1
Coded values for each variable of FCCD for biomass and ethanol production.

Variables	Unit	−1	0	+1
Yeast extract	g l^{-1}	1	3	5
di-potassium hydrogen phosphate	g l^{-1}	0.1	1.05	2
Sodium di-hydrogen phosphate	g l^{-1}	0.1	1.05	2
Magnesium sulphate	g l^{-1}	0.1	0.55	1
Ammonium sulphate	g l^{-1}	0.1	1.05	2