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Short Communication

An interactive study of influential parameters for shikimic acid production using statistical approach, scale up and its inhibitory action on different lipases

Garima Rawat, Priyanka Tripathi, Sweta Yadav, R.K. Saxena *

Department of Microbiology, University of Delhi South Campus, New Delhi 110 021, India

HIGHLIGHTS

- Statistical optimization for the production of shikimic acid from *C. freundii* GR-21.
- A higher amount of 16.78 g L^{-1} shikimic acid was obtained in 60 h after RSM.
- Optimized process was successfully scaled in 14 L bioreactor with similar yields.
- Shikimic acid produced shows successive inhibition against pancreatic lipase.
- Shikimic acid produced inhibits activity of lipases sourced from different microbes.

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ABSTRACT

Shikimic acid is the promising candidate as a building block for the industrial synthesis of drug Tamiflu used for the treatment of Swine flu. The fermentative production process using microbes present an excellent and even more sustainable alternative to the traditional plants based extraction methods. In the present study, the fermentative production of shikimic acid by *Citrobacter freundii* GR-21 (KC466031) was optimized by process engineering using a statistical modeling approach and a maximum amount of 16.78 g L^{-1} was achieved. The process was also scaled up to 14 L bioreactor to validate the production of shikimic acid. Further, the potential of anti-enzymatic nature of purified shikimic acid was evaluated for different lipases wherein, shikimic acid inhibited the hydrolysis of triglycerides by 55–60%. Shikimic acid also profoundly inhibited pancreatic lipase activity by 66%, thus providing another valuable therapeutic aspect for treating diet induced obesity in humans.

1. Introduction

Chemists have long been aware of the attainable assets of using chiral building blocks as starting materials in the synthesis of natural products. Shikimic acid (3,4,5 trihydroxy cyclohexene carboxylic acid), a widely occurring primary plant metabolite, has been identified in past for only its limited use as an optically active synthetic precursor in multistep chemical synthesis of cyclohexene substituted skeletons. More recently, its applications as a chiral template in the synthesis of antiviral drug (Tamiflu) for the treatment of Swine flu has gained the impetus (Bochkove et al., 2011; Johansson and Liden, 2006). Shikimic acid acts as a key starting material for the manufacturing of drug Oseltamivir phosphate (Tamiflu) which is a neuraminidase inhibitor prevents the spread of virus in the body. Oseltamivir is an effective treatment against Swine flu and it is also used in prophylaxis (Ghosh et al., 2012). Shikimic acid finds many applications in the synthesis of a variety of industrially important chemical products which have innovative and multifunctional roles such as in dermo-cosmetic preparations, anti-enzymatic activity and an exfoliating agent for stratum corneum (Guglielmini, 2010). Indeed, shikimic acid being an anti-enzymatic in nature also plays a key role in cosmetic industry, it provides a new route to improve deodorization with maximum efficacy and as sebum controller (Guglielmini, 2010; Kanwar et al., 2005).

The biological production of shikimic acid involves the shikimate pathway which is pervasive in microorganisms & plants and provides precursors for the biosynthesis of primary metabolites such as the aromatic amino acids (Ghosh et al., 2012). Currently, main commercial supply of shikimic acid is from the Chinese plant Star anise (*Illicium vernum*) but the process is ponderous and expensive which eventually impels the microbial route for the production of shikimic acid from renewable resources. During global pandemic of influenza, the limited supply and high cost were held

* Corresponding author. Tel.: +91 01124116559.
E-mail addresses: garima_205@yahoo.com (G. Rawat), rks.micro2012@gmail.com (R.K. Saxena).

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the major reasons behind the shortage of drug (Rawat et al., 2013a,b). With the worldwide rapid development, a breakthrough is being expected in the field by creating new protocols for the higher production of shikimic acid. Thus, an alternative sustainable approach of fermentation process utilizing microorganism has gained the importance to achieve large scale production of this expensive chemical. Several studies were performed on different microorganism mainly with metabolically engineered strain *Escherichia coli* to obtain shikimic acid (Escalante et al., 2010; Frost et al., 2002; Knop et al., 2001).

In the present study, statistical approach of medium optimization and scale up in a 14 L bioreactor was attempted to achieve an improved shikimic acid production by *Citrobacter freundii* GR-21. Statistical optimization of culture conditions resulted in a 2.21-fold increase in the yield of shikimic acid. Furthermore, the shikimic acid produced was purified and its efficacy like its anti-enzymatic property against different lipase mainly pancreatic lipase was evaluated which shows successive inhibition in the range of 55–66%, providing a new dimension of its therapeutic applications.

2. Methods

2.1. Bacterial strain

A shikimic acid producing strain of *C. freundii* GR-21, with accession No. KC466031 was used for the studies. Stock culture was maintained in 50% (v v^{-1}) glycerol and stored at −20 °C in a deep freezer.

2.2. Shikimic acid production

The chemically defined medium optimized earlier by "one-variable-at-a-time-approach" for shikimic acid production (Rawat et al., 2013a,b) was used in the present study. The production medium (50 mL in 250 mL shake flasks) consist of (g L^{-1}): Glucose, 50; K_2HPO_4, 1; Asparagine, 40; $MgSO_4{\cdot}7H_2O$, 0.4; L-Phenylalanine, 0.15; L-Tyrosine, 0.15; L-Tryptophan, 0.15; *p*-Aminobenzoic acid, 0.005; $FeSO_4{\cdot}7H_2O$, 0.0099; $CuSO_4$, 0.0005; $MnSO_4$, 0.0072; $ZnCl_2$, 0.0025; $CaCO_3$, 20; pH 6.0. Each flask was inoculated with 1% inoculums (v/v) (O.D. 1.87) and incubated at 30 °C and 200 rpm for 72 h. The shikimic acid and growth was determined using the culture filtrate.

2.3. Analytical methods

The cell growth (optical density) was monitored at 660 nm by UV–Vis spectrophotometer (UV 1700, Shimadzu). The concentration of shikimic acid and other byproducts formed in production medium were determined by HPLC (Shimadzu Corp., Kyoto, Japan) equipped with analytic column Aminex HPX-87H (Bio-Rad, USA) and RI-detector 10 (Shimadzu Corp., Japan). The mobile phase was 5 mM H_2SO_4 with flow rate of 0.6 ml/min maintained at the column temperature of 55 °C.

2.4. Statistical approach

In our previous studies, results revealed that the major variables influencing the performance of the culture in terms of shikimic acid yield and cell growth were glucose, asparagine, magnesium sulphate ($MgSO_4{\cdot}7H_2O$), potassium-di-hydrogen phosphate (KH_2PO_4) and calcium carbonate ($CaCO_3$). These components were chosen for further optimization using statistical approaches *viz.* Plackett–Burman (PB) and Response surface methodology (RSM).

2.4.1. Evaluation of the variables using Plackett–Burman design

The screening of most significant factors affecting shikimic acid production was performed using PB design. The importance of the seven variables, namely, glucose, asparagine, KH_2PO_4, $CaCO_3$, $MgSO_4{\cdot}7H_2O$, inoculum level and agitation rate for shikimic acid production was investigated. Physical factors *viz.* pH and temperature were considered as independent variables in this study. Each variable is represented at two levels, −1 for low level and +1 for high level (Table 1a). Design expert software 6.0 (Stat Ease Inc., Minneapolis, USA) was used to generate a set of 12 experiments. The experimental design and obtained data were analyzed statistically.

2.4.2. Response surface methodology (RSM)

Based on the Plackett–Burman studies, a response surface methodology of face centered central-composite design (FCCCD) was employed to determine the optimal level of selected variables which significantly influenced the production of shikimic acid. The levels of each factor and the design matrix are given in Tables 1a and 2.

Design expert software 6.0 (Stat Ease Inc., Minneapolis, USA) was used for the experimental designs and subsequent regression analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test. The average of shikimate activity was taken as dependent variable or response (*Y*).

2.5. Shikimic acid production in 14 L bioreactor

The shikimic acid production was scaled up in a 14 L bioreactor (Bioflow IV, NBS, USA) with a working volume of 8 L under the RSM optimized culture conditions. The medium was inoculated with 6% inoculum raised in seed culture (OD660 1.85) and fermentation was carried out at 30 °C, pH 6 and 200 rpm for 96 h. Dissolved oxygen level was saturated to 100%. Samples were withdrawn periodically at an interval of 6 h for analysis.

Table 1a
Statistical approach of medium optimization for shikimic acid production.

Variables	Glucose (g L^{-1})	KH_2PO_4 (g L^{-1})	Asparagine (g L^{-1})	$CaCO_3$ (g L^{-1})	Inoculum level (v v^{-1})	Agitation rate (rpm)	$MgSO_4$ (g L^{-1})
(i) Maximum and minimum ranges of seven parameters used in the Placket Burman run during the signal parameters for shikimic acid production							
High level (+1)	40	0.5	30	10	2	150	0.2
Low level (−1)	60	1.5	50	30	6	250	0.6

Variables (g L^{-1})	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Coded
(ii) Experimental ranges and levels of the three independent variables used in RSM in terms of actual and coded factors										
Glucose	6.36	−α	20	−1	40	0	60	+1	73.64	+α
Asparagine	41.59	−α	45	−1	50	0	55	+1	58.41	+α
$CaCO_3$	13.18	−α	20	−1	30	0	40	+1	46.82	+α

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