Optimization of Bioprocess Variables for Fungal Lipase Production using Statistical Experimental Design: A Mini Review

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Abstract— **Lipases hold the important role in wide spectrum of biotechnological applications. Lipases are unique, not only due to their ability to perform hydrolysis of fats into fatty acids and glycerol at the water-lipid interface, but also can reverse the reaction in non-aqueous media. Currently, the studies revealed that fungi are the one of choice for lipase production. Some important lipase-producing fungal genera include *Aspergillus, Penicillium, Rhizopus, Candida, Geotrichum, etc.* The development of response surface methodology applied to optimize fungal lipase production is discussed.**

Keywords—extracellular lipase; enzyme; RSM

I. Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a group of enzymes that catalyze the hydrolysis of triglycerides to intermediate and short chain di and monoglycerides, free fatty acids and glycerol [1]. In addition, lipases are also involved in a wide range of conversion reactions that include esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis in non aqueous media [2].

Lipases are commonly used in the processing of fats and oils, food processing, leather, textile, detergents and degreasing formulations, paper manufacture, synthesis of fine chemicals, production of pharmaceuticals, cosmetics, etc. [3]. In fact, the industrial practices require the efficiency in case of time and cost of bioprocesses. Therefore, with the purpose of getting the maximum yield and minimum cost of lipases, the optimization of lipases production is necessary. It is possibly to be achieved by choosing inexpensive substrate and inducer as well as efficient method of optimization and downstream processing [4].

The greatest difficulty in optimization of culture conditions for lipase production is the presence of interactive effects of medium compositions and culture condition factors. In case of conventional method of lipase optimization, the independent variable is studied while maintaining all the other factors at a fixed level, consequently it requires more data set of experiments which results in time consuming and backbreaking work [5]. On the other hand, statistical experimental designs are systematic and more efficient in case of time and cost for bioprocess optimization can overcome these shortages. Statistical approach methods such as response surface methodology can provide a systematic and efficient plan for experimentation to achieve certain goals, so that several control factors are simultaneously studied. There are less review articles on fungal lipases and its optimization of production, this review is a mini compilation report on the optimization of fungal lipase production using statistical method.

II. Fungal Lipase

Looking into the source of lipases, microorganisms are the preferred sources due to several industrial potentials. Recently, the extensive research works have been carried out on plant lipases, animal lipases and microbial lipases, particularly bacterial and fungal lipases. In the Figure 1, it shown the biodiversity of lipases with biological origin and shows the number of characterized and uncharacterized lipases [6]. In addition, many researches have explored fungi as valuable sources of lipase due to their versatility of its properties, ease of mass production, thermal stability, pH stability, broad substrate specificity, retained activity in organic solvents, and its low cost extraction procedure. Moreover, most fungal lipases are of considerable commercial importance for its bulk production [7]. It is easy to find the species of fungi which able to produce lipases in several habitats, including soils contaminated with oils, wastes of vegetable oils, dairy product industries, seeds, and deteriorated food [8, 9]. Some of the major lipase-producing fungi are of the genera *Mucor, Rhizopus, Geotrichum, Rhizomucor, Aspergillus, Humicola, Candida, Ashbya, Beauveria, Fusarium, Acremonium, Alternaria, Eurotrium, Ophiostoma* and *Penicillium*. The genera *Pichia, Hansenula* and *Saccharomyces* are reported to produce lipase as well. Among the different biological sources of the lipases studied, filamentous fungi are thought to be the best source for industrially useful lipases because these lipases are usually extracellular and soluble [10,11]. There are few reports concerning about molds with alkalophilic and thermostable potential lipases. For instance, the thermophilic *Mucor pusillus, Rhizopus homothallicus*, and *Aspergillus terreus* are well known as the producer of thermostable extracellular lipase. Additionally, *Mucor* sp. produces an extracellular, inducible, alkalophilic, and thermostable lipase [12]. *Rhizopus* species is mainly divided into three groups, *Rhizopus oryzae, Rhizopus microsporus,* and *Rhizopus stolonifer* [13]. The

lipase gene from *R. stolonifer* has been reported with 84% amino acid sequence identity to *R. oryzae* lipase. The LIP2 lipase from the *Yarrowia lipolytica* (YLLIP2) is reported as an ideal candidate for enzyme replacement therapy due to its unique biochemical properties due to its highest activity at low pH values and unrepressed by the bile salts. YLLIP2 belongs to the same gene family as *Thermomyces lanuginosus* lipase, a well-known lipase with many applications in the field of detergents and biotechnological processes [14]. In addition, among *Candida albicans, Candida antarctica, Candida rugosa, Geotrichum asteroids, Geotrichum candidum, Geotrichum asteroids, Trichosporon fermentas, Saccharomycopsis lipolytica,* and *Yarrowia lipolytica* (formally *Candida paralipolytica)* are reported to produce multiple lipase forms [15].

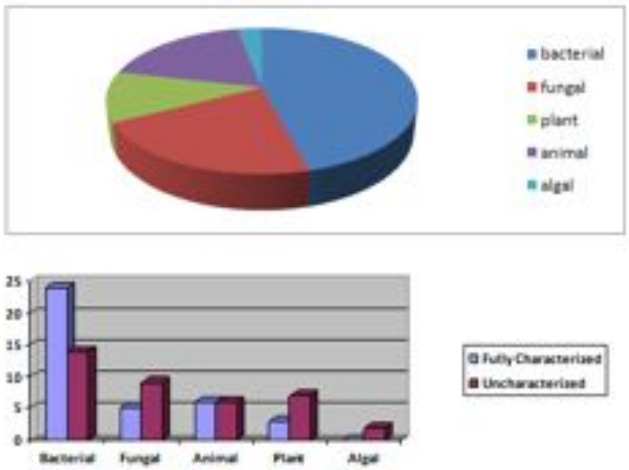

Fig. 1. Lipases biodiversity and comparison of characterized and uncharacterized lipases

Generally, fungal lipases constitute an important group of biotechnologically important enzymes because of the versatility of its properties and ease of mass production. Fungal lipases are versatile in its enzymatic properties and substrate specificity, which make it very attractive for industrial applications. Moreover, fungal lipases have gained special industrial attention due to they comprise of a variety of different applications including production of biopolymer and biodiesel, pharmaceuticals, agrochemicals, cosmetics and flavours, etc. [16, 17]. However, there are less review articles on fungal lipases, such as a compilation of reports on the fungal lipase production, process operation, purification, and its industrial applications.

The lipase production by fungi was developed by bioprocesses, mainly, using submerged fermentation such as the screening of high lipase producers, successful substitution of synthetic media by agro-industrial residues, scale-up of process and the use of mathematical models as a tool for process optimization. Nowadays, lipases like most specialty and industrial enzymes are increasingly produced via recombinant DNA technology as well [18].

III. Optimization of Lipase Production by Statistical Approach

In term of optimization of enzyme production, there are two common methods used, the conventional and statistical method of optimization. In fact, the conventional method has several limitations such as costly, laborious and time consuming. The conventional procedures of optimization described as altering one variable at a time (OVAT) while all other parameters are kept constant [19]. Moreover, it requires more sets of experimental data and can not provide further information about the variables interactions [20]. By contrast, the statistical experimental approach to optimize a system provides more systematic and efficient plan for bioprocess and interactions among variables in the systems as well [21]. The statistically designed experiments consist of several well-planned steps of individual experiments conducted together as follows:

- Selection of responses that will be observed
- Identification of factors and levels, different treatments at which these factors will be set in different individual experiment, consideration of blocks and define the data analysis procedure
- Conducting designed experiments,
- Analysis of the data using statistical software
- Prediction of the experimental performance including individual factor contribution, relative factor interaction, determination of optimum levels, ANOVA, and performance under optimum conditions
- Validation of the experiment and statistical model

Those steps of statistical experiment are usually used in the response surface methodology (RSM) and further developed in many kind of design of experiments.

A. Response Surface Methodology (RSM)

Term of response surface methodology (RSM) is described as a collection of mathematical and statistical techniques for empirical model building. The o\bjective of design of experiments using RSM is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests in a system called runs. In the system, changes are made in the input variables in order to identify the output response and the reason of changes in it. Originally, RSM was developed to model experimental responses and then migrated into the modelling of numerical experiments. The difference is in the type of error generated by the response. In RSM, the errors are assumed to be random. Additionally, the purpose of application of RSM in the design optimization is for lowering the expensive experimental cost and also to reduce their numerical noise [22]. Design of experiment is an important aspect of RSM usually abbreviated as DoE. The choice of design depends on the properties it is required or desired to have. Some of the design properties considered in the early development of RSM include the following, orthogonality, rotatability and uniform precision. Two important models are commonly used in RSM. These are special cases of model includes the first-degree model (d = 1) and the second-degree

model (d = 2). Designs for fitting first-degree models are called first-order designs and those for fitting second-degree models are referred to as second-order designs. The most common first-order designs are 2k factorial (k is the number of control variables), Plackett–Burman, and simplex designs, whereas the most frequently used second-order designs are the 3k factorial, central composite, and the Box–Behnken designs. Central composite design (CCD) is perhaps the most popular of all second-order designs.

The lipase production by microorganisms, especially by bacterial and fungal strains, the experimental designs using statistical approach for optimization of lipase have been studied both in whole-cell and extracellular lipase production. Response surface methodology was employed to optimize the extracellular lipase production by *Burkholderia* sp. HL-10. The optimum concentration of olive oil, tryptone and Tween-80 were determined by using a faced-centered central composite design (FCCCD) [23]. A combination of Plackett-Burman design with Box-Behnken factorial design was used for the optimization of lipase production by *Rhizopus orizae* KG-10 [24]. RSM was carried out as well for the optimization of lipase production from *Aspergillus terreus* using face centered central composite design (FCCCD) [19].

B. Further Development of RSM (Taguchi's Contribution to Experimental Design)

A full fractional design have a large number of experiments involves all possible combination of factors in the system. This method tends to be tedious, laborious and time consuming. Therefore, selection of only small set from all of the possibilities to reduce the number of experiments is conducted, known as a partial fractional design. Although this method is well known, there are no general guidelines for its application or the analysis of the results obtained by performing the experiments. Considering by this difficulties, Dr. Genichi Taguchi formulated a special set of general design guidelines for factorial experiment; he introduced the idea of robust parameter design [21, 25]. Taguchi's methods [26] study the parameter space based on the fractional factorial arrays from DoE, called orthogonal arrays. Among various statistical experimental designs, Taguchi experimental design offers solution to overcome the drawbacks by which many factors can be examined simultaneously and much quantitative information can be obtained with a few experimental trials [27]. A few reports are available on the application of Taguchi's method in the field of biotechnology. The basic principle of this method serves as screening filters which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments [28]. Taguchi method of DOE involves establishment of large number of experimental situation described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments [29]. Taguchi methods utilize two-, three-, and mixed level fractional factorial designs and similar to the familiar fractional factorial designs. Taguchi design has applied in the biotechnological area such as to optimize several fermentation processes for improving production of different secondary metabolites, in molecular biology such as PCR optimization, in the waste water treatment and bioremediation and in the medical technology fields such as tumour identification, vaccine production, diagnostic uses, etc. [21].

Furthermore, variables or factors are arranged in an orthogonal array (OA) in term of Taguchi design. The orthogonal array properties are such that between each pair of columns each combination of levels (or variables) appears an equal number of times. Due to an orthogonal layout, the effects of the other factors can be balanced and give a relative value representing the effects of a level compared with the other levels of a given factor. In orthogonal array experiments, the number of test runs is minimized, while keeping the pair-wise balancing property [25].

Taguchi proposed that the input variables in an experiment were of two types, (1) control factors: easy to control and (2) noise factors: difficult to control. Taguchi design has four steps [21] include planning phase to identify the conditions and the control factors such as parameter or variable such as temperature, pH, agitation, aeration, dilution rate, inoculums, substrate concentration, moisture content, etc. The conducting phase is performing the experimental design in random order using special orthogonal arrays (OA) such as L4, L8, L9, L12, L16, L18, L27 and L64. The experiment of Taguchi design includes a number of trial (experimental) conditions. The symbol indicates the size of experimentation, e.g. L9 has 9 trials. The total degree of freedom available in an OA is equal to number of trials minus one. In the optimization of cell-bound lipase production by *Rhodotorula mucilagenosa* [30], Taguchi experimental design, a standard orthogonal array (OA) L9 (3^4) with 8 degrees of freedom, was used to examine the following four factors: carbon sources (A), nitrogen sources (B), initial pH (C) and surfactants (D). The levels of the factors studied included palm oil, coconut oil and soybean oil as carbon sources; peptone, tryptone and NH_4NO_3 as nitrogen sources; Gum Arabic, Tween 80 and Triton X-100 as surfactants; and pH 4, 5 and 6 as initial pHs of the culture medium. Another example is optimization of xylitol production using *Candida* sp. by plan L18 (21 x 37) as an OA [31]. The analysis phase includes analysis of experimental data and prediction of optimum conditions. The last, implementation phase is conducting the experiment to support verification of results that obtained and solution implementation. The Taguchi experimental design and other response surface methodology approach were used, for example, Teng and Xu [32] were used Taguchi method L18 (2^1 x 3^7) for the initial optimization to obtain significant factors that influence the *Rhizopus chinensis* whole-cell lipase activity yield. The factors were selected to test the effect on the lipase production using response surface methodology, Box-Benhken design (4 factors and 3 levels). Nuylert and Hongpattarakere [30] were studied the cell-bound lipase from *Rhodotorula mucilagenosa* P11I89 for use as a methanol-tolerant, whole-cell biocatalyst for production of fatty acid methyl ester (biodiesel, FAME). The optimization of enhancement of transesterification activity in their study were

used a combination of Taguchi experimental design L9 (3^4) and central composite design (CCD). RSM was employed to study the interaction of the parameters which obtained from Taguchi method. Factorial design (2^3) with 6 axial points and 6 central points was carried out. In fact, Taguchi method has certain limitation in practice. The optimal solutions were only obtained within the specified setting levels. In other words, once the parameter setting is determined, the range of optimal solutions is constrained. The method is unable to find the real optimal values when the specified parameters are continuous in nature. RSM can overcome this limitation due to its statistical techniques applicability to the experimental design, model construction, evaluating the effects of factors and screening optimum condition of factors for desirable response [30, 32].

IV. Conclusion

To overcome the drawbacks of conventional method on optimization of lipase production, the statistical experimental designs such as Taguchi experimental design and response surface methodology (RSM) can be applied. It is important to study the optimization of lipase production by fungal strain in order to obtain the new lipase with better quality; low cost produced and wider range of application in the industrial field.

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