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Optimization of chemical and physical parameters affecting the activity of pectin lyase and pectate lyase from *Debaryomyces nepalensis*: A statistical approach

Sathyanarayana N. Gummadi *, D. Sunil Kumar

Department of Biotechnology, Indian Institute of Technology-Madras, Chennai 600036, India

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Abstract

Debaryomyces nepalensis was previously isolated, which is capable of producing pectin lyase and pectate lyase using pectin as sole source of carbon. The parameters affecting the activity of these enzymes are categorized into two, viz., chemical (amount of substrate and enzyme) and physical (pH of reaction mixture and temperature of incubation). The effect of these parameters on pectin lyase and pectate lyase was studied using central composite design. The optimal conditions of amount of substrate and enzyme (culture broth) were found to be 545 μl (∼1 g/l pectin) and 123 μl for pectin lyase and 707 μl (∼1.1 g/l polygalacturonic acid) and 96 μl for pectate lyase. The optimum pH and temperature for reaction was found to be 6.4 and 35 °C for pectin lyase and 7.5 and 32 °C for pectate lyase. After optimization, the activity of pectin lyase and pectate lyase was increased by 1.3- and 1.4-fold, respectively.

Keywords: Pectin lyase; Pectate lyase; Pectin; Polygalacturonic acid; Transelimination reaction; Physical parameters; Chemical parameters; Response surface methodology; Central composite design

1. Introduction

Pectin is one of the most widely available polysaccharide in nature next to cellulose, starch and chitin. The basic unit of pectin is α-D-galacturonate units linked through α-1,4-glycosidic linkages. The side chains of pectin molecule consist of rhamnose, galactose, arabinose and xylose. The carboxyl groups of galacturonate are esterified with methanol and based on the degree of esterification the pectic substances were differentiated into protopectin, pectin, polygalacturonic acid and pectinic acid [1]. Hence, for the efficient degradation of pectic substances combined action of different pectinolytic enzymes is required. Pectinases have wide variety of applications including juice and oil extraction, clarification of juice and wines, macerate and liquefaction of vegetable and plant tissues [2–5]. Pectic transeliminases or pectic lyases are one among the complex group of pectinases, which degrade pectic substance by transelimination mechanism yielding unsaturated oligogalacturonates. Pectin lyase (PL) and pectate lyase (PGL) are the two most important pectic lyases, which degrade pectin and polygalacturonic acid, respectively. Recently, pectic lyases have been exploited for many industrial applications such as retting and degumming of plant fibers, coffee and tea fermentation and bioscouring of cotton [6–9]. In general, PL was produced by fungi whereas bacteria produce PGL [1,8]. Since applications of pectic lyases are in an increasing trend [3,7–9], production of these enzymes with good catalytic properties by a single strain is required. In this context, we previously isolated *Debaryomyces nepalensis* from rotten apple capable of producing PL and PGL by utilizing pectin as sole source of carbon and nitrogen. The present strain has been deposited in National Collection of Yeast Cultures (NCYC) with accession number (D3893). Very few strains such as *Candida boidinii* [10], *Paenibacillus* sp. and *Bacillus* sp. [11] are known to produce both PL and PGL in appreciable amounts. However, the activities obtained in the present study are much higher than those quoted by *C. boidinii* and *Bacillus* sp. [10,11]. Hence, this isolate can be exploited for large-scale production of pectic transeliminases.

The production of PL and PGL by this isolate was quantified by measuring the activity using the methods available in the

* Corresponding author. Tel.: +91 44 2257 4114; fax: +91 44 2257 0509.
E-mail address: gummadi@iitm.ac.in (S.N. Gummadi).

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literature [12,13]. In order to study further on production aspects by this isolate, it is important to measure the activity of PL and PGL at optimal conditions of reaction and physical parameters. It is known that activity measurement is strongly influenced by amount of substrate and enzyme (chemical parameters), pH of reaction mixture and temperature of incubation (physical parameters). The general practice of determining these optima is by varying one parameter while keeping the other at an unspecified constant level. The major disadvantage of this single variable optimization is that it does not include interactive effects among the variables; thus, it does not depict the net effects of various parameters on enzyme activity. It has also been reported in literature that physical and chemical parameters affecting the activity of enzyme were optimized separately [14–16]. In this study, we optimized the chemical and physical parameters influencing the activity of PL and PGL separately using response surface methodology.

2. Materials and methods

2.1. Chemicals

Apple pectin (Sigma) was used as the substrate for PL and polygalacturonic acid (Sigma) was substrate for PGL. The other chemicals were of analytical grade procured in India.

2.2. Microorganism and maintenance

The isolate was identified as *D. nepalensis* (99.8% sequence identity) based on 26S rDNA D1/D2 analysis and the strain is deposited in National Collection of Yeast Cultures, Norwich, UK, with accession number D3893. The isolate was maintained in YEPD agar plates and sub cultured for every 2 weeks.

2.3. Production of PL and PGL

A loopful of the strain from YEPD agar plates was transferred to 5 ml sterile YEPD medium and incubated on rotary shaker at 180 rpm and 30 °C. After 12 h, 2% (v/v) of the seed culture was transferred into a 100 ml Erlenmeyer flask containing 25 ml of lemon medium (LM) and incubated at 180 rpm and 30 °C. LM had the following composition (g/l): $MgS0_4{\cdot}7H_20$, 0.1; NH_4Cl, 2; Na_2HPO_4, 6; K_2HPO_4, 3; NaCl, 5; lemon peel, 5. The initial pH was adjusted to 7.0 before sterilization.

2.4. Enzyme assay

Supernatant from the culture broth was used as the source for enzyme assay. PL and PGL activity was assayed by measuring the formation of unsaturated oligogalacturonates at 235 nm [17]. PL assay mixture consists of 600 μl of substrate (3 g/l pectin) in 100 mM citrate phosphate buffer (pH 5.2) and 100 μl of enzyme (culture broth) [12]. But PGL assay mixture consists of 600 μl of substrate (2.4 g/l polygalacturonic acid) in 75 mM Tris–HCl buffer (pH 8.0) with 1 mM $CaCl_2$ and 100 μl of enzyme [13]. The assay mixture was incubated for 3 min at 30 °C and the increase in absorbance at 235 nm was measured using Perkin-Elmer UV–vis spectrophotometer. One unit of enzyme activity was defined as amount of enzyme, which produces an increase of 1 U of A_{235} of the reaction mixture per minute [10,18,19].

2.5. Experimental design

The reaction parameters were optimized using a statistical experimental design technique called the response surface methodology and central composite design (circumscribed) was used [20,21]. The parameters were divided into two groups, chemical (amount of substrate and enzyme) and physical (pH and temperature). Experiments were carried out in two separate sets to optimize these parameters.

According to this design, the total number of treatment combinations is $2^k + 2k + n_o$, where 'k' is the number of independent variables and n_o is the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = \frac{X_i - X_0}{\delta X} \tag{1}$$

where x_i is dimensionless coded value of the variable X_i, X_0 is the value of the X_i at the center point and δX is step change. A 2^k-factorial design with four axial points ($\alpha = 1.414$) and six replicates at the center point with a total number of 14 experiments was employed for chemical parameters. The number of center point replications can also be chosen to verify any change in the estimation procedure, which will also be a measure of precision described by the following equation:

$$n_o = \lambda_4(\sqrt{F} + 2)^2 - F - 2k \tag{2}$$

where F is the number of points in factorial portion, i.e., first four experiments in experimental design (run numbers 1–4 in Table 2) and λ_4 is the mixed fourth order moment [19]. The total number of center point replications obtained after substituting the values in Eq. (2) is five, but six replications were performed to reduce error.

The behavior of the system was explained by the following quadratic model:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_{ij} \tag{3}$$

where Y is the predicted response, β_0 the offset term, β_i the linear effect, β_{ii} the squared effect and β_{ij} is the interaction effect. This equation was optimized for maximum value to obtain the optimum conditions using the 'Solver Tool' of Microsoft Excel (XP version).

2.6. Optimization of reaction parameters

Enzyme activity was considered as the dependent output variable whereas amount of substrate and enzyme were considered as independent variables. The different levels of amount of substrate and enzyme considered for the design is given in Tables 1 and 2 for PL and PGL, respectively. 2^k-factorial design with four axial points and six replicates at the center point with a total number of 14 experiments was employed (Tables 1 and 2).