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Original Research Paper

Statistical optimization of cultural conditions of an halophilic alpha-amylase production by halophilic *Streptomyces sp*. grown on orange waste powder

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ABSTRACT

A newly isolated strain from the water of the salt marsh of Ain Mlila in Algeria identified as *Streptomyces* sp. 20r was investigated for its potential to produce amylolytic enzymes. The production optimization of α-amylase using an agroindustrial residue (orange waste) as the sole carbon source was performed with statistical methodology based on Plackett–Burman experimental designs. Among the various parameters screened, the effects of various carbon and nitrogen sources were investigated. The production of the α-amylase, proteins and biomass was achieved using the statistical method of Plackett–Burman design at $N=24$, namely 24 experiments and 23 factors in which 19 are real ones and 4 dummy. The production medium is prepared using orange waste powder at 30 °C under agitation. Statistical analysis showed that the changes in substrate concentration (5–15%), NaCl (0–6.5%), inoculum size (5–10%) and pH (5–9) were positively affecting the production and giving the highest level of α-amylase activity corresponded to 8.26 U/mL in submerged fermentation after 5 days of cultivation.

1. Introduction

α-amylases is one of the most important enzymes in present day biotechnology, this enzymes are gaining more importance because their spectrum of application has widened in many fields such as clinical, medicinal pharmaceutical and analytical chemistry. Besides their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile and paper industries (Agger et al., 2001). Amylases represent one of the three largest groups of industrial enzymes and account for approximately 25% of the total enzyme sales worldwide (Li and Yu, 2011). With the development of effective techniques large scale production of α-amylase becomes an attractive business (Zangirolami et al., 2002). Maximum enzyme production is one of the most important goals in biotechnological processes by optimizing the cultural conditions such as inoculum size, temperature, pH, agitation, aeration and dissolved oxygen etc.

α-amylases can be derived from several sources, including plants, animals and microorganisms. Microbial α-amylases have a broad spectrum of industrial applications as they are more stable than with plant and animal and they can be obtained cheaply (Grupta et al., 2003), also the major advantage is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). Several microbial α-amylases are available commercially and they have almost completely replaced the chemical hydrolysis in over 75% of starch hydrolyzing processes due to many advantages, not least its highest yields (Tonkova, 2006). It has been found in several microorganisms like bacteria (Chakraborty et al., 2012), fungi (Djekrif-Dakhmouche et al., 2006) and actinobacteria (Sivakumar et al., 2012). Actinobacteria, specifically *Streptomyces*, are one of the most investigated groups because they constitute a potential source of biotechnologically interesting substances. α-amylases are widely distributed in the species of *Streptomyces* (Singh et al., 2011). Starch hydrolysis, catalyzed by α-amylase, is one of the most important large scale uses of Actinomycetes in terms of enzymatic processes. The fact that some enzymes have the particular capacity to convert starch into shorter polymers of glucose units has made α-amylase the subject of several studies in recent years for this kind of process. Production of α-amylase using synthetic media is very expensive and uneconomical. Those media therefore have to be replaced with more economically available agro-industrial residues to reduce the cost (Balkan and Ertan, 2007). For these reasons, certain agricultural, industrial and environmental wastes were investigated for their ability to induce α-amylase production. Designing an appropriate fermentation

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medium is of crucial importance because the medium composition can significantly affect the product yield. For commodity products, the cost of the medium can substantially affect the overall economics of the process (Silva and Roberto, 2001).

In Algeria, orange-processing industry rejects hundreds of thousands of tons of waste per year, which cause serious environmental problems (Djekrif-Dakhmouche et al., 2006). The accumulation of large amounts of orange waste as well as environmental considerations and to avoid health hazards due to the unsatisfactory disposal methods require the necessity to find alternative solutions for the recovery of such wastes. Any waste could be considered as raw material as long as there is an option to develop methods for its valorization according to current environmental legislation, (Möller et al., 2001). High added value products can be produced using orange peel waste as a potentially valuable low cost resource (Rivas et al., 2008; Balu et al., 2012). According to Rivas et al. (2008) orange peel waste contains 16.9% soluble sugar, 9.21% cellulose, 10.5% hemi-cellulose and 42.5% pectin as the most important components. Among the different methods cited in the literature for the recovery of orange waste, their use in the production of enzyme is the most interesting (Siles and Thompson, 2010).

Submerged fermentation (SmF) has been traditionally used for the production of industrially important enzymes (Approximately 90%) because of the ease of handling and greater control of environmental factors such as temperature and pH. It is well established that extracellular enzyme production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physicochemical factors such as pH and inoculum density, thus the optimization of media components and cultural parameters is the primary task in a biological process. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production (Djekrif-Dakhmouche et al., 2006; Cotârlet, 2013). It is well known that medium optimization is approached by either empirical or statistical methods, but the classic or empirical methods have several limitations toward complete optimization (Djekrif-Dakhmouche et al., 2006). The recent strategy is statistical optimization, which allows rapid screening for a number of factors and factor interactions, reflecting the role of each component. Statistical methods have been applied for the optimization of α-amylase (Dey et al., 2001; Abou-Elela et al., 2009; Cotârlet, 2013). A statistical approach has been employed in the present study for which a Plackett–Burman design is used for identifying significant variables influencing α-amylase production under SmF by *Streptomyces sp*.

2. Materials and methods

2.1. Selection of amylase-producing actinomycetes

The Actinomycetes strains used in this study were isolated from samples of water, soil and tree barks collected at two sites located in the northeast of Algeria (Kitouni et al., 2005). Stock cultures were maintained on agar in inclined tubes and spore suspensions were prepared in liquid Bennett medium after cultivation (30 °C/5 days) in this same medium. Spores were maintained in 50% glycerol, 50% Bennett medium (v/v) at −20 °C. Fourteen strains were tested for the production of α-amylase. Cells were spotted in duplicates on malt yeast extract agar (Shirling and Gottlieb, 1966) containing (g/L): Beef extract 3.0, peptone 10.0, soluble starch 2.0, agar 15.0, pH 7.2. Amylase-producing strains were identified, after an incubation period of 3 days at 30 °C, by the addition of lugol solution 0.1% and observing a clearing zone surrounding the culture.

2.1.1. Halo assay

For selecting the most producing of α-amylase, cells were spotted on starch (0.2%)/NB plate and digestion of starch was detected with I_2/KI solution to form halo and the colonies with the largest halo-forming zone were chosen for further investigations (Tatsinkou et al., 2005). In the present study the strain 20r was chosen and the sequence of its 16S rRNA gene was determined and submitted to GenBank under accession number KP314280.

2.2. Substrate and culture media

Orange waste was procured from the UNAJUC a juice production firm of Ramdhan Djamel, Skikda. Algeria. The chemical composition of the waste according to the analyzes of the firm is as follows (%): total sugar 44.5 ± 1.65; crude protein 3.71 ± 0.05; crude fat 0.39 ± 0.001 and Ash 3.46 ± 0.27. The waste is air dried and then passed through a sieve of 0.250 mm diameter and stored in sealed boxes: 5% and 10% of the suspension of orange waste are prepared with distilled water. They are well mixed and centrifuged (6000 rpm). The supernatant is used as a basal medium after dilution with 0.1 M phosphate buffer at pH 5 and its enrichment with various substances. In order to determine the factors significantly influencing α-amylase production the Plackett and Burman design was used. The composition of the production medium varies according to the design matrix.

2.3. Inoculum preparation

Streptomyces sp. (20r), 5 days old, were harvested from plates by adding ten milliliters of distilled water. The spores were dislodged with a platinium loop under aseptic conditions. Then it was, appropriately diluted for the required density of spores and used as the master suspension (Solis-pereira et al., 1993).

2.4. Fermentation conditions

Streptomyces sp. (20r) strain was cultivated in 250-mL Erlenmeyer flasks containing 50 mL of a growth medium, as described above, supplemented with a carbon and nitrogen sources at different concentrations according to Table 1 generating 24 different runs. The contents were thoroughly mixed; the cotton plugged flasks were autoclaved at 121 °C (15 psi) for 20 min. After cooling the medium, they were inoculated with 10 and 5% of a spore suspension ($DO_{600}=0.68$), kept on a rotary shaker at permanent conditions for 5 days at 30 °C, 140 rpm (orbital shaker New Brunswick Scientific Co., New Jersey, USA). Their whole content filtered to separate cells from the supernatant. The contents of the flasks were centrifuged at 6500 rpm at 4 °C for 20 min, filtered through a Whatman filter N°2 and the crude supernatants used for enzymatic assays.

2.5. Experimental design and statistical analysis

The present study was aimed at screening the important medium components with respect to their main effects by Plackett–Burman design. The Plackett–Burman experimental design is a two-factorial design, which identifies the critical physico-chemical parameters required for elevated α-amylase production by screening n variables in $n+1$ experiments (Plackett and Burman, 1946).Twenty three variables chosen for the present study which nineteen real variables and four dummy.

The experimental design for the screening of the variables is given in (Table 2). The columns represent different variables and the rows represent different experiments. To estimate the experimental error four variables (X3, X12, X16 and X19) are designed as dummy variables