Optimization of Glucoamylase Production by *Colletotrichum* sp. KCP1 Using Statistical Methodology

Vimal S. Prajapati, Ujjval B. Trivedi, and Kamlesh C. Patel

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Abstract Glucoamylase is a key enzyme used in the food processing as well as in commercial production of glucose from starch. A natural fungal strain identified as *Colletotrichum* sp. KCP1 using 18S rDNA partial genome sequencing has been studied for optimization of glucoamylase production. Media components were screened and optimized through the statistical approach for the synthesis of glucoamylase in solid state fermentation using wheat bran as the substrate. The medium components influencing the enzyme production were identified using Plackett-Burman design. Among various variables screened along with wheat bran as major growth substrate, starch, whey, and casein acid hydrolysate were found to be most significant. The optimum concentrations of these significant parameters were determined employing the response surface central composite design, revealing starch concentration (1.5 g), whey (0.1 mL), and casein acid hydrolysate (0.1 g) per 5 g of wheat bran for highest enzyme production.

Keywords: glucoamylase, *Colletotrichum* sp. KCP1, Plackett-Burman design, response surface methodology, solid state fermentation

Introduction Traditionally, glucoamylases have been produced by submerged fermentation (SmF). In recent years, however, solid-state fermentation (SSF) processes have been increasingly applied for the production of this enzyme (1). SSF compared to SmF is more simple, requires lower capital, has superior productivity, reduced energy requirement, simpler fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater, has easier control of bacterial contamination and requires low cost for downstream processing (2,3). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is therefore most important (4). Amylase production and physicochemical parameter optimization using wheat bran has been studied by SmF and SSF (5). Tea waste and copra waste have been reported as substrates for glucoamylase production using *Aspergillus* sp. (6,7). Glucoamylase production by *Aspergillus* sp. using rice flakes manufacturing waste along with wheat bran and rice powder under SSF has been reported by Anto et al. (8). Glucoamylase is the major starch-degrading enzyme secreted by *Colletotrichum gloeosporioides* (9). Statistic experimental design has been employed for the production of intermediate temperature stable α amylase from *Aspergillus oryzae* (10).

Conventional approaches for increased microbial metabolite production usually employ manipulation of nutritional requirements, physical parameters, and genetic makeup of the producing strain (11). Development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium, and trace element sources. Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time while keeping the others at fixed level. However, statistical method offers several advantages over conventional method being rapid and reliable, short lists significant
nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time, glassware, chemicals, and manpower (12). Initial screening of the ingredients is done to understand the significance of their effect on the product formation and then a few better ingredients are selected for further optimization (13). Statistical approach has also been used even for efficient recovery of amylase (14). Fungi from the genus *Colletotrichum* are ascomycetes found in environment, especially in association with the plant, either as pathogens, symbionts, or endophytic. *C. gloeosporioides* was reported to produce amylolytic enzymes through fermentation in semi solid medium composed of residues of the processing to produce amylolytic enzymes through fermentation in surface methodology.

In the present study a statistical approach has been employed in which a Plackett-Burman design is used for identifying various nutrients as significant variables influencing glucoamylase production by *Colletotrichum* sp. KCP1 a natural and novel isolate. The levels of the significant variables are further optimized using response surface methodology.

### Materials and Methods

#### Strain isolation and identification

Fungal culture isolated from farm soil samples collected from Vallabh Vidyanagar, Anand, Gujarat, India on potato dextrose agar (PDA) (Himedia, Mumbai, India) was screened for amylase production on starch (Himedia) agar plate. Culture was maintained at 4°C on Bushnell Hass agar (BHA) (Himedia) slants containing 1% starch (Himedia). Bushnell Hass mineral salt solution has the following composition (g/L): MgSO$_4$ 0.2; CaCl$_2$ 0.02; KH$_2$PO$_4$ 1.00; K$_2$HPO$_4$ 1.00; NH$_4$NO$_3$ 1.00; FeCl$_3$ 0.05 (pH 5.5). Genomic DNA of the isolate was extracted and the 18S rDNA gene was amplified using universal primers (F: 5'-CTG GJT GAT CCT GCC AGT AG-3', R: 5'-CCG CGG CTG CTG GCA CCA GA-3'). Amplification was carried out in a thermal cycler (2720; Applied Biosystems, Foster City, CA, USA) with reaction profile: initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 45 s, extension at 72°C for 45 s, and finally extension at 72°C for 5 min. The purified PCR product was sequenced and the phylogenetic relationship of the isolate was determine by comparing the sequence data with the existing sequences available through the gene bank database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

#### Identifying the significant variables using Plackett-Burman design

The present study was aimed at screening of the important medium components with respect to their main effects by Plackett-Burman design. This experimental design is a 2 factorial design, was used to identify the critical parameters required for prominent glucoamylase production by screening n variables in n+1 experiments (17). The variables chosen for the present study were glucose, starch, whey, sucrose, yeast extract, soyabean meal, and casein acid hydrolysate concentration while having wheat bran as a common substrate (Table 1). The experimental design for the screening of the variables is presented in Table 2. The Plackett-Burman design assumes that there are no interactions between the different media constituents. All the variables were denoted as numerical factors and investigated at 2 widely spaced intervals designated as -1 (low level) and +1 (high level). The effects of individual parameters on glucoamylase production were calculated by the following equation:

$$E(X_i) = 2(M^+ - M^-)/N$$

where, $E(X_i)$ is the effect of parameter under study; $M^+$ and $M^-$ are responses (glucoamylase activities) of trials at which the parameter was at its higher and lower levels, respectively; $N$ is the total number of trials.

Experimental error was estimated by calculating the variance among the dummy variables as

$$V_{eff} = \frac{\sum (E_d)^2}{N}$$

where, $V_{eff}$ is the variance of the effect of level; $E_d$ is the effect of level for the dummy variables; and $N$ is the number of dummy variables used in the experiment. The standard error (SE, $E_{s}$) of concentration effect was the square root of variance of an effect, and the significance level ($p$-value) of each concentration effect was determined using the Student’s $t$-test:

$$t(X_i) = E(X_i)/E_{s}$$

where, $E(X_i)$ is the effect of variable $X_i$.

#### Response surface methodology (RSM)

The levels of the significant parameters and the interaction effects between various medium constituents which may influence the glucoamylase production significantly were analysed and optimized by response surface central composite design (CCD). RSM is useful for small number of variables (up to 5) but is impractical for large number of variables, due to high number of experimental runs required. The concentrations of the 3 major components starch, casein acid hydrolysate, and whey (identified by Plackett-Burman design) were optimized, keeping temperature, pH, and inoculum size constant.

According to the design, the total number of treatment