Optimization of fusaric acid production by *Fusarium oxysporum* f. sp. *lycopersici* using response surface methodology

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**Abstract**

Production of fusaric acid was maximized by employing response surface methodology with two level factorial design involving potato infusion (X1) and sucrose (X2) as variables, after optimizing the carbon and nitrogen source. The p-value for each factor was <0.05 suggesting that these factors have significant effect on the production of fusaric acid. The optimized medium consisted 100 g/l potato infusion, 10 g/l sucrose for the production of 12.8 mg/l fusaric acid. Besides, different process parameters like pH and inoculums size were also standardized. Optimum pH and inoculums size were found to be 6.5 and 6 mm agar plug respectively. This is the first report of using RSM (Response Surface Methodology) for optimizing a medium for FA production. This technique can be used in increasing FA production at industrial scale for commercial product without augmentation of costly additives.

**Keywords:** Fusaric acid; *Fusarium oxysporum* f.sp. *lycopersici*, response surface methodology.

**Introduction**

Media optimization is normally carried out by varying one parameter at a time while keeping the others constant. RSM is a technique for studying the effect of several factors acting together and affecting the responses by varying them in a number of experiments (Maddon et al., 1977). RSM had been successfully applied in the optimization of medium composition for the production of biomass by Cunninghamamella sp. 2A1 (Sulaiman et al., 2005), optimization of growth medium for the production of *CG Tase* from *Bacillus* sp. (Rahman et al., 2004; Ibrahim et al., 2005) and optimization of culture medium for production of glucosyltransferase by *Aspergillus niger* (Lee et al., 1997). Fusarium species produce a range of toxic compounds such as fusaric acid (FA), fumonisins, beauvericin, enniatin, moniliformin and trichotheccenes (Abbas et al., 1991; Bacon et al., 1996; Capasso et al., 1996; Zonno et al., 1996; Amalfitano et al., 2002; Idris et al., 2003). Fusaric acid is a well-known phytotoxin that is produced by several Fusarium species, particularly pathogenic strains of *F. oxysporum* causing wilt diseases of a great variety of plants (Gaumann, 1957; Kern, 1972). Although fusaric acid is not generally regarded as a mycotoxin, some attention will be given here to fusaric acid production by *F. oxysporum* because fusaric acid as well as certain other phytotoxins such as lycomarasmin and lycomarasmic acid produced by *F. oxysporum* (Kern, 1972) are chelating agents and may be involved in certain diseases of abnormal bone development in animals. In addition, fusaric acid is toxic to mice (intra peritoneal LD50 80 mg/kg) and death caused by the lethal dose has been attributed to its hypotensive effect (Hidaka et al., 1969). The ability of fusaric acid to cause significant decreases of blood pressure has also been observed in cats, dogs, rabbits and rats and has been attributed to the inhibition of dopamine-3-hydroxylase (Hidaka et al., 1969; 1971).

Fusaric acid has been administered to humans in clinical trials as an antihypertensive agent (Ibrahim et al., 2005) in the treatment of Parkinson's disease (Hidaka, 1971; Matta et al., 1973) and at dosage rates up to 1200 mg/day in the treatment of drug addiction (Pozuelo et al., 1976). Bacon et al. (1996) surveyed 78 different stains of Fusarium fungi and reported that all the cultures tested produced fusaric acid. The author suggested that, since the production of fusaric acid is so widespread, this compound should be used as a marker toxin for Fusarium contamination. This study is the pioneer work which reports the application of RSM to optimize fusaric acid production using a local fungal isolate, *Fusarium oxysporum* f. sp. *lycopersici* with assessment of the process and nutrient parameters for its commercialization.

**Material and methods**

**Culture conditions and storage**

Standard culture of *Fusarium oxysporum* f.sp. *Lycopersici* (FOL) obtained from Indian Type Culture Collection, I.A.R.I; New Delhi (F-1322) was maintained on potato sucrose agar (Hi-Media Mumbai, India) and incubated at 26°C for 96 hrs. The culture was stored at 4°C and sub-cultured every month.

**Fungal culture filtrate**

To obtain fungal culture filtrate, flasks containing 100 ml of potato-sucrose broth were inoculated with 6 mm diameter mycelia disc of inoculum and incubated at 26°C. Filtrate was obtained by filtration through 4 layers of cheese cloth, twice through Whatman No. 1 paper and centrifugation at 3000 g for 30 min to sediment spores and mycelia and stored at 5°C in sterile bottles.

**Extraction of fusaric acid (FA)**

FA from culture filtrates was extracted following the method of Barna et al. (1983). Briefly pH of the filtrate was adjusted to 3.9-4.0 with 2N HCl and FA was extracted thrice with ethyl acetate. Organic extracts were...
pooled and evaporated at room temperature. Samples were dissolved in
1000 µl of 80% methanol and stored at 20°C until further use.

**Qualitative and quantitative analysis of Fusaric acid**

Samples were applied on TLC plates (TLC silica gel 60 F254, Merck) together with FA standard (Sigma). The plates were developed in n-butanol, acetic acid, ethyl acetate, water (3:2:2:2, v/v) solvent, dried at 80°C and FA was detected under UV light (λ=254).

The UV spectrophotometric assay for FA was performed according to Stefan (2005) using UV grade solvent, dried at 80°C and FA was quantified by UV spectrophotometric method.

**Experimental design (CCD)**

Experimental design was determined using design expert software version 7.0 (State-Ease Inc., Minneapolis, USA). A full factorial central composite design (CCD) was used for two independent factors with six replicates of the central points and six axial points, leading to a total of 13 sets of experiments. Low and high factor settings were coded as -1 and +1 respectively, the centre points were coded as 0 and the design is extended up to +α respectively, the centre points were coded as -1 and +α (Table 1).

The value of alpha represents the distance from the centre of the design space to an axial. The media composition for these experimental designs was Potato infusion (Variable): Sucrose (Variable). Final pH of the medium was adjusted to 6.5 after sterilization. Fusaric acid production was evaluated at 10th day of incubation through TLC method. The average fusaric acid concentration was taken as the dependent variable or response(Y). Regression analysis was performed on the data obtained and used to fit the following second-order polynomial equation:

\[ Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 A^2 + \beta_4 B^2 + \beta_5 AB + \beta_6 AC + \beta_7 BC + \beta_8 AB^2 + \beta_9 A^2B + \beta_{10} A^2C + \beta_{11} A^3 + \beta_{12} B^3 + \beta_{13} C^3 + \beta_{14} A^2BC + \beta_{15} AB^2C + \beta_{16} A^2BC + \beta_{17} AC^2B + \beta_{18} A^2C^2 \]

**Selection of media**

To determine the production of FA in relation to the mycelial growth of *F. oxysporum lycopersici*, 7 liquid media were screened for FA production (i) Potato dextrose (PDB, Hi-media, Mumbai) (ii) Sabouraud broth (Hi-media, Mumbai) (iii) Czapek Dox Broth (CDB, Hi-media, Mumbai) (iv) Tomato juice (v) Corn meal (CDB, Hi-media, Mumbai) (vi) Potato sucrōse. 2 agar discs of a 3 mm *F. oxysporum* culture were transferred to each of the 250 ml conical flasks containing 200 ml medium and the mycelial dry weight was recorded. The culture filtrates were directly extracted as mentioned above and FA contents were quantified by spectrophotometric method.

**Effect of pH**

Fungal culture was grown on potato sucrose agar plates for 96 hrs (log phase). Different diameters of agar plugs (1 mm diameter) of fungal culture and incubated at 26°C. Culture filtrate was harvested at 4, 6, 8, 9, 10, 11 and 13 days by filtration through Whatman No.1 filter paper and used for FA quantification as previously outlined.

**Effect of Inoculum size**

Fungal culture was grown on potato sucrose agar plates for 96 hrs (log phase). Different diameters of agar plugs were inoculated with 6 agar plugs (1 mm diameter) of fungal culture and incubated at 26°C. Culture filtrate was harvested at 4, 6, 8, 9, 10, 11 and 13 days by filtration through Whatman No.1 filter paper and used for FA quantification as previously outlined.

**Time course for fusaric acid production**

100 ml of potato sucrose medium in 250 ml flasks were inoculated with 6 agar plugs (1 mm diameter) of fungal culture and incubated at 26°C. Culture filtrate was harvested at 4, 6, 8, 9, 10, 11 and 13 days by filtration through Whatman No.1 filter paper and used for FA quantification as previously outlined.

**Effect of pH**

The complex medium used in the experiment contained potato infusion (200 g) and sucrose (20 g) in 1000 ml of deionized water. 100 ml of liquid medium was poured into each of the 250 ml flasks and the medium adjusted to different pH of 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using 1 M NaOH or 1 M HCl. Each pH medium was dispensed into 250 ml conical flasks and all flasks were autoclaved at 121°C for 15 min. The flasks were then inoculated with 6 of incubation through TLC method. The average fusaric acid concentration was taken as the dependent variable or response(Y). Regression analysis was performed on the data obtained and used to fit the following second order polynomial equation:

\[ Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 A^2 + \beta_5 B^2 + \beta_6 C^2 + \beta_7 AB + \beta_8 AC + \beta_9 BC + \beta_{10} AB^2 + \beta_{11} A^2B + \beta_{12} A^2C + \beta_{13} BC + \beta_{14} AB^2C + \beta_{15} A^2BC + \beta_{16} A^2BC + \beta_{17} AC^2B + \beta_{18} A^2C^2 \]
Where \( Y = \text{predicted response}, \beta_0 = \text{regression coefficient}, \beta_1, \beta_2, \beta_3 = \text{linear effect}, \beta_{11}, \beta_{22}, \beta_{33} = \text{squared effect}, \beta_{12}, \beta_{23}, \beta_{13} = \text{interaction effect}. \) The contour plots were then generated by design expert software with the above model to obtain the optimum concentration of the medium components. The optimal concentrations of factors were obtained by a numerical optimization procedure and analyzing the response surface plots (Myers et al., 1995).

**Result**

**Effect of incubation time**

Culture filtrate harvested at different intervals of time showed varying concentrations of fusaric acid. Extraction of fusaric acid from 100 ml of culture filtrate showed increase in fusaric acid gradually from 3rd day (2.5 ml), reached the maximum at 10th day (6.5 ml) and declined later (Table 2). These findings were confirmed by both qualitative and quantitative methods using thin-layer chromatography (TLC) and Spectrophotometric analysis. In TLC analysis, a dark intense band with \( R_f \) value 0.59 was obtained in standard as well as samples at 254 nm which was analyzed spectrophotometrically after elution revealed that maximum yield of fusaric acid was on 10th day of incubation. Fig. 1 shows a bell shaped curve indicating a correlation between the time of incubation and fusaric acid production. As the incubation time increased, the fusaric acid level in the medium also increased gradually and it reached the maximum on 10th day with 11 mg/l concentration and finally attained the stationary phase after 19th day of incubation. Very little variation was observed between potato sucrose and tomato juice broth. Therefore, subsequent studies were performed with potato sucrose broth to get the maximum fusaric acid production.

**Growth rate and selection of media**

Potato dextrose, Czapek-Dox and Sabouraud media are the basic media of growth for fungi. In addition to this Corn meal, Potato sucrose and Tomato juice were also looked for the optimum biomass production of the fungus. Significant difference was observed between Potato sucrose and other medium compositions. Initiating from the 3rd day (Fig. 2), the biomass production doubled after 8 days of incubation, reached the maximum at 19th day and finally attained the stationary phase after 19th day of incubation. Very little variation was observed between potato sucrose and tomato juice medium. However, the potato sucrose broth was found to be more suitable due to its simple and cost effective composition than tomato juice broth. Therefore, subsequent studies were performed with potato sucrose broth to get the maximum fusaric acid production.

**Effect of pH**

The effects of pH variation on fusaric acid level are shown in Fig. 3. Maximum fusaric acid yield was observed around acidic pH with the highest value of 11.0 mg/l at pH 3. On increasing the pH that is towards alkalinity, the fusaric acid level found to be depressed with least production of 8.9 mg/l at pH 8. The results suggest that the optimum pH of the medium should be 3 to have a maximum fusaric acid production.

**Effect of Inoculum size**

Alteration in inoculums size from 1 mm agar plug to 12 mm agar plug showed a significant variation in fusaric acid production. Fig. 4 shows the relationship between the fusaric acid production and inoculums size. The relation was found to be positive from 1 mm to 4 mm where as on increasing further to 12 mm it responded negatively. The fusaric acid obtained at 1 mm and 6 mm agar plug was similar (10 mg/l). However, maximum level was obtained using 4 mm of inoculums size (10.9 mg/l). The results imply that the fungus FOL required 4 mm agar plug for maximum fusaric acid production.

**Model selection**

The sequential model sum of squares for the fusaric acid showed that the linear coefficient was significant (p-value <0.0001) and the model is not aliased. P-value for two-factor interaction (2FI), quadratic and cubic terms for
fusaric acid production were >0.05, meaning that the interactions among factors were not significant. This indicates that the linear model was accurate in describing or predicting the pattern of significant factors for the production of fusaric acid from Fusarium oxysporum f. sp. Lycopersici.

Model fitting

ANOVA was used to evaluate the adequacy of the model. The Fisher F-test with a very low probability value (<0.0001) for response (fusaric acid production) demonstrated a high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient ($R^2$) (Haalland, 1989). The $R^2$ value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of 1 represents the ideal case at which 100% of the variation in the observed value can be explained by the model (Khuri et al., 1987). In this case, the value of $R^2$ for fusaric acid was 0.9160 indicating that only 1-7% of the total variations were not explained by the model. The value of the adjusted $R^2$ is also high, which indicates a high significance of the model. A higher value of the correlation coefficient signifies an excellent correlation between the independent factors. An insignificant lack of fit indicated that the model fits the data. The lack of fit tests compares the residual error to the pure error from replicated design points. The lack of fit F-value of 0.21 for fusaric acid implies that it is not significant relative to the pure error. On the other hand, a non-significant lack of fit represents a good model which fits the data.

Table 3 summarizes the response for each individual experiment along with the predicted response. Regression analysis was then performed on the data obtained. The regression equation obtained after the analysis of variance (ANOVA) gives the growth occurred as a function of the initial concentrations of potato infusion and sucrose. The coefficient of determination ($R^2$) was calculated to be 0.9825, which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 99% of the variability in the response could be explained by the model.

$$Y = +9.72 - 0.33A - 0.66B + 0.78AB + 0.96A^2 + 0.36B^2$$

Where $Y$ = fusaric acid, $A$ = potato infusion, $B$ = sucrose

The 3-D response surface curves were then plotted to understand the interaction of the medium components and the optimum concentration of each component required for optimum growth. The response surface curves (Fig. 5) shows the relative effects of both variables. The fusaric acid level was found to be negatively linked with the potato infusion and sucrose component with optimum production 13.0 mg/l at concentration of 100 g/l of potato infusion and 10 g/l of sucrose respectively.

Numerical optimization of factors

Based on Table 3 (run no. 11), the highest concentration of fusaric acid (12.78 mg/l) from Fusarium oxysporum f. sp. Lycopersici was obtained when the concentration of potato infusion and sucrose were 100 and 10 g/l respectively. To obtain the maximum concentration of fusaric acid, the factor levels and response were set at the desired goal using Design expert’s numerical optimization under desirability equal to 1. Optimal concentration of potato infusion and sucrose

Fig. 5. Response surface plot (3-D) of fusaric acid production as a function of inoculum size and concentration of potato infusion and sucrose.
was established at 100 g/l and 10 g/l for fusaric acid respectively. This solution gives the predicted response of fusaric acid 12.78 mg/l. From 3 replications of experiment, fusaric acid 13 mg/l was achieved.

Discussion

Fusaric acid, a major toxin secreted by all Fusarium spp. and it is the key factor with many applications like its use as chelating agent, antihypertensive agent etc. As per our knowledge, no report is available on optimization of process parameters and media composition for maximum production of Fusaric acid using statistical methods. This study is the 1st step towards optimization of a medium for maximum fusaric acid production by applying CCD method to increase the fusaric acid level with augmentation of simple additives and minimal cost. The classical, ‘one-at-a-time’ factorial design experiments, where a single factor is varied while others are kept constant are often expensive and time consuming and do not taken into account has possible interaction of various independent factors that would skew the results. For these reasons, statistical methods have been developed to reduce the cost and duration of experiments that also allow for the observation of any interacting factors in the final process response. One such highly successful method, as 1st described by Box and Wilson (1951) is the ‘response surface methodology’ (RSM), which mathematically identifies the optimal response by using a combination of experimental factors. RSM is a very useful and handy technique since it encompasses fewer experimental trials to identify and quantify the multiple variables with their interaction (Liu et al., 2007). The results indicated that the application of these statistical methods not only helped us to find the optimum levels of the most important factors considered with minimum amount of manpower and time, but also proved to be useful and satisfactory in optimizing medium for the fusaric acid production. Duarte et al. (2003) reported that defined culture media such as Czapek-Dox and Sabouraud did not induce greater production of toxic metabolites when compared with Potato Sucrose and production of Fusarium spp. Toxic metabolites on PS-broth reached a peak after 25 days of static incubation at 25°C under illumination. In the present investigation, maximum fusaric acid production was achieved using inoculums of sizes 4 mm respectively. An interesting observation in the present study is the loss of pigmentation while growing the fungus in Czaphox, Potato sucrose, Corn meal and Tomato juice media. However, Potato dextrose and Sabouraud media showed pigmentation. In a similar study, Claydon (1977) showed a complete inhibition of pigment formation when cultures of Fusarium solani were grown on Czapek-Dox. Optimum growth conditions for Fusarium oxysporum f. sp. lycopersici have been identified in this study. The temperature and pH requirements were found to be similar to other Fusarium fungi. A simple micronutrient medium (Potato infusion & sucrose) sustained maximum fusaric acid production. Employing these results provide optimized conditions for utilizing Fusarium oxysporum f. sp. lycopersici in in-vitro and in-vivo studies for the selection of resistant cultivars and maximum production of fusaric acid commercial application.

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References


