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Comparison of the production of fusaric acid produced by *Fusarium oxysporum* f. sp. *lycopersici* in different cultures with HPLC method

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Abstract

Fusarium oxysporum f. sp. *lycopersici* is an important pathogen of tomato and produces a variety of mycotoxins, pigments, and phytohormones. Fusaric acid is one of the oldest known secondary metabolites produced by *F. oxysporum* and some other *Fusarium* species. In this research we performed qualitative and quantitative analysis of fusaric acid production in three medium with HPLC method. PSB (Potato sucrose broth) medium, CYB (Czapek yeast extract broth) and Potato sucrose and tomato juice medium were selected. PSB medium showed the highest concentration of fusaric acid in equal conditions. Also other metabolites except of fusaric acid less produced in this medium.

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Introduction

Fusarium species produce a range of toxic compounds such as fusaric acid (FA), fumonisins, beauvericin, enniatin, moniliformin and trichothecenes (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, rabbitsand rats and has been attributed to the inhibition of dopamine-3-hydroxylase (Hidaka *et al.*, 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 strains 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. In this research we try to perform qualitative and quantitative analysis of fusaric acid production in three medium and select the best of them.

Material and methods

Culture conditions and storage

Standard culture of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) obtained from Science and Research Branch, Islamic Azad University Type Culture Collection, Tehran, Iran (F272) was maintained on potato sucrose agar and incubated at 26 °C for 96 hrs. The culture was stored at 4°C and sub-cultured every month.

Culture Medium

Three mediums were tested for FA production. PSB medium consisted of 100 g potato infusion and 10 g sucrose and 1000 ml Distilled water (A). Final pH was 6.5 (Pritesh *et al.*, 2010). CYB (Czapek yeast extract broth) medium consisted of 3 g $NaNO_3$,1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 5 g Yeast extract, 30 g Sucrose, 1000 ml Distilled water (B). Final pH was 6.0-6.5 (Samson and Pitt, 1985). Potato sucrose and tomato juice medium consisted of 58 g potato infusion, 5.8 g sucrose, 50 ml tomato juice and 950 ml Distilled water (C). Final pH was 6.0-6.5. Flasks containing 100 ml of three different culture mediums were inoculated with 6 mm diameter mycelia disc of inoculums and incubated at 22°C for 21 days in shaker incubator (50 rpm).

Fungal culture filtrate

To obtain fungal culture filtrate, filtrate was obtained by filtration 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.

Fusaric acid extraction

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. The organic phase was removed and dried under vacuum, the residues dissolved with 5 ml methanol and stored at -20 °C until further use.

Qualitative and quantitative analysis of fusaric acid

TLC

Samples were analyzed by a modified TLC as described by Bacon *et al.* (1996). The residue was applied on thick silica gel 60F-254 with fluorescent indicator. The plates were developed in n-butanol: acetic acid: ethyl acetate: water (3:2:2:2, v/v). Plates were subsequently dried and FA was detected under UV light (λ=254).

The UV spectrophotometric assay for FA was performed according to Stefan (2005) using UV grade methanol with λ max 260 nm.

Two-dimensional TLC

Standard FA and some samples were analyzed by Two-dimensional TLC method. The residue was applied on HPTLC plate without fluorescent indicator.The plates were horizontally developed in n-butanol: acetic acid: ethyl acetate: water (3:2:2:2, v/v). Then, plates were developed in methanol vertically (Bacon *et al.*, 1996).

HPLC

The residue was dissolved in 5ml of methanol and analyzed by HPLC equipped with a reverse phase column packed with nucleosil 120 – 5 C18 and set at 50°C. The samples were eluted with linear methanol, K_2HPO_4 1% and water (75:15:10). The fraction containing the FA standard from the HPLC was collected and the presence of FA was qualitatively and quantitatively confirmed (Regina *et al.*, 2002).

Results

TLC

In TLC analysis, a dark intense band with Rf value 0.78 was obtained in standard as well as all samples at 254 nm. In samples that cultured in A medium created 2 dark intense bands, in B medium created 3 dark intense band and in C medium created 3 dark intense bands.

Two-dimensional TLC

Standard FA and some samples were analyzed by Two-dimensional TLC method. Standard FA created one intense band on plate and samples created 3-4 intense bands horizontally and 3-4 intense band vertically in that one of them was in the same of standard FA in RF value.

Table 1. Concentration of FA (mg/l) in different media.

Medium	Concentration of FA (mg/l)
PSB	1624.24
CYB	1303.72
PSB+T	600.02

HPLC

100, 500 and 1000 mg/l of standard FA dissolved in methanol analyzed by HPLC equipped with a reverse phase column packed with nucleosil 120–5 C18 and set at 50°C. The samples were eluted with linear methanol, K_2HPO_4 1% and water (75:15:10). Then all extracted compounds which dissolved in methanol were injected into the HPLC system. R*etention time* (RT) of standard FA was used for presence of fusaric acid and concentration of the substance measured by the area under the peak (Figures 1-4; table 1).

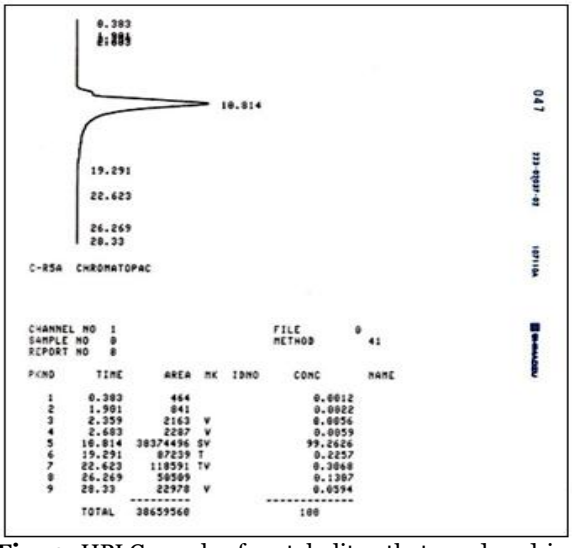

Fig. 1. HPLC graph of metabolites that produced in PSB medium.

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.

What is important in this study to determine the maximum amount of fusaric acid produced in three different media, PSB medium showed the highest concentration of fusaric acid in equal condition. Also other metabolites except of fusaric acid less produced in this medium. If we require purification of this metabolite is also recommended in this medium.

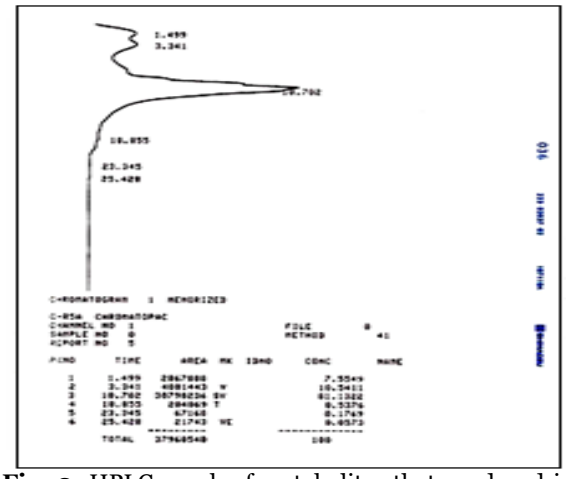

Fig. 2. HPLC graph of metabolites that produced in CYB medium.

The study results confirmed divided Petrish *et al.* (2010) with the difference that the HPLC method is far more accurate assessment of the methods used in their paper. 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.

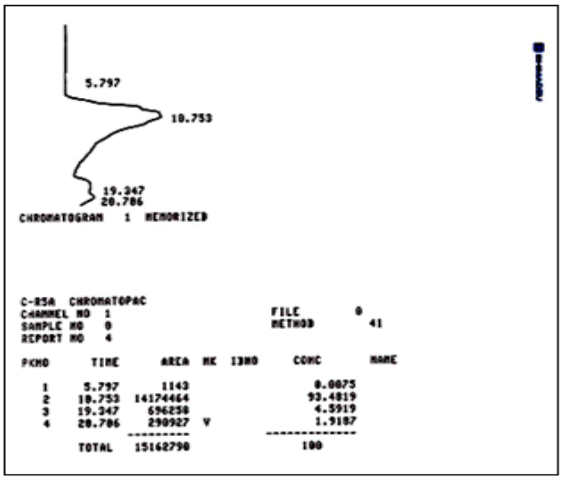

Fig.3. HPLC graph of metabolites that produced in PSB+ tomato juice medium.

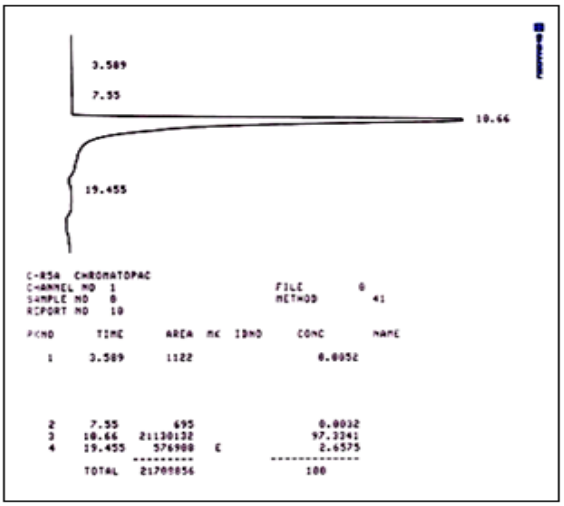

Fig. 4. standard FA solved in ethanol (1000 ppm).

Since the host of this form of pathogen is tomato, expected to add to the tomato extract more favorable conditions for the production fusaric acid. The results did not confirm this. An interesting observation in the present study is the loss of pigmentation while growing the fungus in medium with tomato juice However, Potato dextrose and Potato sucrose and CYB 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. 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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