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Production and Determination of Nigerloxin by *Aspergillus niger*

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ABSTRACT

An extensive study was carried out to develop a simple and accurate method for extraction and determination of nigerloxin. Nigerloxin has four UV- absorption maxima at 230 (Є = 15,588), 279 (Є = 21,200), 318 (Є = 5,096) and 400 nm (Є = 3,312) wavelengths in methanol, but can be accurately quantified by spectrophotometer at 279 nm as recommended wavelength. The study describes the enhancement of nigerloxin production with *Aspergillus niger;* a fungus that has the potential to produce nigerloxin in different ratios of wheat bran/sugarcane-bagasse. Nigerloxin production was increased by raising the bran level and reached its maximum in bran:bagasse ratios of 4:1 (w/w). Supplementation of the medium with nitrogen showed that nigerloxin production increased especially with glycine (0.5%). The use of phosphorus at 0.25% stimulated nigerloxin production, however further rise in phosphate led to decrease of nigerloxin yield. Incorporation of phosphorus and nitrogen (glycine) together stimulated nigerloxin production. The production was also increased with addition of 0.5% succinic acid to the medium. Nigerloxin was produced after 3 days by *A. niger* in bran/bagasse medium and the optimum production was recorded after 7 days at 30°C. Supplementing the bran medium with 20% potatoes and pineapple peels enhanced nigerloxin production more than bagasse addition.

Key Words: Nigerloxin; *A. niger;* Wheat-bran:sugarcane-bagasse; Nitrogen:phosphorus ratios; Potatoes; Pineapple peels

INTRODUCTION

The filamentous fungus *Aspergillus niger* is able to accumulate and excrete high concentrations of several organic acids such as citric acid (Nguyen *et al.*, 1992; Kirimura *et al.*, 2001; Haq *et al.,* 2001, 2003), gluconic acid (Sakurai *et al*., 1989; Sankpal *et al*., 1999; Sankpal & Kulkarni, 2002; Ikeda *et al.*, 2006), gibberellic acids (Hasan, 1994) and different extracellular enzymes. It has been recently discovered that *A. niger* has ability to produce a new molecule (nigerloxin) in wheat bran medium (Rao *et al*., 2002a, b).

Nigerloxin (2-amido-3-hydroxy-6-methoxy-5-methyl-4- (prop-1'-enyl) benzoic acid), a new fungal metabolite, is an inhibitor against two different enzymes lipoxygenase and rat lens aldose reductase with free radical-scavenging properties (Rao *et al*., 2002b). Lipoxygenases are involved in the pathogenesis of some diseases, such as allergy, atherosclerosis and cancer (Samuelsson *et al*., 1987).

The present study was carried out for define simple and accurate method for analysis and determination of nigerloxin. Also, to study the production of nigerloxin by *A. niger* in medium containing different ratios of wheat-bran/sugarcane-bagasse, nitrogen/phosphorus ratios and organic acids. Inexpensive raw materials such as potatoes and pineapple peels were evaluated for nigerloxin production as well.

MATERIALS AND METHODS

Nigerloxin production in bran by *Aspergillus niger* van tieghem. *Aspergillus niger* van tieghem was isolated from soil and used in this investigation. Four grams of wheat bran (particles in a size range of 0.6 - 2 mm using Canadian standard sieve) in 100 mL Erlenmeyer flask were moistened with 6 mL distilled water and autoclaved. Then the medium was inoculated with a spore suspension of the organism and incubated at 30°C for 5 days. After 24 h of incubation the cultures were disturbed, for homogenous distribution of the fungus, after which the flasks were left to stand for subsequent 4 days. Different samples were used for nigerloxin assay as described below.

Extraction of nigerloxin. Ethyl acetate (30 mL) was added to dry cultures in each flask and agitated on a rotary shaker at 150 rpm and 30°C for 1.5 h. The mixture was then filtered using cheesecloth and the cultures were re-extracted with 20 mL ethyl acetate. The ethyl acetate layer was filtered with filter paper on anhydrous sodium sulphate. The extract was concentrated to dryness.

Assay of nigerloxin. Nigerloxin was separated by Thin-Layer Chromatography (TLC) by using toluene-acetone-methanol (5:3:2, v/v/v) as the developing solvent. TLC analysis was performed on Silica Gel 60-coated plates, with a fluorescence indicator (UV 6 LC-12 W-Vilber Lourmat). Nigerloxin yellow orange spots were visualized under long wavelength of UV light at

R_f 0.75 value. Nigerloxin band was marked under UV irradiation, scraped off and quantitatively eluted with 7 mL methanol by vortexing. After centrifugation at 3000 rpm for 3 min the supernatant was evaporated to dryness. The extracted nigerloxin was purified several times by TLC, weighted and analyzed with an infrared spectrophotometer (model 470 Shimadzu Corporation) and with NMR spectrometer (EM-360, Varian instrument division). The UV spectra of methanol solution of nigerloxin were obtained with UV-VIS scanning spectrophotometer (model UV 2101 PC Shimadzu) and the absorbance at each λ_{max} was noted. Extinction coefficient (Є) of each wavelength was calculated from the calibration curve at different concentrations from 1 to 30 µg mL^{-1}. The average value of absorbance and concentration was used for calculation the Є value with the following formula:

$$Є = \frac{A.\ Mwt}{Conc}$$

The nigerloxin concentration in samples was estimated by spectrophotometer (Unicam, Helios gamma) at 279 nm as recommended wavelength and calculated from the following formula:

$$\text{Nigerloxin conc (µg/mL)} = \frac{A.\ Mwt.\ F}{Є}$$

A = Absorbance, Mwt = Molecular weight, F = Dilution factor.

Nigerloxin production with varying wheat-bran:sugarcane-bagasse ratios. Four grams of different ratios of wheat-bran/sugarcane-bagasse (particles in a size range of 0.6 - 2 mm) in 100 mL Erlenmeyer flask were processed, extracted and assayed for nigerloxin as described previously.

Effect of amino acids. Amino acids (alanine, asparagine, aspartic acid, glutamine, glycine, lucine & tyrosine) were used separately at 0.5% level (w/w). Four grams of wheat bran/bagasse (4: 1 w/w) in 100 mL Erlenmeyer flask were supplemented with 0.5% amino acids solution in 6 mL distilled water, autoclaved, inoculated and processed as described previously.

Effect of P:N ratios. Potassium dihydrogen phosphate and glycine were used at 0.25 and 0.5% level (w/w). Four grams of the wheat bran/bagasse were treated with P:N solution in 6 mL distilled water, autoclaved, inoculated and processed as described previously.

Effect of organic acids. Different organic acids (ascorbic acid, benzoic acid, citric acid, malic acid, oxalic acid, pyruvic acid, salicylic acid, succinic acid) were used separately at 0.5% concentration (w/w). Four grams of the wheat bran/bagasse medium containing phosphate/glycine at 0.25: 0.5% ratio were treated with the organic acids solution in 6 mL distilled water, autoclaved, inoculated and processed as given above.

Effect of incubation periods and temperatures. Medium of 4 g wheat bran:bagasse (4: 1 w/w) were moistened, autoclaved, inoculated and incubated at 25 and 30°C for 3, 5, 7 and 10 days. The medium was processed as described previously.

Nigerloxin production in natural waste products. Four grams of different ratios of pineapple and potatoe peels in addition to bran and bagasse were used for nigerloxin production by *A. niger* after 7 days at 30°C. The medium was processed as described previously.

Statistical analysis. Data in ttriplicate was subjected to statistical analysis using L.S.D. test (Gomez & Gomez, 1984).

RESULTS AND DISCUSSION

Nigerloxin determination. Nigerloxin has four UV-absorption maxima at 230, 279, 318 and 400 nm wavelengths in methanol (Fig. 1), which can be used to quantify nigerloxin by constructing standard curve. The extinction coefficients (Є) used for determination of nigerloxin concentrations were 15,588 at 230 nm, 21,200 at 279 nm, 5,096 at 318 nm and 3,312 at 400 nm (Fig. 1). But nigerloxin can be quantified more accurately by constructing standard curve at 279 nm as recommended wavelength (Fig. 2).

Nigerloxin production in different ratios of bran/bagasse. Nigerloxin production in fermentation medium containing different wheat bran:sugarcane-bagasse ratios by *A. niger* was monitored (Table I). *A. niger* had potential to produce nigerloxin and the production was increased by raising bran level. Nigerloxin reached its maximum (2.13 mg g^{-1} dry substrate) in 4:1 bran:bagasse ratios (w/w). Sugarcane-bagasse alone was a poor substrate for nigerloxin production. The bagasse contains cellulose, hemicellulose and lignin (Lee, 2005) and it may not provide all the nutrients needed by the organism for nigerloxin production. However, bran contains starch, protein and some minerals (Rao *et al*., 2005).

Effect of amino acids. Supplementation of bran/bagasse (4:1 w/w) with 0.5% amino acids (alanine, asparagines, aspartic acid, glutamine, glycine, lucine & tyrosine) was studied (Table II). The results showed that most amino acids increased nigerloxin production by *A. niger* and optimum production was noted when glycine was used as a supplement.

Effect of P:N ratio. The response of *A. niger* for nigerloxin production in the presence of different ratios of phosphorus (KH_2PO_4) and nitrogen (glycine) was studied (Table III). Phosphorus at 0.25% stimulated nigerloxin production; however, a greater rise in phosphorus level led to decreased nigerloxin yield. Nitrogen in the form of glycine stimulated the production at 0.25 and 0.5%. Incorporation of both phosphorus and glycine together stimulated nigerloxin (Table III). Rao *et al.* (2005) noticed that *di*-sodium hydrogen phosphate (Na_2HPO_4) at 1% concentration did

Table I. Production of nigerloxin in bran/bagasse by *A. niger* after 5 days of incubation at 30°C

Bran : bagasse ratio	Nigerloxin (mg/g dry substrate)	Activation[a] %
5 : 0	1.87	-
4 : 1	2.13	14
3 : 2	1.53	0
1: 1	1.41	0
2 : 3	0.90	0
1: 4	0.63	0
0 : 5	0.13	0

[a]Activation in nigerloxin production compared with bran only

Table II. Effect of amino acids on production of nigerloxin in bran/bagasse (4: 1 w/w) by *A. niger* after 5 days of incubation at 30°C

Amino acids (0.5%)	Nigerloxin (mg/g dry substrate)	Activation[a] %
Control	2.13	-
Alanine	2.31	8
Asparagine	2.13	0
Aspartic acid	2.17	2
Glutamine	1.94	0
Glycine	2.51*	18
Lucine	2.22	4
Tyrosine	2.15	1

* Mean significant increase compared to the control.
[a]Activation in nigerloxin production compared with control

Table III. Effect of P/N ratio on production of nigerloxin in bran/bagasse by *A. niger* after 5 days of incubation at 30°C

Phosphorus: glycine ratio [a]	Nigerloxin (mg/g dry substrate)	Activation[b] %
0 : 0	2.13	-
1 : 0	2.41*	13
2 : 0	2.00	0
0 : 1	2.43*	14
0 : 2	2.48*	16
1 : 1	2.41*	13
1 : 2	2.51*	18

* Mean significant increase compared to the control (without P/N).
[a]1= 0.25%, 2= 0.5%. [b]Activation in nigerloxin production compared with control.

not affect nigerloxin yield.

Effect of organic acids. The result showed that succinic acid and citric acid at 0.5% concentration stimulated nigerloxin production in medium of bran:bagasse (4: 1 w/w) containing 0.25% phosphate and 0.5% glycine, although the other organic acids reduced its production (Table IV). With the supply of organic acids, the yield of nigerloxin could be enhanced to 2.62 mg compared to 1.87 mg g^{-1} dry un-supplemented wheat bran. In this respect Rao *et al.* (2005) found that nigerloxin production was the highest when wheat bran was supplemented with 5% (w/w) tri-sodium citrate.

Effect of incubation period and temperature. The effects of incubation period and temperature on nigerloxin production by *A. niger* in bran:bagasse (4:1 ratio w/w) medium was studied. Nigerloxin was produced after 3 days and enhanced by increasing both

Table IV. Effect of organic acids on production of nigerloxin in bran/bagasse (containing P/glycine 1:2) by *A. niger* after 5 days of incubation at 30°C

Organic acids sources	Nigerloxin (mg/g dry substrate)	Activation[b] %
Control [a]	2.51	-
Ascorbic acid	1.73	0
Benzoic acid	0	0
Citric acid	2.56	2
Malic acid	2.17	0
Oxalic acid	0.61	0
Pyruvic acid	2.00	0
Salicylic acid	0	0
Succinic acid	2.62	4

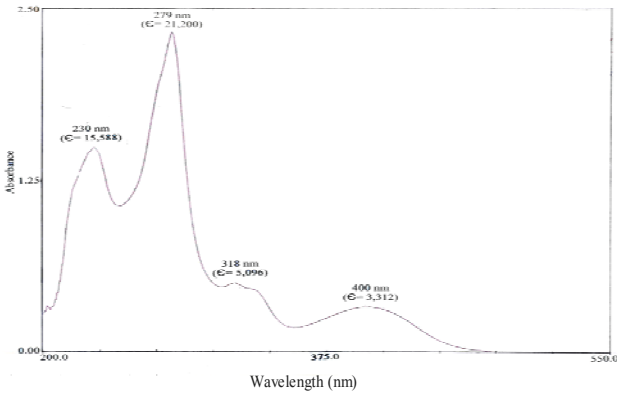
[a] Control and all treatments contain P/glycine (0.25: 0.5 %)
[b]Activation in nigerloxin production compared with control

Table V. Production of nigerloxin in different raw materials by *A. niger* after 7 days of incubation at 30°C

Natural waste products	Ratios	Nigerloxin (mg/g dry substrate)	Activation[a] %
Bran/bagasse	4: 1	2.95	-
Pineapple	5: 0	0.55	0
Bran/pineapple	4: 1	3.58*	21
Potatoes	5: 0	2.36	0
Bran/potatoes	4: 1	3.90*	32
Bran/potatoes/bagasse	3: 1: 1	3.08	4

* Mean significant increase compared to bran/bagasse as control.
[a]Activation in nigerloxin production compared with control.

Fig. 1. Absorption spectrum of nigerloxin in methanol (absorbance vs. Wavelength)



incubation period and temperature (Fig. 3). The results revealed that for 7 days 30°C was more favorable incubation temperature for optimum nigerloxin yield.

Nigerloxin production in natural waste products. The use of inexpensive raw materials, pineapple and potatoe peels in addition to bagasse and bran, for nigerloxin production enhanced the economic efficiency of the production process. Nigerloxin production by *A. niger* in different raw materials after 7 days was studied (Table V). Bran:potatoes (4:1 w/w), was the most favorable, giving a nigerloxin yield of 3.9 mg g^{-1} dry weight, followed by bran:pineapple under optimum conditions. Use of bran:potatoes peels (4:1 w/w)

Fig. 2. Standard curve of nigerloxin in methanol at 279 nm

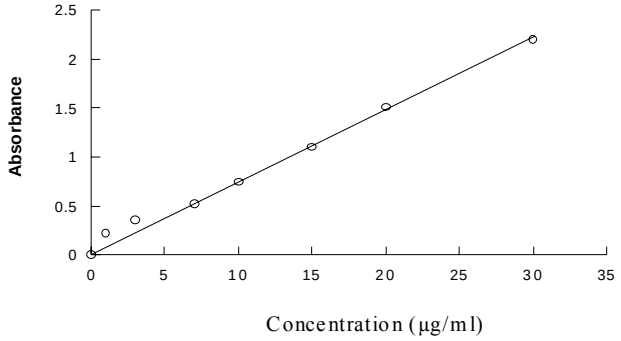
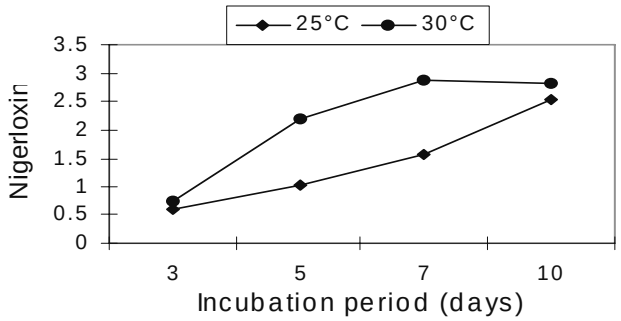


Fig. 3. Effect of incubation periods and temperatures on production of nigerloxin (mg/g dry substrate) by *A. niger* in bran/bagasse (4: 1 w/w) medium



increased nigerloxin yield by 108% after 7 days.

CONCLUSION

Nigerloxin can be quantified accurately by spectrophotometer at 279 nm as recommended wavelength. *A. niger* has potential to produce nigerloxin and the production was increased by raising the bran level and reached its maximum in bran:bagasse ratios of 4:1 (w/w). Supplementation of bran:bagasse with 0.5% glycine, 0.25% phosphorus and 0.5% succinic acid increased nigerloxin production and the maximum yield reached to 2.62 mg as compared to 1.87 mg g^{-1} dry un-supplemented bran. This production was optimal at 30°C and after 7 days of incubation. The use of inexpensive raw materials, potatoes peel (20%) in addition to bran, raised nigerloxin by 108%.

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