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ORIGINAL PAPER

Studies on the production of nigerloxin using agro-industrial residues by solid-state fermentation

Dasagrandhi Chakradhar · Saleem Javeed · Avinash P. Sattur

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Abstract Nigerloxin, a new and potent lipoxygenase inhibitor, was discovered in our laboratory through solid-state fermentation of wheat bran by *Aspergillus niger* V. Teigh (MTCC-5166). The aim of this study is to investigate the possibility of using different agro-industrial residues as nutritional supplements along with wheat bran to enhance the production of nigerloxin. Nigerloxin produced by SSF was quantified spectrophotometrically at 292 nm. The results indicate that the inhibitor production was influenced by the type of solid substrate supplemented, moisture content, pH and size of the inoculum. Individually optimized supplements were tested in different combinations to determine their effects on nigerloxin production. A twofold increase in the production of nigerloxin (4.9 ± 0.3 mg gds^{-1}) was achieved by supplementing wheat bran with 10% w/w sweet lemon peel and 5% v/w methanol at optimized process parameters, that is, an initial moisture content of 65% v/w and incubation period of 6 days with an initial inoculum size of 2 ml (8×10^5 spores gds^{-1}). Nigerloxin production was stable between pH of 4 and 5.

Keywords Agro-industrial residues · Solid-state fermentation · *Aspergillus niger* V. Teigh (MTCC-5166) · Nigerloxin · Lipoxygenase inhibitor

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Introduction

Solid-state fermentation (SSF) offers numerous advantages for the production of bulk chemicals, enzymes and secondary metabolites [11, 12, 16, 29, 30]. This is mainly because of lower energy requirements and less wastewater. An attractive aspect of SSF from the environmental point of view is its utilization of several agricultural byproducts as substrates, such as wheat bran, coconut oil cake, groundnut oil cake and rice bran for the production of value-added products [18].

Lipoxygenases are a family of non-heme iron containing dioxygenase involved in the pathogenesis of some diseases, such as allergies, atherosclerosis and cancer [27]. During the course of our screening for enzyme inhibitors from microbial sources, we discovered a new metabolite, designated as nigerloxin. Nigerloxin was found to be a potent inhibitor of lipoxygenase (LOX-1) and aldose reductase with an IC_{50} value of 79 and 69 μM, respectively, in in-vitro studies. The inhibitor exhibited a reversible and mixed type of inhibition against LOX-1 with a K_i value of 13 μM. The radical scavenging property of nigerloxin (ED_{50}-66 μM) was also reported and compared well with that of known standard antioxidant molecules [21]. Hence, this molecule can be considered to be a lead molecule for the development of potential therapeutic agents to treat late diabetic complications, such as retinopathy, neuropathy and nephropathy.

Nigerloxin production was previously improved twofold by using various nutritional biochemical supplements [22]. Attempts were also made to use a few agricultural wastes like sugarcane bagasse, pineapple peel and potatoes [10]. However, a detailed optimized study using various agro industrial wastes for nigerloxin production was not carried out. The present investigation was focused on the use of

different agro-industrial residues for the production of nigerloxin and development of biomass.

Materials and methods

Culture

The fungus used for this study was isolated from honey bee wax of the local forest habitat and deposited in Microbial Type Culture Collection (MTCC-5166). Briefly, 1 g of bee wax was extracted with 10 ml of sterilized distilled water for 5 min at room temperature and subjected to the pour plate technique on Czapek solution agar (Difco^{TM}) and incubated at 28 ± 2°C for 5 days. The morphological and cultural characteristics of the strain were identified according to the method previously described [28]. The taxonomic position of the strain was related to *Aspergillus niger* V. Teigh based on descriptions of previous literature [23]. The strain was further maintained on potato dextrose agar (Hi Media, Mumbai, India) at 4°C and sub-cultured once every 3 weeks.

Inoculum preparation

A. niger V. Teigh (MTCC-5166) was grown on potato dextrose slants for 5 days to achieve complete sporulation. A loopful of spores were scraped and transferred into 500-ml Erlenmayer flasks containing 100 ml potato dextrose broth. The flasks were incubated at 30°C in a shaker incubator (200 rpm). Cells were harvested after 48 h, washed with sterile distilled water and resuspended in 10 ml of sterile distilled water. This cell suspension was used as an inoculum.

Solid state fermentation

Experiments were conducted in 250-ml Erlenmeyer flasks containing 5 g of sterilized substrate. The substrate was moistened with distilled water to bring the initial moisture level to 60% v/w. After thorough mixing, the flasks were autoclaved at 121°C for 1 h and cooled to room temperature. It was inoculated with 2 ml of cell suspension (6×10^5 spores gds^{-1}) and incubated at 30°C for 5 days. Unless otherwise mentioned, these conditions were maintained throughout the experiment.

Nigerloxin extraction and determination

Ethyl acetate (50 ml) was added to each flask and agitated on a rotary shaker at 150 rpm for 2 h. The mixture was then filtered using cheesecloth, and the cultures were re-extracted with 50 ml of ethyl acetate. The ethyl acetate layer was filtered using Whatman no. 1 filter paper on anhydrous sodium sulphate. The extract was concentrated to dryness under reduced pressure using a rotary evaporator (Hiedolph-Laborota 4003/G3).

Nigerloxin was separated from the crude extract by thin layer chromatography (TLC) using benzene:acetone (4:1 v/v) as the developing solvent. TLC analysis was performed on silica gel 60-coated plates with a fluorescent indicator. Nigerloxin was visualized as yellow–orange under long wavelengths of UV light at R_f 0.55. The inhibitor spot was scraped off the TLC plate and quantitatively eluted with 3 vol of methanol. After a brief centrifugation at 3,000 rpm for 3 min, the supernatant was separated and evaporated to dryness. The methanol solution of nigerloxin was then subjected to spectral analysis using UV–VIS spectrophotometer (Shimadzu UV 1601) and compared with standard nigerloxin.

The concentration of nigerloxin in the samples was determined at the recommended wavelength of 292 nm and calculated using the formula:

$$\text{Nigerloxin concentration (mg/g dry weight)} = \frac{A\text{Mwt}F}{E}$$

A, Absorbance; *Mwt*, molecular weight; *F*, dilution factor; *E* extinction value.

Biomass estimation

Biomass was estimated by determining the *N*-acetyl glucosamine released by the acid hydrolysis of chitin, present in the cell wall of the fungi [26]. Then 0.5 g of fermented substrate previously dried at 110°C for 2 h in a hot-air oven was subjected to acid hydrolysis with 1 ml of concentrated sulphuric acid. To this, 1 ml acetyl acetone reagent was added and incubated in a boiling water bath for 20 min. After cooling, 6 ml ethanol was added followed by 1 ml Ehrlich reagent (Sigma) and incubated at 65°C for 10 min. After cooling to room temperature, the *N*-acetyl glucosamine released was read at 530 nm against the reagent blank. *N*-acetyl glucosamine (Sigma) was used as the external standard. A standard calibration curve was prepared by plotting different initial dry weights of biomass extracted from a known quantity of dry fermented substrate against the corresponding *N*-acetyl glucosamine content measured spectrophotometrically at 530 nm. A correlation equation thus obtained was used to determine the dry fungal biomass in SSF. All experiments were done in triplicates, and mean ± SD was reported.

Analytical methods

One gram of the fermented substrate was extracted with 5 ml of distilled water at 32 ± 1°C on an orbital shaker at

250 rpm for 1 h. The extract was filtered through a Whatman no. 1 filter paper. The filtrate was then centrifuged at 6,000 rpm for 10 min. The supernatant was used for the determination of glucoamylase activity [2]; 0.5 ml of this filtrate was then incubated with 0.5 ml of 1% w/v soluble starch in 0.1 M sodium acetate buffer at pH 5.0 at 55°C for 15 min. The reducing sugar released was measured [14]. One unit (U) of glucoamylase activity was defined as the amount of enzyme that released 1 μM of reducing sugar as glucose, per minute, under assay conditions and expressed as U gds^{-1}. Total and residual sugars were estimated using phenol sulphuric acid method [4]. To fix the initial moisture content of the solid medium, supplemented wheat bran was soaked with the desired quantity of additional water. After soaking, the sample was again dried at 55°C for 1 h. The dry weight was recorded, and percentage moisture was calculated as follows: Percentage moisture of solid medium = (Initial weight of substrate) − (Final weight of substrate)/(Initial weight of substrate) × 100 per dry weight.

Screening of agro-industrial residues for enhanced production of nigerloxin and optimization of fermentation parameters

Nigerloxin production was optimized by supplementing different agricultural residues previously dried at 50°C for 24 h. Several fermentation parameters and culture conditions were altered and the effects observed after 5 days of incubation at 28°C, unless otherwise stated.

1. The effect of different agro-industrial residues like paddy husk (PH), paddy straw (PS), corn cob (CC), saw dust (SD), coir waste (CW), sugar cane bagasse (SB), groundnut oil cake (GC), coconut oil cake (CK), palm oil cake (PC), sweet lemon peel (SP), pineapple peel (PP), lemon peel (LP) and banana peel (BP) were studied separately and in combination with basal wheat bran medium for the optimum production of nigerloxin. The effect of addition of organic solvent (methanol) on the production of biomass and nigerloxin was also studied. The best supplements, producing optimum yields of nigerloxin, were combined in various combinations with wheat bran to study their effect on inhibitor production. Only the supplement that showed maximum production of nigerloxin gds^{-1} was taken for further studies.
2. The effect of initial moisture content on the production of nigerloxin and amyloglucosidase activity in the supplemented wheat bran medium was studied by adjusting the media with different ratios of moistening agent (distilled water). At the end of the 5th day, the amount of nigerloxin produced and enzyme activity in each treatment were determined.
3. The effect of initial pH of the supplemented wheat bran medium on the production of nigerloxin and biomass was determined by altering the initial pH of the fermentation media with the addition of moistening agent adjusted to the required pH with an acid or an alkali. The effect of the inoculum size on the production of nigerloxin and biomass was also determined by taking different volumes of the mycelial suspension (stock 10 ml). Different volumes of broth culture (0.1–4 ml) were used for inoculating the fermentation medium. At the end of 5 days, the amount of nigerloxin produced was determined along with the biomass.
4. A time course study of nigerloxin production in supplemented wheat bran medium up to 7 days was studied with all optimized parameters. The amount of nigerloxin produced was estimated daily, until 7 days, and the results were compared with the control wheat bran.

Results and discussion

Nigerloxin (2-amino-3hydroxy-6-methoxy-5-methyl-4-(prop-1′-enyl) benzoic acid) (Fig. 1) with a molecular weight of 265 and molecular formula $C_{13}H_{15}NO_5$ was discovered in our laboratory as a potent inhibitor of rat eye lens aldose reductase and lipoxygenase possessing a free radical scavenging property [21]. The inhibitor is produced only by solid-state fermentation and not in submerged fermentation conditions. The production of the inhibitor is directly related to sporulation of the culture.

Determination of nigerloxin by spectrophotometric method

The calibration curve of nigerloxin was constructed by taking the absorption maxima at different concentrations of nigerloxin in methanol (1 mg/ml stock).

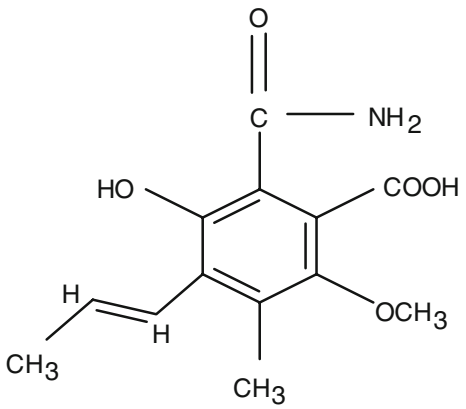

Fig. 1 Structure of nigerloxin

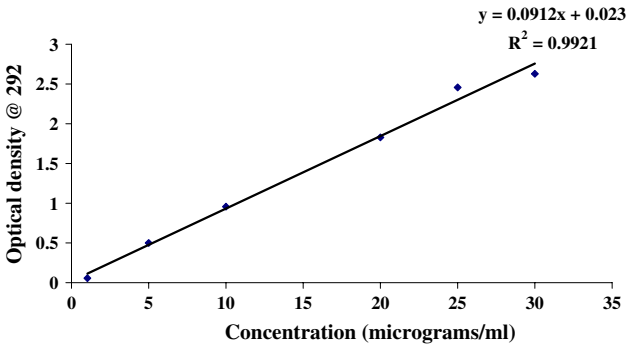

Fig. 2 Standard curve of nigerloxin

Nigerloxin has four UV-absorption maxima at 234-, 292-, 359- and 389-nm wavelengths in methanol. The extinction coefficients (E) used to determine nigerloxin concentrations were 13,200 at 234 nm, 26.800 at 292 nm, 6,230 at 359 nm and 2,100 at 389 nm. Nigerloxin can be quantified more accurately by constructing a standard curve at 292 nm as the recommended wavelength (Fig. 2).

Determination of biomass in SSF

Fungal dry weight and *N*-acetyl glucosamine content were determined to obtain a suitable relationship for biomass estimation of *A. niger* V. Teigh (Fig. 3). The evidence of a linear relationship was observed, and its correlation is shown in the equation: $Y = 456.3X - 25.80$ ($R^2 = 0.9925$). The biomass dry weight can be calculated by the formula (BD_W) = [(Glucosamine concentration + 25.80)/456.3]. This equation was correlated to the fungal dry weight, and hence used as an indirect method for estimation of fungal biomass.

Selection of substrates for higher yields of nigerloxin

Tropical agro-industrial residues, such as PH, PS, CC, SD, CW, SB, GC, CK, PC, SP, PP, LP and BP, were evaluated for selecting the best substrate for nigerloxin production. The highest production of nigerloxin was achieved with wheat bran (2.3 ± 0.35 mg gds^{-1}) (Table 1). Others either yielded negligible (PH, PS and CC) or poor yields (SB, GC, SP and PP). Hence, wheat bran was used for further supplementation studies, and the value (2.3 ± 0.35 mg gds^{-1}) was used as a control in further studies. These results are in agreement with the previous findings [24] suggesting the use of wheat bran as the best choice of solid substrate both in terms of biomass and the secondary metabolite production by SSF.

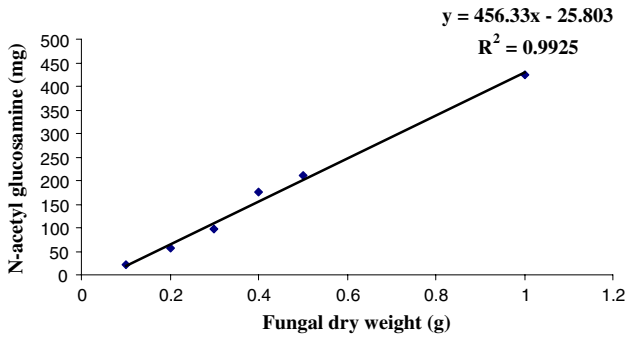

Fig. 3 Correlation between fungal dry weight and *N*-acetyl glucosamine content

Table 1 Growth and nigerloxin production on various agro-industrial residues by *A. niger* V. Teigh

Substrate	Biomass (mg gds^{-1})[a]	Nigerloxin yield (mg gds^{-1})[a]
Paddy husk	220 ± 5.5	0.4 ± 0.15
Paddy straw	170 ± 4.3	0.2 ± 0.11
Corncob	330 ± 6.5	0.5 ± 0.13
Sawdust	80 ± 1.4	0.0 ± 0.00
Coir waste	80 ± 2.2	0.0 ± 0.00
Sugarcane baggage	430 ± 8.7	0.7 ± 0.21
Groundnut oil cake	350 ± 7.3	0.5 ± 0.10
Coconut oil cake	280 ± 6.5	0.0 ± 0.00
Palm oil cake	220 ± 3.7	0.0 ± 0.00
Sweet lemon peel	338 ± 7.6	1.3 ± 0.25
Pineapple peel	226 ± 5.8	0.7 ± 0.15
Lemon peel	132 ± 4.7	0.0 ± 0.00
Banana peel	65 ± 2.2	0.0 ± 0.00
Wheat bran	176 ± 7.4	2.3 ± 0.35

[a] Results are mean ± SD of three determinations

When different agro-industrial wastes were supplemented at 10% w/w with wheat bran, the results were interesting (Table 2). Wheat bran supplemented with 10% w/w CC resulted in 13% activation of nigerloxin production when compared to control wheat bran. Among the oil cake supplementations, GC proved to have a beneficial role on nigerloxin production in terms of an increase in yield by 23.4%. A similar finding by Ellaiah et al. [6] suggested that groundnut oil cake supplementation at 10% w/w resulted in a 14% increase in the production of neomycin. A significant increase in the production of nigerloxin (55%) associated with the highest biomass (571 ± 3.2) produced per gram of dry substrate was achieved in wheat bran medium supplemented with SP.

As CC, GC and SP have shown positive effects on nigerloxin production, their effect on nigerloxin production by supplementation to wheat bran at different ratios was also carried out (Fig. 4). It was seen that with CC supplementation up to 20% w/w, there was a steady

Table 2 Evaluation of different agro-industrial residues supplemented with wheat bran for the enhanced production of biomass and nigerloxin

Supplement (10% w/w)	Mean ± SD[a]				
	Total sugar (mg gds^{-1})	Residual sugar (mg gds^{-1})	% Sugar utilized	Biomass (mg gds^{-1})	Nigerloxin (mg gds^{-1})
PH	116 ± 3.5	63 ± 0.2	46.9 ± 1.1	310 ± 1.5	2.16 ± 0.1
PS	102 ± 2.7	59 ± 0.2	42.1 ± 0.5	304 ± 0.2	2.08 ± 0.3
CC	132 ± 1.0	56 ± 1.2	58.4 ± 0.1	457 ± 1.6	2.64 ± 0.1
SD	75 ± 1.2	44 ± 1.5	40.0 ± 1.2	464 ± 1.3	2.02 ± 0.1
CW	72 ± 1.4	40 ± 1.2	44.4 ± 0.6	280 ± 0.5	1.09 ± 0.2
SB	112 ± 1.1	62 ± 0.5	44.6 ± 1.5	475 ± 2.2	2.13 ± 0.2
GC	134 ± 3.3	41 ± 1.2	69.4 ± 1.7	514 ± 1.5	2.92 ± 0.5
CK	73 ± 1.8	58 ± 0.3	20.5 ± 1.1	345 ± 2.4	2.15 ± 0.1
PC	126 ± 0.5	72 ± 1.1	34.9 ± 0.3	341 ± 1.6	2.02 ± 0.1
SP	163 ± 0.1	40 ± 1.8	75.4 ± 0.7	571 ± 3.2	3.71 ± 0.4
PP	111 ± 1.2	48 ± 1.4	56.9 ± 1.3	374 ± 1.2	2.35 ± 0.1
LP	99 ± 0.7	63 ± 0.3	36.3 ± 1.1	248 ± 1.1	1.03 ± 0.3
BP	112 ± 1.3	69 ± 1.9	38.3 ± 0.4	297 ± 1.6	2.15 ± 0.3
CW	143 ± 1.6	63 ± 1.3	55.2 ± 1.4	433 ± 2.2	2.24 ± 0.2

PH paddy husk, *PS* paddy straw, *CC* corncob, *SD* sawdust, *CW* coir waste, *SB* sugarcane bagasse, *GC* groundnut oil cake, *CK* coconut oil cake, *PC* palm kernel cake, *SP* sweet lemon peel, *PP* pine apple peel, *LP* lemon peel, *BP* banana peel, *CW* control wheat bran

[a] Mean ± SD values of three replicates for each treatment is presented

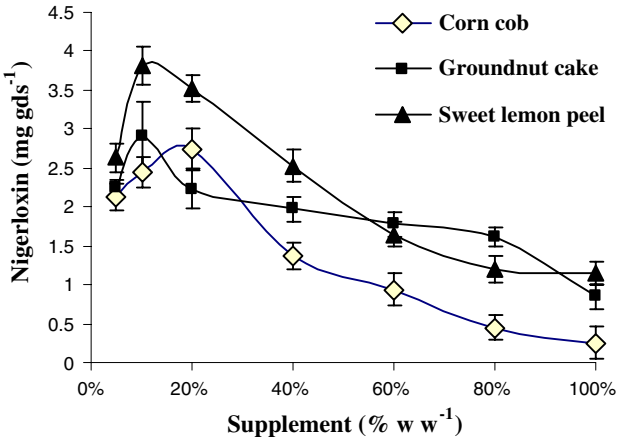

Fig. 4 Effect of corn cob, groundnut oil cake and sweet lemon peel supplementation with wheat bran at different ratios on the production of nigerloxin

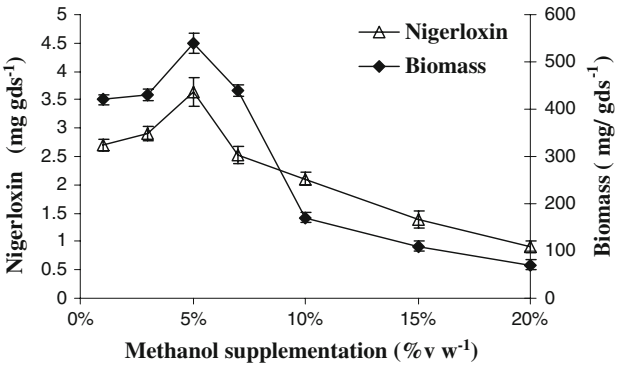

Fig. 5 Effect of methanol supplementation to wheat bran on biomass and nigerloxin production

increase in nigerloxin production and a further increase in supplementation resulted in deleterious effects on nigerloxin production. GC had shown a beneficial role on nigerloxin production with an optimum ratio of 10% w/w. Maximum yield of nigerloxin (3.82 ± 0.5 mg gds^{-1}) could be obtained in wheat bran medium supplemented with SP at 10% w/w. This improved yield of nigerloxin could be due to the availability of some of the organic acids, trace elements and amino acids in a readily available form that might have stimulated the cultures to produce more nigerloxin. However, excess supplementation has produced a deleterious effect on the production of nigerloxin.

The beneficial role of primary and secondary alcohols on the production of some of the primary metabolites by solid-state fermentation is well established. Specifically, the effect of methanol treatment for enhanced production of citric acid was observed in SSF of apple pomace by *A. niger* [9]. Hence, it was worth investigating whether methanol supplementation could increase the sporogenic activity of fungi and thereby enhance nigerloxin accumulation. A steady increase in the production of nigerloxin and biomass was observed with the addition of methanol up to 5% v/w (Fig. 5). A further increase of methanol drastically reduced the yields of nigerloxin as well as biomass. The probable reason for the reduced production of nigerloxin could be because of delayed sporulation of the

Table 3 Individual and combined effects of supplementation to wheat bran on nigerloxin production

Supplementation (% w/w)	Nigerloxin (mg gds^{-1})[a]
None	2.24 ± 1.3
20% Corncob	2.74 ± 0.7
10% Groundnut cake	2.91 ± 1.1
10% Sweet lemon peel	3.71 ± 0.4
5% Methanol[b]	3.55 ± 0.4
20% Corncob + 10% groundnut oil cake	2.37 ± 0.5
20% Corncob + 10% sweet lemon peel	2.17 ± 0.1
20% Corncob + 5% methanol[b]	2.54 ± 0.1
10% Groundnut oil cake + 10% sweet lemon peel	3.12 ± 0.6
10% Groundnut oil cake + 5% methanol[b]	2.31 ± 0.3
10% Sweet lemon peel + 5% methanol[b]	4.62 ± 0.3
20% Corncob + 10% groundnut oil cake + 5% methanol[b]	2.13 ± 0.5
20% Corncob + 10% groundnut oil cake + 10% sweet lemon peel	2.54 ± 0.8
10% Groundnut oil cake + 10% sweet lemon peel + 5% methanol[b]	3.72 ± 1.1
20% Corncob + 10% groundnut oil cake + 10% sweet lemon peel + 5% methanol[b]	2.63 ± 0.6

[a] Mean ± SD of three replicates for each treatment was represented

[b] 5% methanol supplemented in v/w

fungus due to unfavorable conditions created by excess addition of methanol compared to control wheat bran.

The individual optimized levels of CC (20% w/w), GC (10% w/w), SP (10% w/w) and methanol (5% v/w) were combined at various possible combinations to check if the nigerloxin yield could be enhanced (Table 3). Wheat bran supplementation with 10% w/w GC and 5% v/w methanol caused a 1.4-fold increase in nigerloxin production. However, supplementation with 10% w/w SP and 5% v/w methanol resulted in a 2.2-fold increase. These results are in agreement with earlier data [10] that represented a 100% activation in nigerloxin production by potato supplementation to the bran medium. However, all other combinations of supplementation have not resulted in any significant improvement in the production of nigerloxin. This finding is in agreement with previous observations [25] suggesting that the addition of extra nutrients to the substrate resulted in inhibition of product formation.

Effect of initial moisture content on nigerloxin production

The moisture content of any substrate is of utmost importance in a solid-state fermentation system, which controls the growth of microorganisms, metabolite production and enzyme activity [15, 19, 31, 32]. Glucoamylases are important enzymes for the liquefaction of starchy materials [1]. Previous reports [8, 20] suggest that wheat bran with additional nutrients is a useful medium for the production of glucoamylase and other enzymes in SSF process. From our previous studies, we noticed that the glucoamylase activities are significantly higher during spore germination of *A. niger*. The effect of initial moisture content of the substrate on the production of nigerloxin and glucoamylase activity is presented in Fig. 6. Maximum nigerloxin production of 4.71 mg gds^{-1} was observed at 65% v/w of initial moisture content. A decrease in nigerloxin yield was noticed when the moisture level was higher or lower than optimum. This result was in agreement with earlier findings of Johns et al. [13], who reported that an initial moisture content of less than 40% resulted in a reduced product yield, but that of 50–60% v/w resulted in the highest microbial compound. Higher initial moisture in SSF leads to sub-optimal product formation because of a reduced mass transfer process and decreased initial moisture level results in reduced solubility, oxygen transfer and low availability of nutrients to the culture [3]. In this study, at low initial moisture contents low glucoamylase activity was observed. Higher activity (190 ± 18.2–220 ± 15.2 U gds^{-1}) of these hydrolyzing enzymes at 55–65% v/w moisture has resulted in effective utilization of sugars present in the substrate, which could be attributable to

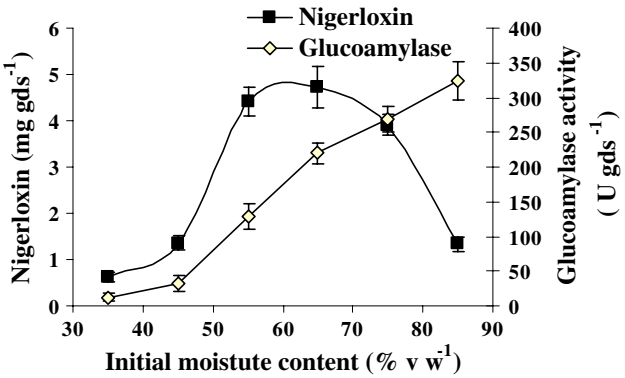

Fig. 6 Effect of initial moisture content of the supplemented wheat bran (SWB) on the glucoamylase activity and nigerloxin production

the high yields of nigerloxin at an initial moisture content of 50–65% v/w. A further increase in moisture level caused a rise in enzyme activity, but reduced nigerloxin yield. Increased glucoamylase activity at high initial moisture up to 80% has been reported by Ellaiah et al. [5]. Higher moisture contents may result in agglomeration of the substrate, thereby restricting the supply of oxygen to micro-organisms and inhibiting pigment production [7]. But in this study, it could be due to the liberation of rapidly reducing sugars as a result of increased enzyme activity at this moisture level, which in turn could have inhibited the spore germination and thereby nigerloxin accumulation. This finding is in agreement with the previous report [22], suggesting that high concentrations of glucose are inhibitory to the production of nigerloxin.

Effect of initial pH of the medium on nigerloxin production

To study the effect of pH, the supplemented wheat bran containing 10% w/w SP with initial pH 4 was adjusted to various initial pH values. The medium contained 5% v/w methanol. The yield of nigerloxin was only 1.22 mg gds^{-1} after 5 days with an initial pH of 2, but a yield as high as 4.5 ± 0.28 mg gds^{-1} was obtained at an initial pH value of 4. Maximum biomass was also observed at this pH (565 ± 25.1 mg gds^{-1}). Nigerloxin production was stable between pH 4 and 5 associated with maximum biomass observed. However, pH 6–7 resulted in decreased production of nigerloxin. A further increase in pH proved to be inhibitory to the production of biomass as well as nigerloxin (Fig. 7).

Effect of inoculum size on the production of biomass and nigerloxin

An inoculum size of 2.0 ml (8×10^5 spores gds^{-1}) resulted in maximum yield of nigerloxin (4.73 ± 0.33) with biomass of 587 ± 21.3 mg gds^{-1} (Fig. 8). A further rise in inoculum

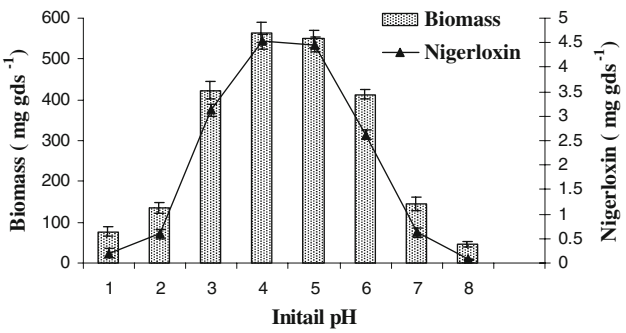

Fig. 7 Effect of initial pH of the supplemented wheat bran (SWB) on biomass and nigerloxin production

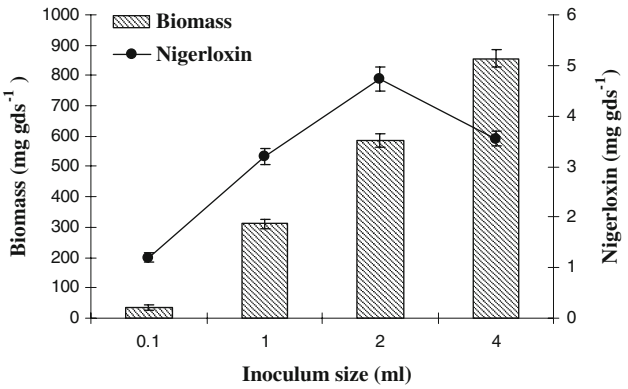

Fig. 8 Effect of inoculum size on the production of biomass and nigerloxin in SSF of supplemented wheat bran (SWB)

size doubled the biomass, but resulted in decreased production of nigerloxin. The present results are in agreement with Pandey et al. [17], which showed that lower and higher inoculum levels caused poor product formation in SSF. A higher inoculum development might have caused aggregation of a huge biomass, which in turn rapidly exhausted the nutrients necessary for product formation.

Time course study of nigerloxin production in optimized wheat bran medium

The production of nigerloxin by *A. niger* V. Teigh with optimized parameters (such as 65% v/w moisture, pH-4, inoculum size of 8×10^5 spores gds^{-1}) was studied for 7 days and compared with control wheat bran medium. The result represents a lag phase extending up to 48 h in both treatments. A significant increase in the production of nigerloxin and biomass was observed at 72 h after fermentation in supplemented wheat bran medium with respect to the control. From Fig. 9, it follows that maximum

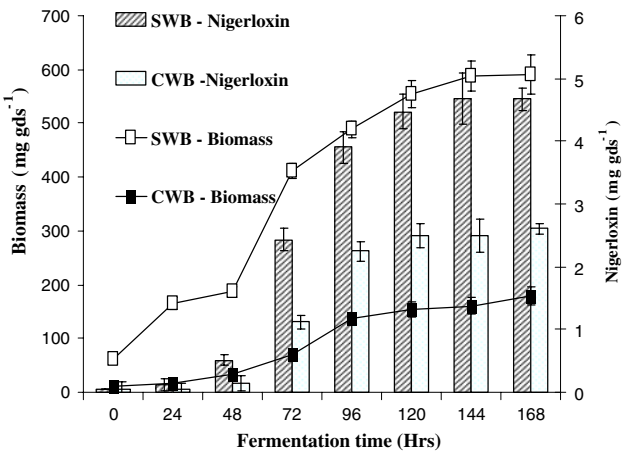

Fig. 9 Time course production of nigerloxin and biomass in control wheat bran (CWB) and supplemented wheat bran medium (SWB)

nigerloxin (4.67 ± 0.17 mg gds^{-1}) production associated with high biomass (591 ± 12.1 mg gds^{-1}) in supplemented wheat bran medium was obtained on the 6th day after fermentation and remained stable till the end of the fermentation. These results are in agreement with the previous finding by [22], which suggested that maximum nigerloxin production was achieved after 5 days. Since the highly sporulated fungi present after 5 days of fermentation, this may be responsible for the overaccumulation of nigerloxin. Hence, the production of nigerloxin is directly correlated to sporulation and biomass production.

Conclusion

Based on the results obtained from this study, the maximum yield of nigerloxin in wheat bran medium could be enhanced from 2.4 ± 0.33 to 4.91 ± 0.37 mg gds^{-1} by supplementing 10% w/w SP and 5% v/w methanol at an initial moisture level of 65% v/w over 6 days of fermentation with an unchanged initial pH (4.0) and an inoculum size of 2 ml (approximately 8×10^5 spores gds^{-1}). The yield is 2.1-fold higher when compared to the control wheat bran medium. Though similar attempts have been made in the past to produce nigerloxin using supplementation, complete optimization was not done. The data generated in this study give a window of possibility to use these results, especially the use of spore catalyst (glucoamylase) and its application in biotransformation studies for the development of nigerloxin into a powerful bioactive molecule with improved biological activities.

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References

1. Arasaratnam V, Balasubramanyam K (1993) Synergistic action of alpha amylase and glucoamylase on raw starch. J Microbiol Biotechnol 7:37–46
2. Bernfield P (1955) Amylase alpha and beta. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 1. Academic press, New York, pp 149–151
3. Carrizales V, Rodríguez H (1981) Determination of specific growth rate of moulds in semi solid culture. Biotechnol Bioeng 23:321–333. doi:10.1002/bit.260230207
4. Dubios M, Gilles KA, Hamilton JK, Roberts DA, Smith F (1956) Calorimetric methods for determination of sugars and related substances. Anal Chem 28:350–356. doi:10.1021/ac60111a017
5. Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002) Optimization of process parameters for glucoamylase production under solid-state fermentation by a newly isolated *Aspergillus* species. Process Biochem 38:615–620. doi:10.1016/S0032-9592(02)00188-7
6. Ellaiah P, Srinivasulu B, Adinarayana K (2004) Optimization studies on neomycin production by a mutant strain of *Streptomyces marinensis* in solid state fermentation. Process Biochem 39:529–534. doi:10.1016/S0032-9592(02)00059-6
7. Gautam P, Sabu A, Pandey A, Szackacs G, Soccol CR (2002) Microbial production of extra cellular phytase using polystyrene as inert support. Bioresour Technol 83:229–233. doi:10.1016/S0960-8524(01)00215-2
8. Ghildyal NP, Lonsane BK, Sreekantiah KR, Sreenivasa murthy V (1985) Economics of submerged and solid state fermentation for the production of amyloglucosidase. J Food Sci Technol 22:171–176
9. Hang YD, Woodams EE (1984) Apple pomace; a potential substrate for citric acid production by *Aspergillus niger*. Biotechnol Lett 6:763–764. doi:10.1007/BF00133071
10. Hasan HAH (2007) Production and determination of Nigerloxin by *Aspergillus niger*. Int J Agric Biol 9:315–318
11. Hasseletine CW (1977) Solid state fermentation. Part 1. Process Biochem 12:24–27
12. Holker U, Hofer M, Lenz J (2004) Biotechnology advantages of laboratory solid state fermentation with fungi. Appl Microbiol Biotechnol 64:175–186. doi:10.1007/s00253-003-1504-3
13. Johns MR, Stuart DM (1991) Production of pigments by *Monascus purpureus* in solid culture. J Ind Microbiol 8:23–38. doi:10.1007/BF01575587
14. Miller GL (1959) Use of dinitrosalicylic acid for estimation of reducing sugars. Anal Chem 31:426–428. doi:10.1021/ac60147a030
15. Pandey A (1992) Recent developments in solid state fermentation. Process Biochem 27:109–116. doi:10.1016/0032-9592(92)80017-W
16. Pandey A, Selvakumar P, Ashakumary L (1994) Glucoamylase produced by *Aspergillus niger* on rice bran is improved by adding nitrogen sources. J Microbiol Biotechnol 10:348–349. doi:10.1007/BF00414878
17. Pandey A, Soccol CR, Mitchell D (2000) New developments in solid state fermentation: I-bioprocess and products. Process Biochem 35:1153–1169. doi:10.1016/S0032-9592(00)00152-7
18. Pandey A, Soccol CR, Rodriguez–Leon JA, Nigam P (2001) Solid state fermentation in biotechnology. Asiatech Publishers Inc, New Delhi, p 221
19. Pandey A (2003) Solid-state fermentation. Biochem Eng J 14:81–84. doi:10.1016/S1369-703X(02)00121-3
20. Ramakrishna SV, Ghildyal NP, Lonsane BK, Ahmed SY, Sreenivasa Murthy V (1982) Wheat bran as substrate in solid state fermentation. In: Biotechnology proceedings, Department of chemical engineering and technology, New Delhi, pp 267–283
21. Rao KCS, Divakar S, Babu KN, Rao AGA, Karanth NG, Sattur AP (2002) Nigerloxin, a novel inhibitor of aldose reductase and lipoxygenase with free radical scavenging activity from *Aspergillus niger* CFR-W-105. J Antibiot 55:789–793
22. Rao KCS, Karanth NG, Sattur AP (2005) Production of nigerloxin, an enzyme inhibitor and a free radical scavenger, by *Aspergillus niger* using solid state fermentation. Process Biochem 40:2517–2522. doi:10.1016/j.procbio.2004.10.008
23. Raper KB, Fennell DT (1965) The genus *Aspergillus*. The Williams and walkins Co, Baltimore, pp 293–310
24. Robinson T, Singh D, Nigam P (2001) Solid state fermentation: a promising technology for secondary metabolite production. Appl Microbiol Biotechnol 55:284–289. doi:10.1007/s002530000565
25. Sabu A, Saritha S, Pandey A, Bogar B, Szakacs G, Soccol CR (2002) Solid state fermentation for production of phytase by *Rhizopus oligosporus*. Appl Biochem Biotech–Part A, Enzyme Eng Biotechnol pp 251–60
26. Sakurai Y, Lee TH, Shiota H (1977) On the convenient method of glucosamine estimation in koji. Agric Biol Chem 41:619–624

27. Samuelson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN (1987) Leukotriens and lipoxins: structures, biosynthesis and biological effects. Sci 237:1171–1176. doi:10.1126/science.2820055
28. Sattur AP, Rao KCS, Divakar S, Karanth NG, Shamala R, Rao AGA (2003) Bioactive compound and its isolation and method of treatment for lipoxygenase inhibition and as free radical scavenging agent. US Patent US 2003/0195252 A1
29. Soccol CR, Iloki I, Marin B, Raimbault M (1994) Comparative production of alpha amylase, glucoamylase and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentations. J Food Sci Technol 31:320–323
30. Vandenberghe LPS, Soccol CR, Pandey A, Lebeault JM (2000) Solid state fermentation for the synthesis of citric acid by *Aspergillus niger*. Bioresour Technol 74:175–178. doi:10.1016/S0960-8524(99)00107-8
31. Wang HL, Swain GW, Hesseltine CW (1975) Mass production of *Rhizopus oligophorus* spores and their application in temph fermentation. J Food Sci Technol 40:168–170
32. Yongsmith B, Kitprechavanich V, Chitrandon L, Chaisrisook C, Budda N (2000) Color mutants of *Monascus* sp. KB9 and their comparative glucoamylase on rice solid culture. J Anal Sci 13:571–575