Strain improvement of *Aspergillus niger* for the enhanced production of asperenone

C. Chidananda · C. Mohan Kumar · A. P. Sattur

Received: 27 August 2007 / Accepted: 5 November 2007 / Published online: 13 June 2008

Abstract The enhancement of production of asperenone (Fig. 1), an inhibitor of lipoxygenase and human platelet aggregation from *Aspergillus niger* CFTRI 1105, was achieved by UV and nitrous acid mutagenesis. Nitrous acid mutants exhibited increased inhibitor production when compared with UV irradiated mutants. I N 41 a first-generation nitrous acid mutant produced 5.1 fold increased asperenone over parent strain. Mutant II N 31 obtained by second-generation nitrous acid treatment produced 60.3 mg asperenone/g biomass, which was 131 fold increase when compared to first generated mutant I N 41 and 670 fold increase over the parent strain. This mutant was stable for several generations on production medium.

Keywords Asperenone · *Aspergillus niger* · Mutagenesis · Nitrous acid · UV irradiation

Introduction

Aspergillus niger, a work horse of industrial microbiology has been subjected to different types of mutagenesis for enhanced production of product such as glucose oxidase [1], pectinase [2], phytase [3], lipase [4], and catalase with glucose oxidase [5].

Lipoxygenases (LOX) are a family of non-heme iron containing dioxygenase, which catalyzes the addition of molecular oxygen to unsaturated fatty acids containing cis, cis, 1,4-pentadiene systems to give cis, trans hydroperoxydiene. The products of these enzymes are responsible for variety of human disorders such as allergies, inflammations, asthma, psoriasis, hypersensitivity [6, 7] and atherosclerosis [8, 9]. Hence inhibitors against this group of enzymes have great potential in food and health sectors.

During the course of search for the bioactive molecule from microbial sources [10–12], we discovered asperenone from *Aspergillus niger* CFTRI-1105 to be inhibitory against soybean lipoxygenase and human platelet aggregation [13]. Asperenone was originally isolated as a fungal pigment by Jefferson [14] in a medium comprising steroids and inorganic salts [15]. Asperenone shows a dose dependent inhibition against 15-LOX with an IC_{50} value of 0.3 mM. The

Fig. 1 Structure of asperenone

C. Chidananda · C. M. Kumar · A. P. Sattur (✉)
Fermentation Technology and Bioengineering Department,
Central Food Technological Research Institute,
Mysore - 570 013, India
e-mail: ftbe@cftri.res.in
Tel: +91 / 821 / 2515792
Fax: +91 / 821517233

compound has no effect on aggregation of human platelet induced by ADP or epinephrine while exhibiting a dose dependent inhibition on platelet aggregation induced by collagen shows an IC_{50} value of 0.23 mM [13].

However the quantity of this potentially useful compounds yield was low at 90 µg/g biomass (w/w), indicating a need to improve the existing strain. In the present investigation we report the enhancement of asperenone production from *Aspergillus niger* CFTRI 1105, which was achieved using UV irradiation and nitrous acid as mutagenic agents.

Materials and methods

Microorganism

The culture used in this study *Aspergillus niger* CFTRI 1105 obtained from CFTRI culture collection was maintained at 4°C on potato dextrose agar slants (HiMedia, Mumbai, India).

Fermentation media

1. Production media: Potato dextrose broth 24 g/L (HiMedia, Cat No-M-403), $NaNO_3$ 3 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L final pH-5.2. Sterilized by autoclaving at 121 °C for 15 min at 15 lbs.

UV mutagenesis

The spore suspension containing 2.1×10^6 colony forming units (cfu)/ml was spread on the potato dextrose agar plates. The induction of mutation was carried out by exposure to UV germicidal lamp (256 nm) kept at a distance of 50 cm for time intervals ranging from 2–12 min, [16, 4]. After irradiation, the plates were wrapped in aluminum foil and kept overnight at 4°C. Later the plates were incubated at 28°C, for 4 days.

Nitrous acid mutagenesis

One ml of spore suspension containing 2.1×10^6 (cfu)/ml was added in 0.9 ml acetate buffer pH 4.4 and 100 µl of 0.5 M sodium nitrite was added to the spore suspension [4]. The suspension was incubated at 30°C for time intervals from 5–45 min. After incubation, the reaction was terminated by addition of 5 volumes of 0.2 M phosphate buffer, pH 7.0. The samples were adequately diluted and plated on potato dextrose agar incubated at 28°C, for 4 days.

Selection of mutants

Selections of the mutants were based upon intensity of the yellow pigment produced on the reverse side of the plate indicating the production of asperenone [14], and also by observing any changes in colony morphology, colony size comparing to that of parent strain. The desired mutants were picked to check for their ability to produce high asperenone content and were maintained on potato dextrose agar slants

Inoculum development and fermentation

To a 5-day old slant 10 ml of sterile 0.1% Tween-20 (v/v) solution was added. Conidial spore suspension was prepared by gentle scraping of the surface of the aerial mycelium with a sterile inoculation loop to liberate spores, 1 ml from this spore suspension (1×10^6) was transferred in to a 500 ml Erlenmeyer flask containing 100 ml production medium and incubated on rotary incubator shaker operating at 250 rpm at 28°C for 4 days. At the end of the fermentation the biomass was filtered through muslin cloth and was dried overnight in an oven at 60°C. The dried biomass was extracted with ethyl acetate (1:10 w/v) and the solvent was evaporated by vacuum distillation. The residue that remained after was suitably diluted in acetonitrile and assayed for asperenone using HPLC.

Analytical detection of asperenone

Asperenone in the crude extracts of the mutants was estimated by HPLC (Shimadzu LC 10-A), C_{18} column (250 × 4.6 mm) with reverse phase, acetonitrile being mobile phase with A flow rate of 0.5 ml/min at 410 nm showed retention time (RT) of 7.7 min. Asperenone concentration in each crude sample was calculated with reference to the standard curve of asperenone.

Results and discussion

Literature search on asperenone showed that it has been isolated independently by two other groups [17, 18] however this major yellow intermycelial pigment was characterized as a methyl derivative of 13-phenyl tri-deca-4,6,8,10,12-penta-3-one and was given the trivial name as asperenone by Jefferson [14]. Biological beneficiary activities of asperenone is not known much except for showing an antifungal activity [18] and lipoxygenase and human platelet aggregation inhibition [13].

A total 56 of mutants (34 of Nitrous acid and 22 of UV induction), were subjected to submerged fermentation

on production medium comprising potato dextrose broth enriched with salt supplements, which was originally used by Jefferson, to increase the yields of asperenone [15]. In our previous study we found out that upon enriching PDB with salts there was a great deal of increase in the asperenone yields. Probably the salts acted as inducers in enhancing the yields. A genetically engineered end product gibberellic acid yield was enhanced in *Aspergillus niger* by addition of inorganic salts and some acids to broth which acted as inducers [19]. The crude concentrate obtained after solvent evaporation was assayed for asperenone content by HPLC. Among these, 5 mutants from UV and 8 from nitrous acid mutations were found to produce more of asperenone, about 4–5 fold increase than the parent strain (Table 1). The nitrous acid mutant I N 41, which produced the highest yields of asperenone (0.46 mg/g biomass), a 5.1 fold increase than that of parent strain (0.09 mg/g biomass) was selected and was subjected for stability studies for 5 generations. It was observed that production of asperenone in all the 5 generations was similar average 0.45 mg/g biomass (Table 2).

To further enhance the production of asperenone, the nitrous acid mutant I N 41 was subjected to second set of nitrous acid mutation. All the second set of nitrous acid mutants showed enhanced asperenone production when compared to first set I N 41 strain, about 75–130 fold increase in the yield than the 1st generation mutants (Table 3). The second-generation mutant II N 31 showed 131.2 fold increases in asperenone when compared to first generation mutant I N 41 and also a 670 fold increase to that of parent strain. Mutant II N 31 was subjected for the stability studies for 5 generation, which showed consistent yield of asperenone average 60.84 mg/g biomass (Table 4).

It was seen that there was a 131.3 fold increased asperenone and 2-fold increase in biomass was observed in mutant II N 31. Mutant II N 31 was subjected for the stability studies on production medium for 5 generations, which showed consistent yield of asperenone average 60.3 mg/g biomass and a biomass yield average 13.1 g/L (Table 4).

In our study with *Aspergillus niger* CFTRI 1105 showed, a higher efficiency of UV to induce mutagenesis when compared with nitrous acid, which is indicated by percentage

Table 1 Mutants obtained by UV and nitrous acid mutagenesis of *Aspergillus niger* CFTRI 1105

Culture	Morphology & colony size in (cm)	Biomass dry weight (g/L)	Asperenone (mg/g biomass)
IU11 1	1.6	7.40 ±0.21	0.10 ±0.01
IU 71	1.2	8.22 ±0.12	0.18 ±0.03
IU 3	1.5	8.71 ±0.02	0.17 ±0.05
IU 51	2.2	10.07 ±0.02	0.18 ±0.07
IU 71	1.9	9.54 ±0.30	0.15 ±0.04
IX 141	1.3	10.47 ±0.24	0.18 ±0.08
IN 62	2.3	7.87 ±0.21	0.36 ±0.09
IN 41	1.8	7.07 ±0.32	0.46 ±0.04
IX 122	1.6	9.4 ±0.02	0.22 ±0.08
IN 82	1.6	7.96 ±0.02	0.18 ±0.04
IN 141	1.5	7.07 ±0.04	0.32 ±0.04
IN 81	1.9	8.33 ±0.05	0.31 ±0.06
Parent strain	1.7	10.01 ±0.39	0.09 ±0.02

I first generation mutation, U = UV mutants, N&X= Nitrous acid mutants. The values represented mean ± SEM of triplicate analysis.

Table 2 Stability studies on mutant IN 41

Generation	Biomass dry weight (g/L)	Asperenone (mg/g biomass)
I	8.65 ±1.2	0.46 ±0.06
II	8.65 ±1.5	0.46 ±0.04
III	8.5 ±1.2	0.45 ±0.03
IV	7.17 ±1.5	0.41 ±0.06
V	8.36 ±1.4	0.47 ±0.07
IN 41	7.01 ±1.1	0.46 ±0.05

IN 41 = 1st generation control. The values represented mean ± SEM of triplicate analysis

Table 3 Mutants obtained from second round of mutation of IN 41

Culture	Morphology Colony size (cm)	Biomass dry weight (g/L)	Asperenone (mg/g biomass)
II N 21	2.3	5.66 ±1.5	37.69 ±0.02
II N112	1.9	5.66 ±1.3	40.37 ±0.05
II N 92	1.2	5.97 ±2.0	48.89 ±0.06
II N 31	1.9	13.25 ±1.7	60.3 ±0.07
IN 41	1.8	7.07 ±1.4	44.46 ±0.08

IN 41= 1st generation control. The values represented mean ± SEM of triplicate analysis

Table 4 Stability studies on the second generation mutant II N 31

Generation	Biomass dry weight (gm/L)	Asperenone (mg/g)
I	13.12 ±1.5	60.40 ±0.02
II	12.56 ±1.9	60.45 ±0.01
III	12.65 ±1.6	61.45 ±0.03
IV	13.12 ±1.7	62.46 ±0.01
V	13.15 ±1.7	59.45 ±0.02
II N 31	13.25 ±1.4	60.44 ±0.04

IN 31= 2nd generation control. The values represented mean ± SEM of triplicate analysis

survival values 40% at 12th min in UV and 30% at 15th min in nitrous acid. Many other morphological changes like colony size, texture and colour difference were recorded in both UV and nitrous acid mutants. However enhanced asperenone production was observed in mutants generated by nitrous acid mutagenesis. This could be due to hydration of the spore prior to induction and facilitating penetration of chemical mutagen, resulting in increased permeability of cells to cause effective induction. The increase in yield of asperenone in mutant II N 31 obtained by second set of nitrous acid mutagenesis further suggests that the chemical mutagen/nitrous acid is more effective. A similar observation was made by [4], where after exposing the spores of *Aspergillus niger* to UV and nitrous acid mutagenesis, the nitrous acid mutant produced enhanced lipase production while the UV mutants did not. The probable reason here also being the hydration of the spores prior to nitrous acid exposure.

Data obtained in our study suggested that mutagenic treatments have caused changes in the biochemical characteristics of *Aspergillus niger* 1105. Detailed and in depth studies are in progress to precisely point the biochemical and genetic modification. Nevertheless, from the point of developing a process for the production of this enzyme inhibitor, the present study is significant in obtaining a stable mutant, which produces asperenone in significant quantities.

Acknowledgements

The authors thank the Director, CFTRI, Mysore, for providing the facilities and encouragement. CC & CMK are

thankful to the Council of Scientific and Industrial Research, India for the award of Senior Research Fellowship.

References

1. Markwell J, Frakes LG, Brott EC, Osterman J and Wager FW (1989) *Aspergillus niger* mutants with increased glucose oxidase production. Appl Microbiol Biotechnol 30: 166–169
2. Minjares-Carranco A, Trejo-Aguilar BA, Auilar G and Viniegra-Gonzalez G (1997) Physiological comparison between pectinase-producing mutants of *Aspergillus niger* adopted either to solid-state fermentation or submerged fermentation. Enzyme Microb Technol 21:25–31
3. Chelius MK and Wodzinski RJ (1994) Strain improvement of *Aspergillus niger* for phytase production. Appl Microbiol Biotechnol 41:79–83
4. Mala JGS, Kamini NR and Puvanakrishnan R (2001) Strain improvement of *Aspergillus niger* for enhanced lipase production. J Gen Appl Microbiol 47:181–186
5. Fiedurek J and Gromada A (1997) Screening and mutagenesis of molds for Improvement of the simultaneous production of catalase and glucose oxidase. Enzyme Microb Technol 20:344–347
6. Schewe T, Rapoport SM and Kuhn H (1986) Enzymology and physiology of Reticulocyte lipoxxygenase: comparison with other lipoxxygenase. Adv Enzymology 58:191–272
7. Prigge ST, Boyington JC, Faig M, Doctor KS, Gaffney BJ and Amzel LM (1997) Structure and mechanism of lipoxxygenases. Biochimie 79:629–636
8. Cornicelli JA and Trivedi BK (1999) 15-lipoxxygenase and its inhibition: a novel therapeutic target for vascular disease. Curr Pharm Res 5:11–20
9. Steinberg D (1999) At last, direct evidence that lipoxxygenase play a role in atherogenesis. J Clin Invest 31:831–883

10. Rao KCS, Divakar S, Rao AGA, Karanth NG and Sattur AP (2002) (a) Lipoygenase inhibitor from *Lactobacillus casei*. Biotechnol Lett 24:511–513
11. Rao KCS, Divakar S, Rao AGA, Karanth NG and Sattur AP (2002) (b) A lipoygenase inhibitor from *Aspergillus niger*. Appl Microbiol Biotechnol 58:539–542
12. Rao KCS, Divakar S, Babu KN, Rao AGA, Karanth NG and Sattur AP (2002) (c) Nigerloxin, a novel inhibitor of aldose reductase and lipoygenase with free radical scavenging activity from *Aspergillus niger* CFR-W-105. J Antibiotic 55:789–793
13. Rao KCS, Divakar S, Rao AGA, Karanth NG, Suneetha WJ, Krishnakantha TP and Sattur AP (2002)(d) Asperenone: an inhibitor of 15-lipoygenase and human platelet aggregation from *Aspergillus niger*. Biotechnol Lett 24:1967–1970
14. Jefferson WE (1967) (a) The isolation and characterization of asperenone, a new phenylpolyene from *Aspergillus niger*. Biochemistry 6:3470–3483
15. Jefferson WE (1967) (b) Steroids and other factors influencing the accumulation of asperenone and fermentation acids by *Aspergillus niger* in replacement cultures. Biochemistry 6:3484–3488
16. Schwartz LJ and Stauffer JF (1966) Three methods of assessing the mutagenic action of ultraviolet radiation on the fungus *Emericellopsis glabra*. Appl Microbiol 14: 105–109
17. Rabache M, Neumann J and Lavollay J (1974) Phenyl polyene D' *Aspergillus niger*: Structure et propriétés de L' asperubrol. Phytochemistry 13: 637–642
18. Ayer WA, Muir DJ and Chakravarty P (1996) Phenolic and other metabolites of *Phellinus pini*, a fungus pathogenic to pine. Phytochemistry 42: 1321–1324
19. Cihangir N (2002) Stimulation of gibberellic acid synthesis by *Aspergillus niger* in submerged culture using a precursor. World J Microbiol Biotechnol 18:727–729