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ORIGINAL PAPER

Enhanced production of gibberellin A_4 (GA_4) by a mutant of *Gibberella fujikuroi* in wheat gluten medium

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Abstract Mutants of *Gibberella fujikuroi* with different colony characteristics, morphology and pigmentation were generated by exposure to UV radiation. A mutant, Mor-189, was selected based on its short filament length, relatively high gibberellin A_4 (GA_4) and gibberellin A_3 (GA_3) production, as well as its lack of pigmentation. Production of GA_4 by Mor-189 was studied using different inorganic and organic nitrogen sources, carbon sources and by maintaining the pH of the fermentation medium using calcium carbonate. Analysis of GA_4 and GA_3 was done by reversed-phase high-performance liquid chromatography and LC-MS. The mutants of *G. fujikuroi* produced more GA_4 when the pH of the medium was maintained above 5. During shake flask studies, the mutant Mor-189 produced 210 mg l^{-1} GA_4 in media containing wheat gluten as the nitrogen source and glucose as the carbon source. Fed-batch fermentation in a 14 l agitated fermenter was performed to evaluate the applicability of the mutant Mor-189 for the production of GA_4. In 7-day fed-batch fermentation, 600 mg l^{-1} GA_4 were obtained in the culture filtrate. The concentration of GA_4 and GA_3 combined was 713 mg l^{-1}, of which GA_4 accounted for 84% of the total gibberellin. These values are substantially higher than those published previously. The present study indicated that, along with maintenance of pH and controlled glucose feeding, the use of wheat gluten as the sole nitrogen source considerably enhances GA_4 production by the mutant Mor-189.

Keywords *Gibberella fujikuroi* · GA_4 · Mutant · Wheat gluten · Fermentation

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Introduction

Gibberellins are an important group of isoprenoid phytohormones that occur in minute amounts in higher plants. They are involved in the development and regulation of different growth processes throughout the life cycles of plants. Different gibberellins (GAs) selectively affect different parts of the plants. To date, 136 GAs have been identified in plants, fungi and bacteria [21], but only a few of them possess biological activity. The most important bioactive gibberellins are GA_1, GA_3, GA_4 and GA_7, which belong to the group of "C_{19} gibberellins" and exhibit their effects during different stages of plant growth, such as seed germination, stem and petiole elongation, leaf expansion, flower induction, and growth of seed and fruit [6].

In addition to higher plants, certain fungi [21] and a few bacteria [13] also produce gibberellins. At present, species belonging to *Fusarium*, *Gibberella* (perfect stage of *Fusarium*), *Sphaceloma*, *Neurospora* and *Phaeosphaeria* have been reported to produce gibberellins. These fungi produce GA_3 and/or GA_4 as the final metabolite [21]. The production of gibberellins by bacteria was reviewed by Bottini et al. [5] but their reported concentrations are very low, normally in the range of nanograms per liter [14]. *G. fujikuroi* strains belonging to the mating population "C" are capable of producing gibberellins in industrially viable quantities [22, 28]. Although *G. fujikuroi* produces GA_3 as the main product, the fungus also produces its precursors gibberellin A_4 (GA_4) and gibberellin A_7 (GA_7) [29]. During the terminal steps of GA_3 biosynthesis, 1,2-GA_4 desaturase converts GA_4 to GA_7 [30] and a nonspecific P450 monooxygenase later oxidizes GA_7 to GA_3 [31]. Along with the gibberellins, *G. fujikuroi* produces other metabolites like sterols, carotenoids, bikaverin and lipids from acetyl-CoA, a common precursor.

Commercially, gibberellic acid (GA_3) is produced by selected strains of *G. fujikuroi* using aerobic submerged fermentation, although solid substrate fermentation has been investigated for its production [8, 19]. Production of GA_3 by *G. fujikuroi* is strongly influenced by dissolved oxygen [10], the type of nitrogen source, the carbon source and the pH of the fermentation medium [6].

GA_4 and GA_7 possess different bioactivities than GA_3 and are immediate precursors of GA_3. Commercially, GA_4 and GA_3 are available in the form of a mixture because it is difficult and uneconomic to separate them from each other [9], and they are more expensive than GA_3, probably because of lower yields in commercial fermentations.

The GA_{4+7} mixture primarily stimulates the flowering and elongation of fruit cells. Growers of apples, pears and grapes use the mixture of GA_4 and GA_7 to produce larger fruits and an early harvest. The amount of GA_4 and GA_7 used varies depending on the crop, but it is generally applied at levels of 5–50 mg l^{-1}. GA_{4+7} is used with "Golden Delicious" apples to effectively prevent abnormal cell divisions in the epidermal layer that lead to undesirable "russetting" [6]. Using GA_{4+7} in combination with benzyladenine enhances the post-production quality of tulip flowers [18]. It is reported that this mixture also increases the yield of hot pepper [1]. Application of this mixture prevents cold-induced leaf chlorosis in Eastern and hybrid lilies. The mixture of GA_{4+7} also promotes seed cone production in numerous *Pinaceae* species. This enables better seed production of economically important forest trees. GA_4 promotes fruit set of apples and it is also used for fruit thinning, to change fruit shape and size, to increase the individual fruit weight, to thicken skin and to prolong shelf life. During the last few years, commercial interest in the production of GA_4 and GA_7 has increased because of their horticulture uses.

A number of researchers have reported high GA_4- and GA_7-producing strains of *G. fujikuroi* that do not produce GA_3. Gibberellin A_4 (GA_4) was initially isolated and identified in culture filtrate of *Phaeosphaeria* sp. L487 [27]. However, a biosynthetic gene study revealed that GA_1 was the final gibberellin metabolite in *Phaeosphaeria* sp. L487 [15, 16]. Another fungus, *Sphaceloma manihoticola*, which causes superelongation disease in cassava [11, 33], produces GA_4 as the major gibberellin without any GA_3 and GA_7; however, the concentration of GA_4 in the culture filtrate of this fungus was less than 20 mg l^{-1} [25]. *Penicillium citrinum* KACC 43900, a newly isolated endophytic fungus of cereal plants, produced 6 μg l^{-1} GA_4 [17]. Another newly isolated fungus, *Fusarium proliferatum* KGL0401, from the root of *Physalis alkekengi* var. *frenchetii*, produced 17.3 μg l^{-1} GA_4 after 7 days of incubation in Hagem's medium [26]. A US patent [9] describes the production of GA_4 using *G. fujikuroi* LTB-1027 in which a mixture of equal quantities of GA_4 and GA_7 was obtained. This patent reports a total gibberellin concentration of 800 mg l^{-1}, where the ratio of GA_{4+7}: GA_3 was 4:1. The main aim of the present study was to investigate the production of GA_4 by Mor-189, a mutant of *G. fujikuroi* selected on the basis of morphological differences and its high GA_4 production.

Materials and methods

Microorganisms

Gibberella fujikuroi 1019, *G. fujikuroi* 665, *G. fujikuroi* 850 and *G. fujikuroi* 1035 were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (Pune 411008, India), and were maintained on potato dextrose agar (PDA) slopes and subcultured every 15 days.

Media

The parent strains and mutants were maintained on potato dextrose agar (HiMedia Mumbai, India) supplemented with 2 g l^{-1} yeast extract. Slopes were incubated at 28°C for 3–4 days and stored at 4°C. The regeneration agar used to grow the survivors after mutagenesis was composed of (values in g l^{-1}) KH_2PO_4 1.5, NaCl 0.5, $MgSO_4.7H_2O$ 0.2, $Na_2MoO_4{\cdot}2H_2O$ 0.05, yeast extract 3.0, glucose 30, soya peptone 3.0, bile salt 1.0, trace mineral solution 1 ml, and agar 20.0 at pH 6.8. The trace mineral solution contained (values in mg l^{-1}) H_3BO_3 100, $MnCl_2{\cdot}4H_2O$ 100, $ZnSO_4{\cdot}7H_2O$ 100, $FeCl_3{\cdot}6H_2O$ 100, $CaCl_2{\cdot}2H_2O$ 1,000, and $CuCl_2{\cdot}2H_2O$ 50, to which a few drops of HCl were added until the solution became clear.

The basal medium used for GA_4 production contained (values in g l^{-1}) KH_2PO_4 1.5, NaCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.2, $Na_2MoO_4{\cdot}2H_2O$ 0.05, defatted soyabean meal 9, glucose 60, and trace mineral solution 1 ml at pH 6.8. Other carbon sources substituted for glucose were used at 60 g l^{-1}, while nitrogen sources were substituted for defatted soyabean meal on an equal nitrogen basis.

Chemicals

GA_3 (G 7645) and GA_4 (G 7276) were purchased from Sigma Chemical Company (St. Louis, MO, USA), and were used as reference compounds for high-performance liquid chromatography (HPLC) and LC-MS analysis. The reference GA_4 and GA_3 were at least 90% pure. HPLC-grade acetonitrile, ammonium dihydrogen phosphate and phosphoric acid (AR grade) were from E. Merck (Mumbai, India). The media ingredients were from HiMedia

(Mumbai, India). Defatted soyabean meal and wheat flour were purchased locally.

Gibberellin analysis

GA_4 and GA_3 were analyzed using an HPLC purchased from Thermo Separation Products (Fremont, CA, USA). Reversed-phase C_{18}, Lichrospher 100, 5 μm particle size columns were used for analysis (Merck KGaA, Darmstadt, Germany). Detection was performed at 205 nm. Quantification was achieved by the external standard method using peak area. Samples were filtered through 0.2 μm membrane filters and directly injected into the HPLC using a 20 μl loop of a Rheodyne injector. If required, the samples were diluted to lower the concentration of GA_4 and GA_3 to below 300 mg l^{-1}. On-line spectral analysis of the GA_3 and GA_4 peaks was performed for the standard and the samples for peak purity confirmation using a UV3000 scanning detector (Thermo Separation Products) with PC1000 and Spectacle software.

A 125 × 4 mm RP C18 column was used to analyze the GA_3. The elution was performed using a mobile phase consisting of 20% acetonitrile in 5 mmol l^{-1} ammonium dihydrogen phosphate at pH 2.5 and a flow rate of 0.6 ml min^{-1}. GA_4 analysis was done using 60% acetonitrile and 1% acetone in 5 mmol l^{-1} ammonium dihydrogen phosphate at pH 2.5 and a flow rate of 1.0 ml min^{-1}, and a RP C18, 250 × 4 mm column. Identification of GA_4 was further confirmed by LC-MS (Waters Alliance 2695 separation module) under the following conditions: source temperature 150°C, desolvation temperature 350°C, collision energy 10 V, capillary voltage 3.51 kV, and polarity ES positive. The mobile phase used was 60% acetonitrile, 0.1% formic and 1% acetone at a flow rate of 1.0 ml min^{-1}. The same column was used for HPLC and LC-MS analysis. The reference Sigma GA_4 was injected at a concentration of 2 mg l^{-1} for comparison during the LC-MS analysis.

Glucose analysis

The glucose in the fermentation broth was estimated by the dinitrosalicylic acid (DNS) method [24].

Selection of the strain for GA_4 production

Tubes containing 5 ml basal liquid medium with soyabean meal (9 g l^{-1}) and glucose (60 g l^{-1}) were inoculated from fresh slopes of *G. fujikuroi* NCIM 1019, *G. fujikuroi* NCIM 850, *G. fujikuroi* NCIM 665, and *G. fujikuroi* NCIM 1035. The tubes were incubated at 28°C for 48 h on a rotary shaker at 220 rpm. The 5 ml liquid cultures were then transferred to 45 ml liquid medium in 250 ml Erlenmeyer flasks and incubated for 7 days as described above. At the end of incubation, the volume of the broth was adjusted to 50 ml with distilled water and filtered under vacuum. The filtrates were analyzed for pH, glucose, GA_3 and GA_4 as described above. The cell mass was washed with 50 ml distilled water under vacuum and the dry cell mass was estimated at 103°C. All of the experiments were done in triplicate.

Mutagenesis

The parent strain, *G. fujikuroi* (NCIM 1019), was grown in 25 ml liquid basal medium for 72 h, and then shaken at 220 rpm and 28°C. The mycelia were filtered over a sterile sintered glass funnel with a pore size of 50–100 μm (Borosil, Mumbai, India). The short mycelial fragments in the filtrate were counted using a hemocytometer, and 20 μl of this suspension were spread on regeneration agar plates, to which 250 mg l^{-1} Pravastatin were added to exert selection pressure. Pravastatin inhibits the synthesis of secondary metabolites because it is a specific inhibitor of hydroxy methyl glutaryl CoA (HMG-CoA) reductase. The plates were exposed to UV radiation from a germicidal lamp (Sankyo Denki Co. Ltd., Japan) at a distance of 10 cm for different time intervals, such as 3, 5, 7 and 10 s. The plates were incubated at 28°C for 5–6 days in the dark until colonies developed. Colonies with different growth characteristics and pigmentations were selected and transferred to PDA slopes after re-isolation.

The mutant Mor-189 obtained during the experimentation was deposited in the National Collection of Industrial Microorganisms, National Chemical Laboratory (Pune, India) as NCIM 1343.

Shake flask screening of mutants for GA_4 production

G. fujikuroi NCIM 1019 was previously selected as the first parent through a screening experiment. Tubes containing 5 ml basal liquid medium were inoculated from fresh slopes of the parent culture and the selected mutants generated during mutagenesis. The tubes were incubated at 28°C for 48 h on a rotary shaker at 220 rpm. Erlenmeyer flasks 250 ml in capacity and containing 45 ml of liquid medium were inoculated from the tube cultures. The flasks were incubated for 7 days and analyzed as described for the earlier experiment.

Effect of pH control on GA_4 production

The parent culture and selected mutants were grown in shake flasks as before. The pH of the medium was adjusted to 5.6 before inoculation. Separately autoclaved 150 mg $CaCO_3$ powder was added to the flasks before inoculation

to maintain the pH at around 5. After 7 days of incubation at 28°C and 220 rpm, the filtrates were analyzed for GA_3 and GA_4, whereas the washed cell mass was used to determine the dry mass.

Effects of different carbon sources on GA_4 production by the mutant Mor-189

Media containing different carbon sources were prepared with 9 g l^{-1} defatted soyabean meal as the nitrogen source. Separately autoclaved glucose, sucrose, soluble starch, insoluble corn starch, dextrin, maltodextrin, amylose, and amylopectin were used on an equal-carbon basis (equivalent to 24 g l^{-1} C) in different sets of flasks. Sterile $CaCO_3$ (150 mg) was added to all of the flasks. The flasks were inoculated with liquid culture of the selected mutant strain, Mor-189, grown in 5 ml liquid medium with respective sugars for 48 h at 28°C, 220 rpm. The flasks were incubated for 7 days and the filtrates were analyzed.

Preparation of wheat gluten

Fractionation of wheat flour to recover wheat gluten was achieved via a conventional laboratory-scale dough process [4]. Wheat flour dough was made and kept in cold water (10°C) for 15 min. This allowed hydration and resulted in gluten agglomeration. A handful of dough was held under running tap water. This dough was squeezed repeatedly under running water until it became a rubbery mass. This rubbery mass was considered to be wheat gluten and the yield was about 10% (w/w). The wet wheat gluten was lyophilized, powdered, and used in the fermentation medium as the nitrogen source. The nitrogen content of the prepared gluten was determined by a flash combustion method using a ThermoFinnigan 1112 series Flash EA elemental analyzer.

Effects of different nitrogen sources on GA_4 production by Mor-189

Liquid basal media containing different inorganic and organic nitrogen sources with an equal basis of nitrogen (equivalent to 0.55 g l^{-1} N) and glucose (24 g l^{-1} C) as the carbon source were prepared in 250 ml Erlenmeyer flasks. The initial pH of the medium was 5.6, and it was maintained above 5 by the addition of $CaCO_3$. Inorganic nitrogen sources like ammonium nitrate, ammonium sulfate and ammonium chloride, as well as organic nitrogen sources like yeast extract, soya peptone, wheat gluten, defatted cottonseed meal, peanut meal and soyabean meal were used on an equal nitrogen basis. The sterile medium, in 250 ml flasks, was inoculated with 5 ml of a liquid culture of the mutant Mor-189 grown for 48 h, and was incubated for 7 days at 28°C and 220 rpm. At the end of incubation period, the samples were analyzed for dry mass and GA_3 and GA_4 contents.

Fed-batch fermentation for GA_4 production by the mutant Mor-189

An agitated fermenter with a working volume of 10 l (Bioflow 110; New Brunswick Scientific Co., NJ, USA) was used for the production of GA_4 and GA_3 by the mutant Mor-189. Liquid basal medium with 4 g l^{-1} wheat gluten and 20 g l^{-1} initial glucose was used for fermentation. An inoculum (10% V/V, 18 g l^{-1} dry cell weight) grown in the same medium for 48 h was used. The fermentation was continued for 168 h. Aeration was performed at 0.5 volume per volume per min (VVM). The agitation rate was varied between 600 and 700 rpm depending upon the dissolved oxygen (DO_2) status. The amount of DO_2 was controlled using the automatic DO_2 controller, which increased or decreased the agitation speed to maintain the DO_2 at 40% air saturation. The pH was maintained at 7.0 by the addition of sterile 5 N NaOH. A separately autoclaved glucose solution (500 g l^{-1}) was fed as the carbon source as required during the fermentation at a predetermined sugar feed rate in order to maintain glucose-limiting conditions in the culture. Samples were withdrawn every 24 h and analyzed for dry mass, residual glucose, and GA_4 and GA_3 contents.

Results

Mutagenesis and screening

The strains of *G. fujikuroi* obtained from NCIM grew well in the fermentation medium with soyabean meal used as the nitrogen source, and the dry cell mass reached about 17 g l^{-1} in all of the cultures. The sugar uptakes in all of the liquid cultures of the four strains studied were similar. The strains produced between 5 and 62 mg l^{-1} gibberellin ($GA_3 + GA_4$) in the basal medium in shake flask cultures in 7 days. *G. fujikuroi* NCIM 1019 produced the maximal total gibberellin (62 mg l^{-1}) and 6 mg l^{-1} GA_4. *G. fujikuroi* 1019 produced only a few microconidia, while the other three strains produced large numbers of micro- and macroconidia on solid media as well as in liquid cultures. *G. fujikuroi* (NCIM 1019) was therefore selected as the parent to use to improve the strain used for GA_4 production. However, *G. fujikuroi* 1019 grew with long mycelial filaments that led to a highly viscous fermentation broth, accumulated a distinct orange water-insoluble pigment, and secreted a deep violet water-soluble pigment in the fermentation broth. During UV mutagenesis, colonies with a variety of morphological characteristics with respect to size,

margin, shape, surface appearance and pigmentation were generated. An exposure time of seven seconds was found to be suitable for achieving a 95% kill rate and was used in subsequent mutagenesis experiments. In our previous study [20] we reported on mutants that were selected based on their short filament length mycelia in the fermentation broth.

Gibberellin production by mutants

The selected mutant strains showed differences in their growth characteristics in the basal fermentation medium. Mor-25 and Mor-189 grew with short, thick, highly branched mycelia in liquid culture, and the broths had lower apparent viscosities than those for other strains and the parent. Table 1 shows that the mutant Mor-189 produced the maximal GA_4 plus GA_3 (412 mg l^{-1}), and that GA_4 accounted for 17% of this mixture. The GA_4 concentration (71 mg l^{-1}) in the broth of Mor-189 was considerably higher than those obtained for the other mutants and the parent. The growths of all of the mutants in basal medium were similar, and this showed that the specific gibberellin productivity of Mor-189 was also the highest (24.2 mg gibberellin g^{-1} dry cell weight) among the mutants studied. The mutant Mor-189 produced sixfold more GA_3 and 23-fold more GA_4 as compared to the parent *G. fujikuroi* NCIM 1019. Mor-189 was therefore selected for further optimization of gibberellin production.

Effect of pH control on GA_4 production

The growths of all of the studied mutants were similar in the shake flasks with or without $CaCO_3$ in terms of dry cell mass, although they showed differences in terms of growth characteristics and viscosity, similar to those seen in earlier experiments. The medium pH remained above 5 in the flasks to which $CaCO_3$ was added. It can be seen from Table 2 that maintaining the pH above 5 increased GA_4 production in all of the mutants studied. Table 2 also shows that maintaining the pH around 5.5 exerted a positive effect on the GA_4 production by almost all of the mutants without any increase in GA_3 production. This increase in GA_4 concentration was highest in the mutants Mor-25 and Mor-189. Although the ratio of GA_4 to total gibberellin was almost the same for all studied mutants, more GA_4 (94 mg l^{-1}) was produced by Mor-189 than the other mutants. Therefore, in subsequent shake flask experiments, the pH was maintained above 5 by adding $CaCO_3$.

Effects of different carbon sources on GA_4 production by the mutant Mor-189

The results presented in Table 3 show that changing the carbon source used hardly affected either the growth of Mor-189 or the GA_3 production. The ratio of GA_4 to total gibberellin varied from 2 to 30% depending upon the carbon source used. Mor-189 produced more GA_4 when glucose, dextrin starch, and sucrose were used in combination with soyabean meal, but produced much less GA_4 in media containing amylose, amylopectin and maltodextrin, although the growth remained relatively constant. There was no correlation between the nature (slowly utilizable or rapidly utilizable) of the carbon source and the ratio of the two gibberellins produced. Glucose was chosen as the best carbon source, as its use resulted in the production of

Table 1 GA_4 and GA_3 production by selected mutants of *G. fujikuroi* in 7-day shake flask experiments

Mutant	GA_4 (mg l^{-1})	GA_3 (mg l^{-1})	Total GA (mg l^{-1})	GA_4/total GA (%)	Dry cell mass (g l^{-1})
G. fujikuroi NCIM1019	3 ± 1	56 ± 2	59	5	18.1 ± 0.6
Car-1	12 ± 2.6	194 ± 4.5	206	5	18.2 ± 0.4
Mor-1	2 ± 0.6	298 ± 5.2	300	0.6	17.7 ± 0.4
Mor-25	45 ± 4.5	275 ± 6.5	320	14	18.2 ± 0.8
Mor-189	71 ± 4.3	341 ± 5.0	412	17	17.8 ± 0.3

Total GA refers to GA_3 + GA_4. Concentrations are averages of three flasks each ±SD

Table 2 GA_4 and GA_3 production by mutants of *G. fujikuroi* in medium with $CaCO_3$ for pH control in 7-day shake flask experiments

Mutant	GA_4 (mg l^{-1})	GA_3 (mg l^{-1})	Total GA (mg l^{-1})	GA_4/total GA (%)	Dry cell mass (g l^{-1})
G. fujikuroi NCIM 1019	6 ± 1.0	71 ± 3.6	77	7.7	19.1 ± 0.4
Car-1	20 ± 2.6	200 ± 6.5	220	9	17.8 ± 0.3
Mor-1	10 ± 1.7	300 ± 6.0	310	3.2	17.7 ± 0.2
Mor-25	80 ± 2.6	300 ± 5.0	380	21	18.2 ± 0.2
Mor-189	94 ± 3.6	350 ± 8.5	444	21	18.1 ± 0.3

Total GA refers to GA_3 + GA_4. Concentrations are averages of three flasks each ±SD

Table 3 Effects of different carbon sources on GA_4 and GA_3 production by mutant Mor-189 in 7 day shake flask experiments

Carbon source	GA_4 (mg l^{-1})	GA_3 (mg l^{-1})	Total GA (mg l^{-1})	GA_4/total GA (%)	Dry cell mass (g l^{-1})
Amylose	10 ± 3	300 ± 10	310	3	17.5 ± 0.2
Amylopectin	9 ± 2	247 ± 7.0	356	2	17.2 ± 0.2
Corn starch (insoluble)	70 ± 4.5	248 ± 9.1	318	22	18.0 ± 0.4
Dextrin type II	82 ± 3.4	211 ± 2.6	293	28	17.9 ± 0.1
Dextrin type III	89 ± 2	200 ± 4.5	289	30	16.9 ± 0.1
Glucose	92 ± 3	330 ± 6.0	422	22	17.1 ± 0.2
Maltodextrin	7 ± 1	240 ± 11.1	241	3	17.4 ± 0.3
Starch	80 ± 8.1	324 ± 10.4	404	19	18.1 ± 0.4
Sucrose	68 ± 1	290 ± 8.7	358	19	16.8 ± 0.3

Total GA refers to $GA_3 + GA_4$. Concentrations are averages of three flasks each ±SD

Table 4 Effect of different nitrogen sources on GA_4 and GA_3 production by the mutant Mor-189 in 7 day shake flask experiments

Nitrogen source	GA_4 (mg l^{-1})	GA_3 (mg l^{-1})	Total GA (mg l^{-1})	GA_4/total GA (%)	Dry cell mass (g l^{-1})
Ammonium chloride	2 ± 1.0	240 ± 5.5	242	0.8	14.3 ± 0.3
Ammonium nitrate	9 ± 1.0	205 ± 4.3	214	4	13.9 ± 0.3
Ammonium sulfate	7 ± 1.0	200 ± 4.5	207	3	14.1 ± 0.3
Soyapeptone	3 ± 1.0	61 ± 5.5	64	4.5	19.1 ± 0.4
Yeast extract	5 ± 1.0	40 ± 3.4	45	11	18.2 ± 0.2
Cottonseed meal	25 ± 2.0	297 ± 10.1	322	7.7	17.9 ± 0.3
Peanut meal	26 ± 1.7	308 ± 10.6	334	7.8	18.1 ± 0.3
Soyabean meal	40 ± 3.4	331 ± 7.2	371	10.7	19.1 ± 0.4
Wheat gluten	210 ± 8.7	73 ± 4.6	283	74	20.0 ± 0.3

Total GA refers to $GA_3 + GA_4$. Concentrations are averages of three flasks each ±SD

considerably higher levels of total gibberellin (422 mg l^{-1}) and GA_4 (92 mg l^{-1}), although the ratio of GA_4 to total gibberellin was 22%.

Effect of nitrogen sources on GA_4 production by Mor-189

The growth of Mor-189 and the production of GA_4 dropped slightly in inorganic nitrogen media (Table 4), presumably because some of the nutrients available in low quantities in complex media were not available in the medium with inorganic nitrogen sources. Although defatted plant meals, yeast extract and soya peptone resulted in rapid growth, they did not support satisfactory GA_4 production. It was observed that Mor-189 produced the highest level of GA_4 (210 mg l^{-1}) in wheat gluten medium, and the proportion of GA_4 in this medium to total gibberellin was 74%, which was significantly higher than seen in all other experiments. Amongst all of the studied nitrogen sources, the highest level of GA_4 was produced by Mor-189 using wheat gluten as the nitrogen source. This high GA_4 production was not simply an effect of differences in cell mass in the shake flasks, because the difference between the dry cell masses generated with different organic nitrogen sources was only marginal. A liquid basal medium with wheat gluten and glucose was subsequently used for GA_4 production.

Fed-batch fermentation for GA_4 production by the mutant Mor-189

Mor-189 grew in the desired short mycelial form in the 10 l fermenter liquid broth in a similar manner to that observed in the shake flasks. The results of batch fermentation using the mutant Mor-189 are presented in Figs. 1 and 2. Mor-189 grew rapidly during the growth phase and reached a dry cell mass of 21 g l^{-1} in 24 h, and then it leveled off until 168 h (when the fermentation was terminated). The mycelium grew in the form of short, thick filaments with very little pigmentation. The DO_2 was maintained in the range 40–50% air saturation during the fermentation time by adjusting the agitation rate. The production of gibberellins began after 20 h, when the increase in the cell mass slowed down. Gibberellins are produced by *G. fujikuroi* only after strict nitrogen limitation is achieved in the nutrient medium [7]. Also, in the present investigation, the gibberellins were only detected after the culture had

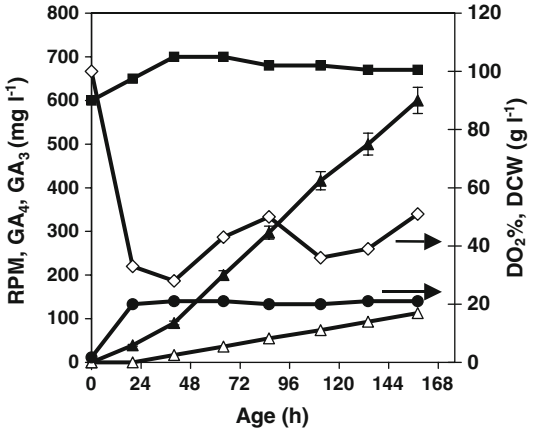

Fig. 1 Production of GA_3 and GA_4 by Mor-189 in a 14 l agitated fermenter. *Filled squares*, agitation speed in rpm; *open diamonds*, DO_2 in % air saturation; *filled circles*, dry cell weight in g l^{-1}; *filled triangles*, GA_4 concentration in mg l^{-1}; *unfilled triangles*, GA_3 concentration in mg l^{-1}

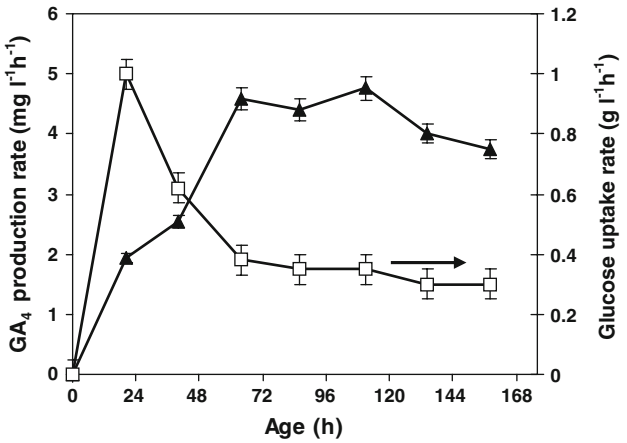

Fig. 2 Performance of mutant Mor-189 in terms of the glucose uptake rate and the GA_4 production rate in a 14 l agitated fermenter. *Filled triangles*, GA_4 in mg l^{-1} h^{-1}; *open squares*, glucose uptake rate in g l^{-1} h^{-1}

reached the stationary stage, as seen from the stable dry cell mass.

The glucose concentration reached zero from an initial level of 20 g l^{-1} during the first 20 h, and the average glucose utilization rate in the fermenter was 1.0 g $l^{-1}h^{-1}$. Most of the glucose used in this period was utilized for the growth of the fungus, because gibberellins were not detected during the first 20 h. After 24 h, a 500 g l^{-1} glucose solution was fed in in one pulse to achieve a concentration of 10 g l^{-1}. The sugar feeding mode was changed to a continuous mode so that the culture experienced glucose-limiting conditions. Between 24 and 40 h, the glucose uptake rate declined to 0.62 g l^{-1} h^{-1}. Glucose utilization later decreased slowly to 0.35 g l^{-1} h^{-1} at 65 h and then remained nearly constant until the end of the batch fermentation. Irrespective of the decline in glucose uptake rate, the GA_4 production rate remained almost the same, and so there was no direct correlation between glucose utilization rate and GA_4 production rate. This is understandable considering that the overall yield of secondary metabolites per gram of sugar fermented is normally very poor. Between 20 and 40 h, the GA_4 production rate was 2.6 mg l^{-1} h^{-1}, which increased to 4.6 mg l^{-1} h^{-1} at 60 h and remained nearly the same until 120 h. Presumably the culture reached high levels of nitrogen limitation and so the repression caused by the nitrogen source decreased further, which led to an increased rate of GA_4 production. The specific GA_4 productivity in the stationary phase of the culture was 0.2 mg g^{-1} DCW h^{-1}, and the specific glucose uptake rate was 16 mg g^{-1} DCW h^{-1}. However, the rate of GA_4 production started to decline after 5 days and reached 3.8 mg l^{-1} h^{-1}, probably because of culture aging. The concentration of GA_4 finally reached 600 mg l^{-1} in 168 h. The combined concentration of the two gibberellins GA_4 and GA_3 finally reached 713 mg l^{-1}, with GA_4 accounting for 84% of the total gibberellin. The production of GA_4 by Mor-189 in the fermenter was 2.8 times higher than that observed in the shake flask, although there was no visible increase in the dry cell mass. This can probably be attributed to better mass transfer in the agitated fermenter as compared to the shake flask.

Analysis of gibberellins

The methods used to analyze the gibberellins were able to satisfactorily resolve GA_3 and GA_4. A 250 mm column was more suited to the analysis of GA_4. Barendse et al. [2] and Gallazzo and Lee [9] have described the chromatographic separation of GA_7 and GA_4. These earlier investigators mentioned that, under the chromatographic conditions they used, GA_7 eluted just before GA_4, with a retention time difference of about a minute. The chromatographic conditions that we used in the present study were similar to those described by these investigators, and so we expected a peak from GA_7 just before the GA_4 peak.

In the present investigation, GA_4 eluted at 12.20 min (Fig. 3). The retention volume of GA_4 was 12.2 ml. In the sample broth, two peaks eluted at 10.38 and 12.26 min, respectively. Online spectral scanning performed between 200 and 350 nm in steps of 5 nm showed a peak purity index of 99% for the standard GA_4, and exhibited maximal absorbance at 205 nm. The peak at 12.26 min in the chromatogram of the sample broth had an identical spectral pattern to the standard GA_4 peak at 12.20 min. The small peak at 10.38 min in the chromatogram of the sample broth exhibited different spectral behavior and was probably not

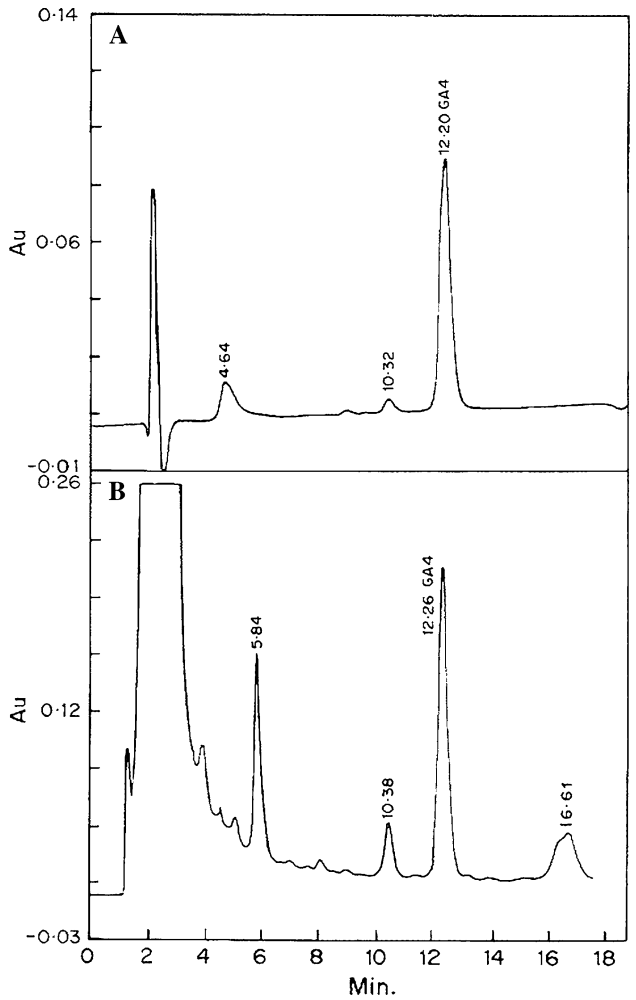

Fig. 3 **a**, **b** Analysis of fermentation broth for GA_4 by HPLC. The analysis was done on an RP C_{18} Lichrospher 100, 250 × 4 mm column with a mobile phase consisting of 60% acetonitrile and 1% acetone in 5 mmol l^{-1} ammonium dihydrogen phosphate at pH 2.5 and at a flow rate of 1.0 ml min^{-1}. Detection was performed at 205 nm. **a** Reference injection; **b** sample injection

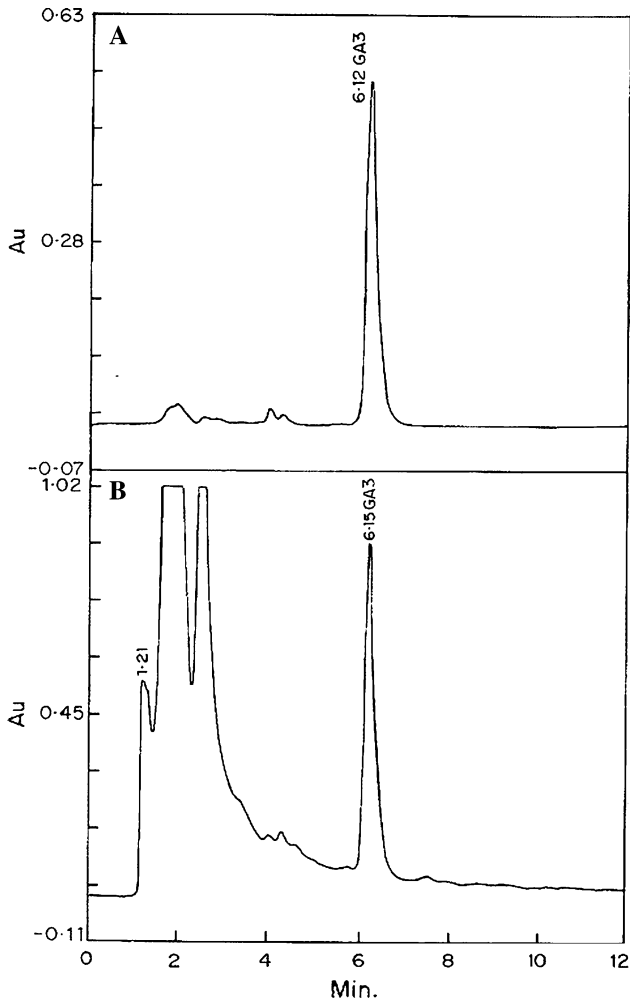

Fig. 4 **a**, **b** Analysis of the fermentation broth for GA_3 by HPLC. The analysis was done on an RP C_{18} Lichrospher100, 125 × 4 mm column with a mobile phase consisting of 20% acetonitrile in 5 mmol l^{-1} ammonium dihydrogen phosphate at pH 2.5 and at a flow rate of 0.6 ml min^{-1}. Detection was performed at 205 nm. **a** Reference injection; **b** sample injection

a gibberellin. Thus, the fermentation broth was substantially free of GA_7.

The analysis of GA_4 was confirmed by LC-MS. Analysis in ES positive mode gave (*m/z*) 333.28 (M + 1), 269.13, 315.18.

Figure 4 shows chromatograms of the reference gibberellic acid (GA_3) and of the fermentation broth of mutant Mor-189. The GA_3 eluted at 6.12 min. The retention volume of the GA_3 peak was 3.6 ml under the present experimental conditions.

Discussion

GA_4 exhibits very high biological activity in terms of promoting fruit growth, appearance and shelf life in fruits with high commercial value like apples, peas, and grapes. Although these biological activities of GA_4 are well documented, its commercial use in agriculture has remained rather limited compared to GA_3, presumably because of limited availability of the product and its very high cost in the market. Procedures for obtaining GA_4 and a mixture of GA_4 and GA_7 by fermentation using *G. fujikuroi* have been patented [9, 12, 32], but little information has been published regarding the production of GA_4 in sufficient quantities.

In our previous study [20], we reported on the use of morphological mutants that have short mycelial lengths in liquid cultures, which led to better oxygen transfer and increased production of GA_3. Mor-189, used in the present investigation, is also a morphological mutant, similar to the mutants described earlier, and has the advantages of a low

viscosity because of the short length of its mycelium and an increased gibberellin yield. Giordano and Domench [10] have described how oxygen availability causes differences in the biosynthesis of fats, pigments and gibberellins by *G. fujikuroi*. They reported that increased oxygen transfer increased the biosynthesis of gibberellins by *G. fujikuroi*. The use of mutants with short mycelial lengths and low viscosities permits the utilization of more concentrated media, and, in turn, a higher cell mass in the fermenter, which can result in a higher volumetric gibberellin productivity.

In shake flasks, it is normally difficult to maintain the pH at any desirable value. The use of sterile $CaCO_3$ in shake flasks allowed us to maintain the pH in the shake flask culture above 5. Borrow et al. [3] reported that the growth and gibberellin production of *G. fujikuroi* were fairly constant over the pH range 4–7, but the composition of resulting gibberellin mixture depended significantly on the pH value. They reported that, GA_3 was the main product at a low pH, while the concentrations of GA_4 and GA_7 were higher at neutral pH. Although maintaining the pH at 5.6 is reported to be beneficial in GA_4 production by mutants selected specifically for its production [9], in the present investigation, maintaining the pH above 5 resulted in marginal increase in the proportion of GA_4 produced by the parent and all the mutants studied. In our earlier investigation [20], we observed that the GA_3 production rate increased immediately after the culture entered the stationary phase, and that the rate lowered considerably later on. In the present investigation, a similar effect was also observed (data not shown). The drop in the GA_3 production rate was assumed to be due to the drop in pH, cell mass aging or catabolite repression. By controlling the pH and carefully controlling the glucose feeding rate so as to achieve glucose limiting conditions during the stationary phase, we could maintain the GA_4 production rate above 4 mg l^{-1} h^{-1} over a considerable length of time between 60 and 144 h, and this resulted in a very high GA_4 concentration in the fermentation broth, which has not been reported previously [9, 11, 17, 25, 26].

In the present study, organic nitrogen sources like plant meals and wheat gluten were found to enhance GA_3 and GA_4 production by mutants of *G. fujikuroi*. In a medium in which wheat gluten was used as the sole nitrogen source, the mutant Mor-189 produced substantially higher quantities of GA_4, and its proportion increased to 74%. Thus, wheat gluten was found to be suitable for GA_4 production by Mor-189.

The genetics and biochemistry of gibberellin production in *G. fujikuroi* has been well studied in recent years. It has been demonstrated that the expression of genes coding for the desaturase involved in the conversion of GA_4 to GA_7 and then to GA_3 share a common regulation that is mediated by the nitrogen catabolite regulatory protein AreA [23]. Our results suggest that the increase in the production of GA_4 is probably due to a substance present either in wheat gluten or produced from wheat gluten during the growth of *G. fujikuroi*. This inhibition was not an effect of the oxygen availability, because in shake flask cultures, as well as in the fermenter, the dry cell masses and mycelial morphologies were similar in media with wheat gluten and other nitrogen sources. This is the first report of the use of wheat gluten to enable the substantially high production of GA_4 by *G. fujikuroi*. Transferring the process from the shake flask level to a 10 l agitated fermenter allowed better control over the growth and GA_4 production by the mutant Mor-189, and we successfully increased the GA_4 concentration from 200 mg to 600 mg l^{-1} in the same fermentation time.

The mutant Mor-189 is deposited in the National Collection of Industrial Microorganisms, National Chemical Laboratory (Pune, India) as NCIM 1343. The other mutants (Car-1, Mor-1 and Mor-25) have also been deposited in the NCIM, as described earlier [20].

Conclusion

In this study, we successfully improved the strain of *Gibberella fujikuroi* used for gibberellin (GA_4) production by mutagenesis and media optimization. The study indicated that, along with the pH, the nitrogen source used was a factor that significant affects GA_4 production by the mutant Mor-189. 2.8-fold more GA_4 was produced in wheat gluten medium after 168 h of fermentation. Studies to define the exact role of wheat gluten in the enhanced GA_4 production exhibited by the Mor-189 mutant of *G. fujikuroi* are in progress.

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