Chapter 5

Gibberellin $A_4$ Production
Abstract:

This chapter describes screening of strains of *Fusarium fujikuroi* obtained from NCIM and mutants selected for higher gibberellin production, for their ability to produce GA₄. The basal medium used for screening of GA₄ producing strain was same as the medium (LMF), optimized for GA₃ production. Mut189, selected for maximal GA₂ production was subsequently chosen based on short filament morphology, relatively higher Gibberellin A₄ (GA₄), total gibberellin production and lack of pigmentation. Mutant Mut189 was investigated further to enhance GA₄ production by optimizing medium constituents and environmental conditions. Under two different culture conditions, Mut189 worked in two different ways. It produced GA₄ as the major metabolite and a small amount of GA₃ as co-metabolite in medium containing wheat gluten as nitrogen and glucose as carbon source. While it produced GA₃ as the major metabolite and small amount of GA₄ as co-metabolite in media containing defatted soyabean meal as nitrogen and glucose as carbon source. The type of gibberellin produced by the Mut189 was dependent on the type of organic nitrogen provided in the medium, in addition to the pH of the fermentation broth. This is the first report of use of wheat gluten for substantially high production of GA₄ by *F. fujikuroi*. 
Chapter 5

5.1 INTRODUCTION

*Fusarium fujikuroi* is the most prolific producer of the gibberellins and GA3 is usually the major gibberellin produced. The most important bioactive gibberellins are GA1, GA3, GA4 and GA7. They belong to “C19-gibberellins” group and exhibit their effect during different stages of plant growth like seed germination, stem and petiole elongation, leaf expansion, flower induction as well as growth of seed and fruit (Bruckner and Blechschmidt, 1991). At present, species belonging to *Fusarium, Speceloma, Neurospora* and *Phaseosphaeria* have been reported to produce gibberellins. These fungi produce GA3 and/or GA4 as final metabolite (MacMilan, 2002). Production of gibberellins by bacteria has been reviewed by Bottini et al., (2004) however; reported concentrations are very low and in the range of nanograms per liter (Kang et al., 2009).

Ascomycetous fungus *F. fujikuroi* belonging to mating population “C” is capable of producing gibberellins in industrially viable quantities (Takahashi et al., 1991; Malonke et al., 2005). Although the main product of gibberellin biosynthesis in *F. fujikuroi* is GA3, it produces its precursors gibberellin A4 (GA4) and gibberellin A7 (GA7) also (Tudzynski, 1999). GA4 and GA7 are also important gibberellins because they possess bioactivities different than GA3 and are immediate precursors of GA3. Usually the published reports on gibberellin biosynthesis have been focused mainly on the screening of microbial strains which produce GA3 with high yield and productivity followed by the optimization of process parameters in batch and fed-batch fermentation. Very few researchers have reported high GA4 and GA7 producing strains of *F. fujikuroi* that do not produce GA3, indicating that the desaturase, for conversion of GA4 to GA7, and the 13-hydroxylase, for conversion of GA7 to GA3, are inactive or missing in these fungi (Rademacher, 1992).

During last few years, commercial interest for production of GA4 has increased because of its horticulture uses. The requirement of the amount of GA4 varies, depending on the crop but is generally applied in the range of 5 to 50 mg/l. Gibberellin A4 (GA4) was isolated and identified in culture filtrate of *Phaseosphaeria* sp. L487 by Sassa and Suzuki (1989). Improved gibberellin fermentation and biosynthetic gene study revealed that not GA4 but GA1 is the final metabolite in this species (Kawaide and Sassa, 1993). Another fungus *Sphaceloma manihoticola*,
causing “super elongation” disease of cassava, produces GA₄ as the major gibberellin component (Zeigler et al., 1979) without producing GA₃ and GA₇. The flanking genes of the *F. fujikuroi* gene cluster are absent in *Sphaceloma*. As a consequence, the biosynthetic pathway ends with GA₄ instead of being further converted to GA₇, GA₁, and GA₃, as occurs in *F. fujikuroi* (Bomke et al., 2008).

The concentration of GA₄ in the fermentation broth of *Sphaceloma manihoticola* however, was only 7 mg/l (Graebe and Rademacher, 1979) and 20 mg/l (Rademacher, 1992) respectively. *Penicillium citrinum* KACC 43900, an endophytic fungus of cereal plants produces 6.03 microgram/l GA₄ (Khan et al., 2008). Another fungus, *F. proliferatum* KGL0401 isolated from the root of *Physalis alkekengi var. frencheti* produced 17.3 microgram l⁻¹ GA₄ after seven days of incubation in Hagem’s medium (Rim et al., 2005). A US patent (Gallazzo and Lee, 2001) describes production of GA₄ using *F. fujikuroi* LTB-1027 in which a mixture of equal quantities of GA₄ and GA₇ were obtained. This patent reports 800 mg/ltotal gibberellin concentration, in which the ratio of GA₄+7: GA₃ was 4:1. As GA₄ and GA₇ are produced in lower yield by commercial fermentations, these gibberellins are about 300 times expensive than GA₃ and therefore are not widely used in agriculture and horticulture. Commercially, they are available in the form of a mixture because their separation from each other is difficult and uneconomic (Gallazzo and Lee, 2001).

The mixture of GA₄+7 primarily stimulates flowering and elongation of fruit cells. Growers of apples, pears and grapes use mixture of GA₄ and GA₇ to produce larger fruits and an early harvest. GA₄+7 mixture is used on “Golden Delicious” apples to effectively prevent abnormal cell divisions in the epidermal layer that lead to undesirable “russetting” (Bruckner and Blechschmidt, 1991). GA₄+7 in combination with benzyl-adenine enhance post-production quality of tulip flowers (Kim and Millar, 2009). It is reported that this mixture also increases yield of hot pepper (Batlang, 2008). Application of this mixture prevents cold-induced leaf chlorosis in Eastern and hybrid lilies. GA₄+7 mixtures also promote seed cone production in numerous *Pinaceae* species. This enables a better seed production for economically important forest trees. GA₄ promotes fruit set of apples and it is also used for fruit thinning, changing fruit shape and size, increasing weight of single fruit, thickening
skin and prolonging the shelf life. During last few years, commercial interest for production of GA_4 and GA_7 has increased because of their horticulture uses.

Amount and the type of gibberellins produced by *F. fujikuroi* is dependent on the genetic constitution of the strain and fermentation conditions like aeration, type of carbon and nitrogen source and pH of the fermentation medium. The main product of gibberellin biosynthesis in *F. fujikuroi* is GA_3, with traces of GA_4 and GA_7. Certain horticultural applications require specifically GA_4. Commercially it is available in the mixture form as GA_4+7 and highly expensive. Very little information has been published describing the production of GA_4 by *F. fujikuroi* and the fermentation conditions. Therefore strains that produce GA_4 in the absence of GA_3 and GA_7 would have considerable importance.

The present chapter describes screening of wild strains of *Fusarium fujikuroi* obtained from NCIM and mutants selected for higher GA_3 productivity for their ability to produce GA_4 and identification of medium constituents and environmental conditions for effective production of gibberellin A_4 (GA_4).

**5.2 MATERIAL AND METHODS**

**5.2.1 Microorganisms**

Four fungal strains namely *F. fujikuroi* NCIM 665, *F. fujikuroi* NCIM 850, *F. fujikuroi* NCIM 892, *F. fujikuroi* NCIM 1019 were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. *F. fujikuroi* mutants, Carl, Mut4, Mut65, Mut189 and Mut226 which were generated during strain improvement programme for increased gibberellin production from glucose, were also used in the present investigation. During the course of study, these cultures were maintained on PDA agar slants (HiMedia Mumbai, India) supplemented with yeast extract at 2 g/l. All media ingredients and analytical methods were same as described in chapter 2 and 3 unless otherwise mentioned.

**5.2.2 Media**

All media ingredients were purchased from HiMedia, Mumbai India. Wheat flour was purchased locally.
1) Potato Dextrose agar (PDA)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Conc. g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato infusion</td>
<td>200</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
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2) Liquid Medium for Fermentation (LMF)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
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</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>Defatted soybean meal</td>
<td>9.0</td>
</tr>
<tr>
<td>Trace mineral solution</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
| pH                       | 6.8 | before autoclaving

3) Trace Mineral Solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·7H₂O</td>
<td>1.0</td>
</tr>
<tr>
<td>MnCl₂·7H₂O</td>
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</tr>
<tr>
<td>CuCl₂·7H₂O</td>
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</tr>
<tr>
<td>FeCl₂</td>
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</tr>
<tr>
<td>H₃BO₃</td>
<td>0.1</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Dissolve the ingredients and add few drops of concentrated HCl till solution becomes clear
4) Liquid Fermentation Medium (LFM)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>20.0</td>
</tr>
<tr>
<td>Trace mineral solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.8 before autoclaving</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Flow Sheet of Work

Screening of Fusarium Strains and Mutants of F. fujikuroi for Production of GA₄

Effect of pH Control on GA₄ Production

Screening of Different Nitrogen Sources for GA₄ Production

Fractionation of Wheat Flour by Laboratory-Scale Dough Process for Wheat Gluten preparation

Utilization of Carbon Sources for Growth and GA₄ Production

GA₄ Production in 10 L Fermenter
5.2.3 Culture conditions

5.2.3.1 Tube cultures

In case of tube cultures, a small mycelial mat from fresh slant culture was suspended in 2 ml sterile physiological saline and teased with a straight inoculating wire and inoculated into 5 ml liquid media in 150 × 25 mm test tubes without rim and the tubes were incubated at 28 °C, 220 rpm, for 48 h.

5.2.3.2 Shake flask cultures

Throughout the experiments for shake flask cultures, 45 ml liquid fermentation medium in 250 ml Erlenmeyer flasks was used. Five ml seed culture grown, as above, was used to inoculate the 45 ml sterile medium thus making the total initial volume 50 ml. The basal medium used for GA₄ production was same as the medium optimized for GA₃ production. A variety of carbon sources were screened for their effect on type of gibberellin produced by the selected mutant and substituted for glucose at 60 g/l and other nitrogen sources substituted for defatted soyabean meal were on equal nitrogen basis. Carbon sources were autoclaved separately for all the experiments and later mixed with other media components. All shake flask experiments were performed in duplicate, for 168 h, unless otherwise mentioned. At the end of the incubation period, final volume of the culture broth was adjusted to 50 ml by sterile distilled water to compensate for evaporative loss of water during incubation. Care was taken that the shake flask cultures do not remain stationary even for a few minutes during sample withdrawal from the flasks.

5.2.4 Screening of cultures for production of GA₄

The cultures obtained from NCIM and the mutants selected for higher GA₃ production were studied for GA₄ production by inoculating respective cultures in 250 ml Erlenmeyer flasks with 50 ml LMF and incubating the flasks at 28 °C, for 168 h. At the end of 168 h incubation, volume of the broth was adjusted to 50 ml with distilled water. At the end of incubation, the culture broth was filtered over Whatman No 1 qualitative filter paper circle under vacuum. The filtrate was collected and stored. The cell mass residue was washed with three volumes of distilled water, observed for pigmentation and used for dry cell weight (DCW) determination at 103
The fermented broth filtrate was analyzed for pH, residual sugar content and GA₄ and GA₃ concentration by two separate high performance liquid chromatography (HPLC) methods described earlier (Chapter 2).

### 5.2.5 Effect of pH on GA₄ production

During the experiments described above, pH of the broth decreased to 2.5. Therefore possibility of decrease in GA₄ production due to decrease in pH was investigated by addition of sterilized 3 g/l calcium carbonate to the flask at the time of inoculation to avoid lowering of pH. The flasks were incubated at 28 °C, for 168 h and samples were analyzed as described earlier. Calcium carbonate powder was weighed in Erlenmeyer flask and autoclaved separately before addition of sterile liquid medium for fermentation. The unutilized CaCO₃ was dissolved off by the addition of 10N HCL. This acid addition was also for the correct estimation of the dry cell mass and converting the Ca-gibberellic acid, if any, into free gibberellic acid.

### 5.2.6 Preparation of wheat gluten

Fractionation of wheat flour to recover wheat gluten was done by a conventional laboratory scale dough process (Borght et al., 2005). Wheat flour dough was made from very fine wheat flour 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, ground finely and used in the fermentation medium as nitrogen source. The nitrogen content of prepared gluten was determined by flash combustion method using Flash EA, 1112 series, Thermo Finnigan elemental analyser. Steps involved in preparation of wheat gluten are summarized in Fig. 5.1.
Chapter 5

Fractionation of Wheat Flour

Conventional Laboratory Scale Dough Process

- Dough in Cold Water (10 °C) for 15 min
- Squeezed Under Running Tap Water
- Rubbery Mass obtained
- Wheat Gluten Yield 10% (w/w)
- Lyophilized and Powdered
- Used as Nitrogen Source

Figure 5.1 Preparation of wheat gluten by conventional laboratory scale dough process

5.2.7 Choice of carbon source for GA₄ production by mutant Mut189

Utilization of carbon sources namely glucose, sucrose, soluble starch, corn insoluble starch, dextrin, maltodextrin, amylase and amylopectin was investigated for growth and GA₄ production by mutant Mut189. LMF media containing different carbon sources were prepared with 20 g/l wheat gluten as the nitrogen source. The carbon sources (equivalent to 24 g C kg⁻¹) were autoclaved separately and later added to remaining constituents of LMF. To investigate effect of control of pH around 5, sterile CaCO₃ (150 mg) was added to the flasks. The flasks were inoculated with seed culture of selected mutant strain Mut-189, grown in 5 ml liquid fermentation
medium with respective sugars for 48 h at 28 °C, 220 rpm. The flasks were incubated for 168 h at 28 °C, 220 rpm. Samples were analyzed for dry cell weight (DCW), pH, residual sugar as well as GA₄ and GA₃ concentration by high performance liquid chromatography (HPLC).

5.2.8 Screening of different nitrogen sources for GA₄ production by Mutl89

The shake flask experiments were performed to evaluate the nitrogen sources for gibberellin (GA₃ and GA₄) production by Mutl89 strain. The effect of nitrogen source was studied in the LMF media with different inorganic and organic nitrogen sources on equal basis of nitrogen (equivalent to 0.55 g/lN) and glucose (24 g/l C) as carbon source. The inorganic nitrogen sources viz. ammonium nitrate, ammonium sulphate, and ammonium chloride and organic nitrogen sources viz. yeast extract, soya peptone, wheat gluten, defatted cottonseed meal, peanut meal and soyabean were surveyed for mutant Mutl89 on which it could grow and produce GA₄. Wheat gluten used was prepared as described above in 5.2.5. The 50 ml media with respective nitrogen sources in 250 ml Erlenmeyer flasks were inoculated with 48 h respective seed cultures described earlier. The shake flasks were incubated at 28 °C, 220 rpm on rotary shaker for 168 h. Samples were analyzed for pH, dry cell mass, GA₃ and GA₄ concentration by HPLC.

5.2.9 Evaluation of GA₄ production in 10 L fermenter by mutant Mutl89

Fermentation batches were carried out with mutant Mutl89 to evaluate fermentation conditions for GA₄ production in optimized liquid fermentation medium (LFM). A small mycelial mat of mutant Mutl89 from fresh slant culture was suspended in 2 ml sterile physiological saline and teased with a straight inoculating wire and inoculated into 5 ml liquid media in 150 × 25 mm test tubes without rim and the tubes were incubated at 28 °C, 220 rpm, for 48 h. This 5 ml seed culture was transferred into a 250 ml Erlenmeyer flask containing 45 ml LFM and incubated at 28 °C for 48 h. Twenty two such flasks each with 45 ml LMF were prepared and inoculated to produce 1 L inoculum necessary for inoculation of the 10 L fermenter. Ten percent (v/v) of the above seed culture, grown in multiple flasks was then transferred into a fermenter.
The fermentation experiments were performed in 14 L laboratory fermenter (Bio­
flow 110, New Brunswick Scientific, USA) with a working volume of 10 L, 
equipped with devices for control and measurement of pH, temperature, dissolved 
oxygen and agitation speed. Temperature was maintained at 28 °C by automatic 
heating or circulating chilled water. The DO₂ was measured with the help of a 
dissolved oxygen probe (Mettler Toledo). Online fermentation data of process 
parameters such as DO₂, pH, agitation and feeding strategy was acquired using NBS 
BioCommand Plus Software.

For the fermentation batches, glucose solution was autoclaved separately in flask and 
later transferred to the fermenter aseptically, as needed. During the fermentation, 
separately autoclaved glucose solution (500 g/l) was fed at predetermined sugar feed 
rate taking care that the culture continues in glucose-limiting condition. The pH was 
controlled at 7.0 by addition of 5 N NaOH. The DO₂ probe was polarized for four 
hours after autoclaving, before the fermenter was inoculated. It was calibrated 
between 0 and 100% air saturation. Zero was adjusted electronically by 
disconnecting the cable for 30 seconds, followed by saturating the medium with 
oxygen by sparging air at 0.5 vvm and 700 rpm, and this DO₂ point was set as 100% 
air saturation. The DO₂ was controlled using the automatic DO₂ controller that 
increased or decreased the agitation speed to maintain DO₂ at 40% air saturation. 
Aeration rate was 0.5 vvm unless otherwise mentioned. The foam formation was 
controlled by periodic addition of food grade silicon oil as antifoam agent. The 
fermentation was continued for 168 h. Samples were analyzed at successive intervals 
for dry cell mass, pH, sugar utilized, GA₄ and GA₃ concentration by Reversed Phase 
HPLC.

5.3 RESULTS AND DISCUSSION

5.3.1 Screening of cultures for production of GA₄

The endeavour was to identify GA₄ producing Fusarium strain, to study the strains 
using various culture conditions and process parameters for further desired 
improvements in GA₄ yield. GA₄ production using biological processes is becoming 
more important because of the growing demand of GA₄ in agriculture and 
horticulture field. Species belonging to Fusarium, Speceloma, Neurospora and
Phaseosphaeria have been reported to produce GA₃ and/or GA₄ as final metabolite (MacMilan, 2002). Amongst the fungi, ascomycetous fungus *F. fujikuroi* belonging to mating population “C” is capable of producing gibberellins in industrially viable quantities (Takahashi *et al.*, 1991; Malonke *et al.*, 2005). Although the main product of gibberellin biosynthesis in *F. fujikuroi* is GA₃, it produces its precursors gibberellin A₄ (GA₄) and gibberellin A₇ (GA₇) also (Tudzynski, 1999).

On the basis of literature review the strains of *F. fujikuroi* obtained from NCIM and the GA₃ producing mutants generated during the mutagenesis programme of GA₃ production as described in Chapter 3 were screened for GA₄ production in LMF. A separate screening for GA₄ producing cultures became necessary because of the distinct difference in the HPLC analysis of GA₃ and GA₄. The four mutant strains namely Carl, Mut4, Mut65 and Mut189 selected for higher GA₃ production, less pigmentation and altered morphology as short, thick branched mycelium were re-evaluated for their GA₄ production ability using glucose as the carbon source. The type strains of *F. fujikuroi* obtained from NCIM and four mutant strains selected earlier grew well in the fermentation medium with soyabean meal as the nitrogen source and their dry cell mass reached about 17 g/l in all the studied cultures (Table 5.1). When the mutants, the parent strain and *Fusarium sp* from NCIM were grown in 50 ml the liquid fermentation medium, there were distinct differences in growth characteristics and products formed. Strains from NCIM grew profusely in a free, long mycelia form in liquid cultures making the broth very viscous. While mutant strains Mut65 and Mut189 grew with short, thick, highly branched mycelium in liquid culture and the broth had lower apparent viscosity as compared to other strains and the parent. The sugar uptake was nearly equal in all liquid cultures of the strains studied. On screening four *Fusarium* strains from NCIM and four GA₃ producing mutants, five cultures were found to produce GA₄ in LMF as detailed in Table 5.1. There was increase in GA₄ production by the mutants Mut65 and Mut189 as compared to the parent strain and other strains. It can be also seen from Table 5.1 that mutant Mut189 produced maximal GA₄ plus GA₃ (412 mg/l) and GA₄ accounted for 17% of the mixture. Mutant Mut189 produced 71 mg/l GA₄ which was considerably higher than that of other mutants and parent (Table 5.1). The growth of all the strains in the basal medium was similar and this showed that the specific gibberellin productivity of Mut189 was also highest (24.2 mg gibberellins g⁻¹ dry cell
weight). Mutant Mut189 produced 23-fold more \( \text{GA}_4 \) as compared to the parent \( F. fujikuroi \) NCIM 1019.

<table>
<thead>
<tr>
<th><strong>Fusarium sp.</strong></th>
<th><strong>Cell Weight g/l</strong></th>
<th><strong>Total GAs mg/l</strong></th>
<th><strong>GA(_3) mg/l</strong></th>
<th><strong>GA(_4) mg/l</strong></th>
<th><strong>GA(_4)/Total GAs %</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>( F. fujikuroi ) NCIM 1019</td>
<td>17</td>
<td>62</td>
<td>56</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>( F. fujikuroi ) NCIM 862</td>
<td>18</td>
<td>29</td>
<td>29</td>
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</tr>
<tr>
<td>( F. fujikuroi ) NCIM 850</td>
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<tr>
<td>Car1</td>
<td>18</td>
<td>206</td>
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<tr>
<td>Mut4</td>
<td>17</td>
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<td>Mut189</td>
<td>17</td>
<td>412</td>
<td>341</td>
<td>71</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 5.1 Screening of \( Fusarium \) strains from NCIM and selected mutants of \( F. fujikuroi \) NCIM 1019 for \( \text{GA}_4 \) production in LFM

5.3.2 HPLC Analysis of \( \text{GA}_4 \)

In the present investigation, \( \text{GA}_4 \) eluted at 12.20 min as shown in Fig. 5.2. The retention volume of \( \text{GA}_4 \) 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 at a difference of 5 nm showed peak purity index of 99% for the standard \( \text{GA}_4 \) and had maximal absorbance at 205 nm. The peak at 12.26 min in the chromatogram of sample broth had identical spectral pattern to the standard \( \text{GA}_4 \) peak at 12.20 min. Barendse and Van de werken (1980) and Gallazzo and Lee (2001) have described chromatographic separation of \( \text{GA}_4 \) and \( \text{GA}_7 \). These investigators have mentioned that under the chromatographic conditions they used, \( \text{GA}_7 \) eluted just before \( \text{GA}_4 \) with the retention time difference of about a minute. The chromatographic conditions that we used in the present investigation like column, mobile phase, temperature and detector were similar to those described by these investigators. In the present investigation, \( \text{GA}_4 \) eluted at 12.20 min (Fig. 5.2) and there was no peak that could be considered to be \( \text{GA}_7 \) before \( \text{GA}_4 \) during the HPLC analysis of fermentation broth samples. A much smaller peak was observed at 10.38 min, which however did not show spectral behaviour like gibberellin during analysis.
with on-line spectral analysis using UV-scanning detector and also was not found to be GA₇ with LC-MS. The fermentation broth was thus substantially free of GA₇.

The 3-D spectral scanning showed a peak purity index of 999 for the standard GA₄ peak and had maximal absorbance at 205 nm. GA₄ peak in the chromatogram of the fermentation broth had identical spectral pattern and peak purity index to that of the standard GA₄.

GA₄ was further confirmed by LC-MS by comparison with mass spectra of authentic GA₄ standard from Sigma. Mass spectra of authentic GA₄ from Sigma Chemical Corporation and a sample of fermentation broth are show in Fig. 5.3 and Fig. 5.3a. Analysis in ES positive mode gave (m/z) 333.28 i.e. (M + 1), 315.18, 269.13.

Figure 5.2 Reversed phase HPLC chromatogram of A) standard compound GA₄ B) a sample of fermentation broth
Chapter 5

Figure 5.3 Mass spectra of authentic GA₄

Figure 5.3a Mass spectra of GA₄ from the fermentation broth

Page 197
5.3.3 Effect of pH control on GA₄ production

In shake flasks it is normally difficult to control pH at any desirable point. The use of sterile CaCO₃ in shake flasks allowed us to maintain pH in the shake flask culture above 5. While in control flasks, without CaCO₃ the pH declined gradually with time and reached in range of 3.0 to 2.5. Growth of all the studied strains was almost similar in the shake flasks with or without CaCO₃, in terms of dry cell mass. Use of hydrochloric acid for dissolution of residual CaCO₃ helped in correct estimation of dry cell mass in the flask. The cultures showed differences in growth characteristics and viscosity similar to that in earlier experiments.

Borrow and their co-workers (1964) had reported that growth and gibberellin production of *F. fujikuroi* were fairly constant over the pH range of 4 to 7 however; the composition of resulting gibberellin mixture significantly depended on the pH value. They reported that at lower pH, GA₃ was the main product while at neutral pH concentrations of GA₄ and GA₇ were higher. In present investigation also the maintenance of pH above 5 using CaCO₃ did not result in any increase in GA₃ production. GA₃ concentrations remained almost equal with and without CaCO₃ addition (Table 5.2). While Gallazzo and Lee (2001) reported the pH control at 5.6 beneficial for GA₄ production by mutants selected specifically for its production. In present study also similar results were obtained. It can be seen from Table 5.2 and Table 5.2a that control of pH around 5.5 exhibited a positive effect on GA₄ production by almost all the studied cultures. Therefore, in subsequent shake flask experiments, pH was controlled above 5 by addition of CaCO₃. This increase in GA₄ concentration was relatively higher in the mutants Mut65 and Mut189. Although percent ratio of GA₄ to total gibberellins was almost equal in these two mutants, GA₄ (94 mg/l) produced by Mut189 was relatively higher than the Mut4 (10 mg/l). The mutant strain Mut189 was used further to study gibberellin A₄ (GA₄) production.
Chapter 5

<table>
<thead>
<tr>
<th>Culture</th>
<th>Dry cell weight g/l</th>
<th>Total GAs mg/l</th>
<th>GA3 mg/l</th>
<th>GA4 mg/l</th>
<th>GA4/Total GAs %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM 1019</td>
<td>18</td>
<td>77</td>
<td>70</td>
<td>7</td>
<td>7.8</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 862</td>
<td>17</td>
<td>80</td>
<td>52</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 850</td>
<td>17</td>
<td>34</td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 665</td>
<td>15</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carl</td>
<td>17</td>
<td>220</td>
<td>200</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Mut4</td>
<td>18</td>
<td>310</td>
<td>300</td>
<td>10</td>
<td>3.2</td>
</tr>
<tr>
<td>Mut65</td>
<td>18</td>
<td>380</td>
<td>300</td>
<td>80</td>
<td>21</td>
</tr>
<tr>
<td>Mut189</td>
<td>18</td>
<td>444</td>
<td>350</td>
<td>94</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 5.2 GA4 production in shake flask with CaCO3

<table>
<thead>
<tr>
<th>Culture</th>
<th>Dry cell weight g/l</th>
<th>Total GAs mg/l</th>
<th>GA3 mg/l</th>
<th>GA4 mg/l</th>
<th>GA4/Total GAs %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM 1019</td>
<td>17</td>
<td>59</td>
<td>54</td>
<td>5</td>
<td>8.5</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 862</td>
<td>18</td>
<td>27</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 850</td>
<td>17</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 665</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carl</td>
<td>18</td>
<td>206</td>
<td>192</td>
<td>14</td>
<td>6.8</td>
</tr>
<tr>
<td>Mut4</td>
<td>17</td>
<td>301</td>
<td>298</td>
<td>3</td>
<td>0.99</td>
</tr>
<tr>
<td>Mut65</td>
<td>18</td>
<td>319</td>
<td>275</td>
<td>44</td>
<td>13.8</td>
</tr>
<tr>
<td>Mut189</td>
<td>17</td>
<td>413</td>
<td>340</td>
<td>73</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Table 5.2a GA4 production in shake flasks without CaCO3

5.3.4 Choice of carbon source for GA4 production by mutant Mut189

Results presented in Table 5.3 show that irrespective of carbon source used, growth of Mut-189 was almost similar. Growth of Mut189 in terms of dry cell mass was in the range of 17-18 g/l. Morphologically no distinct difference was observed in the mycelia of the cultures. Effect of various carbon sources on GA4 production by mutant Mut189 revealed that gibberellin production pattern (i.e. relative concentration of GA4 and GA3) was dependent on the type of carbon source used. The ratio of GA4 to total gibberellin varied from 2% to 30% depending upon the
Chapter 5

carbon source used. Mut189 produced relatively more GA$_4$ when glucose, dextrin, starch, and sucrose were used in combination with soyabean meal. In media containing amylose, amylopectin and maltodextrin Mut189 produced very less GA$_4$, although the growth was almost equal. There was no correlation observed between the slow utilizable or rapid utilizable nature of the carbon source and the ratio of the two gibberellins produced. Although ratio of GA$_4$ to total Gibberellins was comparatively higher for dextrin, glucose was chosen as suitable carbon source as its use resulted in production of considerably higher total gibberellins (422 mg/l) and GA$_4$ (92 mg/l).

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Dry cell Weight g/l</th>
<th>Total GAs mg/l</th>
<th>GA$_3$ mg/l</th>
<th>GA$_4$ mg/l</th>
<th>GA$_4$/Total GAs %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose</td>
<td>17.5</td>
<td>310</td>
<td>300</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>17.2</td>
<td>356</td>
<td>247</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>18.0</td>
<td>318</td>
<td>248</td>
<td>70</td>
<td>22</td>
</tr>
<tr>
<td>(insoluble)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrin type II</td>
<td>17.9</td>
<td>293</td>
<td>211</td>
<td>82</td>
<td>28</td>
</tr>
<tr>
<td>Dextrin type III</td>
<td>16.9</td>
<td>289</td>
<td>200</td>
<td>89</td>
<td>30</td>
</tr>
<tr>
<td>Glucose</td>
<td>17.1</td>
<td>422</td>
<td>330</td>
<td>92</td>
<td>22</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>17.4</td>
<td>241</td>
<td>240</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Starch</td>
<td>18.1</td>
<td>404</td>
<td>324</td>
<td>80</td>
<td>19</td>
</tr>
<tr>
<td>Sucrose</td>
<td>16.8</td>
<td>358</td>
<td>290</td>
<td>68</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 5.3 Choice of carbon sources for GA$_4$ production by mutant Mut189

5.3.5 Screening of different nitrogen sources for GA$_4$ production by Mut189

Nitrogen limitation is the prerequisite for GA$_4$ formation because production of gibberellins starts towards the exhaustion of nitrogen source from the medium. The quality and quantity of nitrogen source used in the medium is known to strongly influence the gibberellin production investigate long ago by Borrow et al., (1964) and therefore C:N of 100:1 was maintained in all the experiments. In the present study, different organic and inorganic nitrogen sources in combination with glucose as carbon source were investigated for the production of gibberellin A$_4$ (GA$_4$). Mut189 produced substantially higher quantities of GA$_3$ in most of the media tested.
but the GA\textsubscript{4} proportion to total gibberellins remained below 10% (W/W). Results of effect of different nitrogen sources on GA\textsubscript{4} production by mutant Mut189 are illustrated in Table 5.4.

<table>
<thead>
<tr>
<th>Nitrogen Sources</th>
<th>Dry cell Weight g/l</th>
<th>Total GAs mg/l</th>
<th>GA\textsubscript{3} mg/l</th>
<th>GA\textsubscript{4} mg/l</th>
<th>GA\textsubscript{4}/Total GAs %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>14</td>
<td>242</td>
<td>240</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>13</td>
<td>214</td>
<td>205</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>13</td>
<td>207</td>
<td>200</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Soyapetone</td>
<td>19</td>
<td>64</td>
<td>61</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>18</td>
<td>45</td>
<td>40</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Soyabean meal</td>
<td>19</td>
<td>371</td>
<td>331</td>
<td>40</td>
<td>10.7</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>18</td>
<td>334</td>
<td>308</td>
<td>26</td>
<td>7.8</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>17</td>
<td>322</td>
<td>297</td>
<td>25</td>
<td>7.7</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>20</td>
<td>283</td>
<td>73</td>
<td>210</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 5.4 Comparison of nitrogen sources for GA\textsubscript{4} production by mutant Mut189

Among the various organic and inorganic nitrogen sources screened, Mut189 showed good growth with complex organic nitrogen sources as presented in Table 5.4. The growth of Mut189 and production of GA\textsubscript{4} was comparatively less in inorganic nitrogen media (Table 5.4) presumably because some of the nutrients available in low quantity in complex media ingredients were not available in the medium with inorganic nitrogen sources. Organic nitrogen sources in the medium also resulted in rapid growth of Mut189. There was no difference in morphology or growth as estimated in terms of dry cell weight when different organic nitrogen sources were used on same nitrogen content basis.

Complex organic nitrogen sources are reported to enhance gibberellin production by \textit{F. fujikuroi} (Fuska \textit{et al.}, 1961). In the present study also, use of complex organic nitrogen sources like plant meals and wheat gluten were found to enhance gibberellin production by mutants of \textit{F. fujikuroi}. However, there was major difference in GA\textsubscript{4} and GA\textsubscript{3} concentration ratio produced by Mut189. Defatted plant meals, yeast extract and soya peptone although resulted in rapid growth, did not support satisfactory GA\textsubscript{4} production. Under identical cultural conditions Mut189 produced 210 mg/l GA\textsubscript{4} in
the medium in which wheat gluten was used as nitrogen source and in this case, the ratio of GA$_4$: Total Gibberellins was 77% while ratio of GA$_4$: GA$_3$ was 3:1.

In the present study, mutant Mut189 produced substantially higher quantities of GA$_3$ in most of the media tested and the proportion of GA$_4$ to total gibberellins remained below 10% (W/W). Surprisingly, in a medium in which wheat gluten was used as the sole nitrogen source, the same mutant produced substantially higher quantities of GA$_4$ (210 mg/l). This was distinctly higher GA$_4$ concentration than all other experiments. The proportion of GA$_4$ in wheat gluten medium was 74% to total gibberellins. It was reported that the addition of a suitable nitrogen source reconstitutes the growth of *F. fujikuroi* and also inhibits gibberellin production (Borrow *et al.*, 1964). The genetics and biochemistry of gibberellin production in *F. fujikuroi* has been well characterized in recent years. It has been demonstrated that the expression of six genes including genes coding for desaturase involved in the conversion of GA$_4$ to GA$_3$ through GA$_7$ share a common regulation mediated by nitrogen catabolite regulatory protein AreA (Mihlan, 2003). Our results suggest that the increase in production of GA$_4$ in a medium composed of wheat gluten might not be an impaired activation of the gene by AreA but probably because of a substance present either in wheat gluten or produced from wheat gluten during growth of *F. fujikuroi* that inhibited activity of 1, 2 GA$_4$ desaturase converting GA$_4$ to GA$_7$. This inhibition was not an effect of oxygen availability in shake flasks because the total cell mass and mycelial morphology were similar in media with wheat gluten and other nitrogen sources.

Thus, wheat gluten was found to be suitable for the GA$_4$ production by Mut189. The study indicated that not only the pH of medium but the type and quality of nitrogen source played equal role in GA$_4$ production by mutant Mut189. Amongst all studied organic nitrogen sources wheat gluten was found to be the most suitable nitrogen source for rapid growth of Mut189 and production of higher GA$_4$ with less GA$_3$. This is the first report of use of wheat gluten for substantially high production of GA$_4$ by *F. fujikuroi*.
5.3.6 Evaluation of GA₄ production in 10 L fermenter by mutant Mutl89

On inoculating the fermenter with 10% v/v Mutl89 inoculum, it was found that the mutant Mutl89 grew rapidly in the first 24 h. It grew in desired short mycelial form similar to that in the shake flasks. The mycelium grew in the form of short, thick filaments with very little pigmentation. The colour of fermentation broth was slightly brownish due to produced water soluble pigment. Mycelium was colourless as appeared from filtered and washed cell mass. Fermentation profile of fed-batch run is shown in Fig. 5.4

![Fermentation profile of F. fujikuroi mutant Mutl89 in LFM medium](image)

**Figure 5.4** Fermentation profile of *F. fujikuroi* mutant Mutl89 in LFM medium

In order to avoid DO₂ limitation, it became necessary to adjust the agitation speed so to maintain in the range of 40-50% air saturation during the batch time except early growth phase. It can be seen that most of the biomass growth was achieved in initial 24 h which later on increased to 21 g/l DCW towards the end of batch. In order to avoid DO₂ limitation, the agitation speed was increased from 600 to 700 rpm at 20 h, which was later maintained between 650 to 700 rpm.

Initial 20 g/l glucose was consumed for the biomass generation during first 20 h and glucose concentration reached zero. Most of the glucose in this period was utilized for the growth of the fungus because gibberellins were not detected in first 20 h. The
production of gibberellins began after 24 h toward end of the growth phase as indicated by arrow in Fig. 5.4. Gibberellins are produced by \textit{F. fujikuroi} only after a strict nitrogen limitation in a nutrient medium is achieved (Borrow \textit{et al.}, 1964; Bu’Lock \textit{et al.}, 1974). In the present investigation also, the gibberellins could be detected only after the culture reached the stationary stage as seen from the stable dry cell mass (Fig. 5.4).

Glucose uptake and GA$_4$ production rate are illustrated in Fig. 5.5. The average glucose utilization rate by the mutant in the fermenter was 1.0 g/l/h. After 24 h, glucose was fed in the form of sterile, 500 g/l glucose solution in one pulse to achieve a concentration of 10 g/l.

![Figure 5.5 Glucose uptake and GA$_4$ production by mutant Mut189](image)

It can be seen from Fig. 5.5 that between 24 and 40 h, the glucose uptake rate declined to 0.62 g/l/h. Glucose utilization later decreased slowly to 0.35 g/l/h at 65 h and then remained nearly constant till end of the batch. Irrespective of decline in glucose uptake rate, GA$_4$ production rate remained almost same and thus, 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 sugar fermented is normally very poor.
Between 20 to 40 h, GA₄ production rate was 2.6 mg/l/h. The rate increased to 4.6 mg/l/h at 60 h and remained nearly same till 120 h. This indicated that as the culture reached severe level of nitrogen limitation, the repression caused by nitrogen source decreased and led to increased rate of GA₄ production. As discussed in Chapter 3 it was observed that the GA₃ production rate increased immediately after the culture entered stationary phase and later the rate lowered considerably. The lowering of GA₃ production rate was assumed to be because of lowering pH, cell mass aging or catabolite repression. Similarly in present investigation, the rate of GA₄ production started to decline after five days and reached 3.8 mg/l/h. By controlling pH and with careful control of glucose feeding rate so as to achieve glucose limiting condition during stationary phase, it was possible to maintain the GA₄ production rate above 4 mg/l/h over a considerable length of time between 60 and 144 h this resulted in a very high GA₄ concentration.

Earlier report on the concentration of GA₄ in the fermentation broth of *Sphaceloma manihoticola* was only 7 mg/l (Graebe and Rademacher, 1979) and 20 mg/l (Rademacher, 1992). Recently, isolated endophytic fungus of cereal plants *Penicillium citrinum* KACC 43900, reported to produces 6.03 microgram/l GA₄ (Khan et al., 2008) while another isolated fungus, *F. proliferatum* KGL0401 from the root of *Physalis alkekengi var. frencheti* produced 17.3 microgram/l GA₄ after seven days of incubation in Hagem’s medium (Rim et al., 2005). A US patent (Gallazzo and Lee, 2001) described production of GA₄ using *F. fujikuroi* LTB-1027 in which a mixture of equal quantities of GA₄ and GA₇ were obtained. This patent reported 800 mg/l total gibberellin concentration, in which the ratio of GA₄+7: GA₃ was 4:1. In present study, concentration of GA₄ finally reached 600 mg/l in 168 h. Combined concentration of the two gibberellins, GA₄ and GA₃, obtained was 713 mg/l, with GA₄ accounting for 84% of the total gibberellins. The production of GA₃ by Mut189 in the fermenter was 2.8 times more compared to that in the shake flask although there was no visible increase in the dry cell mass. Thus present study reveals that higher production of GA₄ than reported earlier.
5.4 CONCLUSIONS

Amount and the type of gibberellins produced by *Fusarium fujikuroi* is dependent on the genetic constitution of the strain and fermentation conditions like aeration, type of carbon and nitrogen source and pH of the fermentation medium. A mutant strain Mut189 was selected based on relatively higher Gibberellin A₄ (GA₄), total gibberellin production and lack of pigmentation. Mut189 is also a morphological mutant and has advantage of lower viscosity because of short length of mycelium and increased gibberellin yield.

Complex organic nitrogen sources like plant meals and wheat gluten enhanced gibberellin production by mutants of *F. fujikuroi*. In a medium in which wheat gluten was used as the sole nitrogen source, the mutant Mut189 produced substantially higher quantities of GA₄ with its proportion increased to 74%. The increase in production of GA₄ is probably because of a substance present either in wheat gluten or produced from wheat gluten during growth of Mut189. The transfer of process from shake flask level to 10 L agitated fermenter allowed better control of the growth and GA₄ production by mutant Mut189. GA₄ concentration increased from 200 mg/l to 600 mg/l in the same fermentation time. It produced 2.8 times more GA₄ in wheat gluten medium after 168h fermentation.

This is the first report of use of wheat gluten for substantially high production of GA₄ by *F. fujikuroi*. The mutant strain of *F. fujikuroi* was successfully improved for gibberellin₄ (GA₄) production by optimization of culture conditions and media constituents. Along with the pH, nitrogen source was significant factor affecting GA₄ production by mutant Mut189.

In conclusion, a simple procedure for obtaining high quantities of GA₄ by incubating the Mut189, mutant strain of *F. fujikuroi* NCIM 1019 in a liquid fermentation medium containing wheat gluten as sole nitrogen source is developed. The biogenetic mechanisms responsible for the accumulation of GA₄ are as yet not fully understood, but may be related to substances contained in the medium and to particular characteristics of the regulation of gibberellin biosynthesis in this strain.
5.5 REFERENCES


Publications from thesis work


Morphological mutants of *Gibberella fujikuroi* for enhanced production of gibberellic acid

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Chemical Engineering Division, National Chemical Laboratory, Pune, India

**Keywords**

fusaric acid, gibberellic acid, high performance liquid chromatography, morphological mutant, viscosity.

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2005/0089: received 28 January 2005, revised 10 July 2005 and accepted 10 July 2005

**Abstract**

Aims: To examine the production of gibberellic acid by selected morphological mutants of *Gibberella fujikuroi* in liquid cultures.

Methods and Results: Mutants of *G. fujikuroi* having different morphological characteristics were selected after UV irradiation. The production of gibberellic acid by mutants that had different hyphal lengths was examined in shake flasks in media with different concentrations of nutrients as well as different volumes of the medium. Fed-batch fermenter study was performed to evaluate the mutant *Mor-25* for growth and production of gibberellic acid. The broth was analysed by high performance liquid chromatography for fusaric acid, the common mycotoxin produced by strains of *Fusarium*. A variety of morphological mutants having different mycelial and soluble pigmentation as well as colony morphologies were generated from *G. fujikuroi* upon exposure to UV radiation. A nonpigmented mutant (*Car-1*) was selected as intermediate parent and later, mutants *Mor-1* and *Mor-25* were selected based on their distinct morphology. The colonies on regeneration agar plates were small, compact and dry. In liquid medium, mutant *Mor-25* grew in a micro-pelleted form and the mycelium had short, highly branched hyphae, curly at tips with thick, swollen cells. Mutant *Mor-25* grew rapidly in a low-cost medium containing defatted groundnut flour, sucrose and salts. In media with higher nutrient concentrations as well as larger volumes, it produced twofold more gibberellic acid than the parent. Fusaric acid, the common mycotoxin, was absent in the fermentation broth of mutant *Mor-25*. The mutants have been deposited in National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India under following culture collection numbers (*Car-1*, NCIM 1323; *Mor-1*, NCIM 1322; and *Mor-25*, NCIM 1321).

Conclusions: Growth of unpigmented, morphological mutants of *G. fujikuroi* that led to lower viscosity in fermentation broth resulted in increased production of gibberellic acid.

Significance and Impact of the Study: The use of morphological mutants that have lower viscosity in liquid cultures for gibberellic acid production is not reported earlier. Similar mutants can be useful for other types of fungal fermentations also.

**Introduction**

Gibberellic acid is an important plant growth promoter. It is used on a large scale for growth of seedless varieties of grapes in India. It is also used for breaking dormancy of several seeds and germination of barley for malt preparation. It is commonly produced by aerobic fermentation using selected strains of *Gibberella fujikuroi*. This secondary metabolite of fungal fermentation has been studied with respect to several aspects. Its production in submerged cultures (Borrow et al. 1964; Bandelier et al. 1997) in solid-state culture (Kumar and Lonsane 1987;
Morphological mutants of Gibberella fujikuroi

G. Lale et al.

Pastrana et al. (1995) and by immobilized cells (Lu et al. 1995; Escamilla et al. 2000; Gelmi et al. 2000) has been described. The effect of a variety of carbon and nitrogen sources in nutrient medium on gibberellic acid (GA3) production has been investigated (Gohlwar et al. 1984). The strains belonging to mating type 'C' produce less conidia and are prolific producers of gibberellins (Giordano et al. 1999). Mutants of G. fujikuroi have been investigated for pigment accumulation (Candau et al. 1991). Utilization of different nitrogen sources by mutants of G. fujikuroi and its effect on GA3 production has been investigated (Sanchez-Fernandez et al. 1997). The biosynthetic pathway, bioconversion of intermediates, specific regulators, inhibitors, effect of nitrogenous compounds on production of GA3, production of pigments like carotenoids and bikaverin, mutants with different pigment profiles and more recently, the genes encoding enzymes in biosynthesis of gibberellic acid by G. fujikuroi have been described and reviewed (Shukla et al. 2003).

In aerobic fermentations involving fungi, their filamentous nature commonly leads to excessive viscosity in the fermentation broth and demands higher agitation and aeration to maintain satisfactory levels of dissolved oxygen (DO). The expenditure on energy for aeration and agitation of such viscous broths is considerably high. It is a common observation that G. fujikuroi also grows in viscous, filamentous form in liquid medium and hence its submerged cultures often become oxygen limited. Rate of growth of G. fujikuroi and production of GA3 in fermenter is governed to a considerable extent by oxygen transfer in the fermenter. It is also reported that G. fujikuroi cultures enter a linear growth phase after initial logarithmic phase (Borrow et al. 1964) presumably because of the oxygen limitation. Any change in morphology of the fungal strain that lowers the viscosity can result in improved oxygen transfer and in turn, increase the GA3 production.

During stationary phase of G. fujikuroi culture several products like bikaverin, carotenoids, gibberellic acid, sterols and lipids are produced from a common precursor, acetyl-CoA. Their concentrations and ratio are governed by availability of oxygen in the culture (Giordano and Domenech 1999). A decrease in production of pigments like bikaverin and carotenoids by G. fujikuroi is likely to be beneficial for production of gibberellins because of the increased carbon flow through the gibberellin pathway as well as requirement of lesser steps during extraction and purification of the gibberellins from fermented broth.

Fusaric acid is a common mycotoxin produced by species of Fusarium that can affect animal and plant health. Strains of Fusarium and Gibberella were found to produce 200–1000 mg fusaric acid per gram of corn (Bacon et al. 1996). The strains of G. fujikuroi belonging to mating type 'C', that are prolific producers of gibberellins also, were found to produce fairly high quantities of this toxic metabolite. It is necessary that the fungal strains to be used for production of gibberellins do not produce such mycotoxins.

The aim of the present investigation was to obtain mutant of G. fujikuroi that does not accumulate pigments, has decreased viscosity of the fermentation broth without compromising gibberellic acid productivity and does not produce fusaric acid.

Materials and methods

Micro-organism

Gibberella fujikuroi (NCIM 1019) was obtained from National Collection of Industrial Microorganisms (NCIM) National Chemical Laboratory, Pune 411008, India. This is a mycelial strain that produces intense coloured bikaverin in solid and liquid media. It also accumulates carotenoids intracellularly. This strain was used as the parent strain.

Media

The parent strain and subsequent mutants were maintained on potato dextrose agar (HiMedia Mumbai, India) supplemented with yeast extract at 2 g l⁻¹ concentration. Slants were incubated at 28°C for 3–4 days and later stored at 4°C.

The parent strain (NCIM 1019) was grown in a liquid medium containing (g l⁻¹) KH₂PO₄ 1·5, NaCl 0·5, MgSO₄·7H₂O 0·2, Na₂MoO₄·2H₂O 0·05, groundnut deffatted cake 11, yeast extract 0·75, sucrose 90, trace mineral solution 1 ml, pH 6·8. The trace mineral solution contained (mg l⁻¹) H₂BO₃ 100, MnCl₂·4H₂O 100 mg, ZnSO₄·7H₂O 100 FeCl₂·6H₂O 100, CaCl₂·2H₂O 1000, CuCl₂·2H₂O 50. A few drops of HCl were added till the solution became clear.

The regeneration agar for growth of survivors after mutagenesis contained (g l⁻¹) KH₂PO₄ 1·5, NaCl 0·5, MgSO₄·7H₂O 0·2; Na₂MoO₄·2H₂O 0·05, yeast extract 3·0, glucose 30, soya peptone 3·0, bile salt 1·0, trace mineral solution 1 ml, agar 20·0, pH 6·8.

Reagents and chemicals

Gibberellic acid (G 7645) and fusaric acid (F 6513) were from Sigma Chemical Company, St Louis, MO, USA and were used as reference compounds for high performance liquid chromatography (HPLC). Reference gibberellic acid was considered to be 90% pure. Acetonitrile, ammonium dihydrogen phosphate and phosphoric acid (AR grade) were from E. Merck (Mumbai, India). The medium
ingredients were purchased from HiMedia (Mumbai, India). Groundnut defatted cake was from a local cattle-feed source.

Growth of the parent and mutagenesis

A tube containing 2.5 ml liquid medium was inoculated from a fresh slant of G. fujikuroi (NCIM 1019) and was incubated at 28°C for 48 h on a rotary shaker at 220 rev min⁻¹. A volume of 20 ml liquid medium in 250 ml Erlenmeyer flask was inoculated from this tube culture and the flask was incubated for 72 h as above. The mycelial culture was filtered over sterile sintered glass funnel G-0, (Borosil, Mumbai, India) to get short fragments in the filtrate. The filtrate was further passed through sterile cotton layer to remove larger fragments. The suspension of very small fragments (1-2 cells) was collected and used further. Fragments were counted using a haemocytometer and the cell number was adjusted to 1x10⁶ per ml. A volume of 10 μl of this suspension was spread on regeneration agar plates. The plates were exposed to UV radiation at 5 cm height for 3-10 s and incubated at 28°C, in dark, till colonies developed. The colonies that showed difference in characteristics in terms of shape, size, surface, colour and soluble pigment production than the parent were selected and transferred to maintenance agar slants.

Shake flask screening

Tubes containing 2.5 ml liquid medium were inoculated from fresh slants of the parent and the two selected mutants generated during mutagenesis. The tubes were incubated at 28°C for 48 h on a rotary shaker at 220 rev min⁻¹. Twenty ml liquid medium in 250 ml Erlenmeyer flask was inoculated from the tube culture and the flasks were incubated for 5 days. At the end of incubation, volume of the broth in the flasks was adjusted to 50 ml with distilled water and then the culture broth was filtered over qualitative filter paper circle under vacuum. The cell mass residue was further washed with three volumes of distilled water, observed for pigmentation and transferred to 104°C for determination of dry mass. The culture filtrates were analysed as described below. The experiments, done in duplicate, were performed twice.

Effect of larger volume and higher strength of medium on GA₃ production

The liquid medium was dispensed in 250 ml Erlenmeyer flasks in 15, 30, 45, 60 and 75 ml quantities and autoclaved. The flasks were inoculated with 10% (v/v) liquid cultures of three selected strains (with approximately 18 g l⁻¹ dry cell mass) grown for 48 h, uniformly, to evaluate the effect of increased medium volume in the flasks. In another set of experiment, they were grown in media with 1, 1.25, 1.5 and 1.75-fold higher nutrient concentrations to evaluate the effect of increased strength of fermentation medium on production of GA₃. The flasks were incubated on rotary shaker at 220 rev min⁻¹, at 28°C, for 5 days. The samples were analysed for dry mass, sugar and gibberellic acid content as described earlier. The experiments, done in duplicate, were performed twice.

Evaluation of mutant Mor-25 in 10 l fermenter

A 10 l working volume fermenter (Bioflo 100; New Brunswick Scientific Co, NJ, USA) was used for evaluation of the Mor-25 mutant. An inoculum grown for 48 h, in 1.25-fold medium was used. The liquid medium contained 1.25-fold concentration of the ingredients. The fermentation was continued for 188 h. DO was controlled at 50% air saturation level using the DO controller. The controller increased or decreased the agitation rate to maintain DO at 50% air saturation. The sugar was fed continuously at predetermined rates to avoid accumulation of the catabolite.

Sugar analysis

The residual sugar in the fermentation broth was estimated by dinitrosalicylic acid (DNS) method after suitable dilution.

HPLC analysis of gibberellic acid

The HPLC equipment was from ThermoSeparation Products, Fremont, CA, USA. A reversed phase, C18, Lichrospher100, 125 x 4 mm, 5-μm particle size column with a 4 x 4 mm guard column obtained from Merck (Merck KGaA, Darmstadt, Germany), was used. The mobile phase composed of 20% acetonitrile in 5 mmol l⁻¹ ammonium dihydrogen phosphate, at pH 2.5 adjusted by H₃PO₄, was used at 0.6 ml min⁻¹. The detection of gibberellic acid was done at 205 nm. Standard graph of gibberellic acid
HPLC analysis of fusaric acid

Fusaric acid was analysed by a modification of the reversed phase HPLC method described by Amalifiano et al. (2002), using RP, C18, Lichrospher100, 125 x 4 mm column. The mobile phase contained 47.5% methanol in 5 mmol L⁻¹ dipotassium phosphate, pH 7.4. The flow rate was 0.6 mL min⁻¹. Standard fusaric acid solutions of 2 and 10 mg L⁻¹ were used for calibration. Fusaric acid solutions of 100-2000 µg L⁻¹ were prepared for analysis of fusaric acid. On-line spectral analysis of standard fusaric acid was performed using the detector and software described above to determine the spectral pattern of fusaric acid using UV3000 detector and Spectacle software. Spectral scan of the peaks in chromatograms of samples having elution time close to the standard fusaric acid peak were performed, similarly. The spectral scanning detector has an ability to determine the spectral behaviour of all the peaks in the chromatogram during the elution itself.

Results

Mutagenesis and screening

The parent strain G. fujikuroi (NCIM 1019) grew in a viscous filamentous form in shake flask. The filtered and washed mycelium looked orange in colour and the filtrate had a red-violet colour. A mutant (Car-1) obtained from the parent G. fujikuroi (NCIM 1019) had lost the pigmentation but was still a very mycelial strain that produced white, cottony, circular colony on regeneration agar. It grew profusely in a free, long mycelial form in liquid cultures making the broth very viscous. The carotenoid accumulation in the cells was negligible as appeared from filtered and washed cell mass. The culture filtrate, unlike parent, did not have the distinct bikaverin colour. Based on the loss of pigmentation, Car-1 was selected and used as the parent in subsequent mutagenesis experiments.

The UV mutagenesis generated a wide variety of mutants. The survivors selected after mutagenesis of Car-1 strain exhibited various morphological changes. The smaller colonies (2-3 mm) on regeneration agar plates that had crusty, uneven margins with rough surfaces and decreased pigmentation were selected because those were expected to have modified morphology in liquid cultures. The liquid cultures of some of these survivors showed growth with short mycelial length and increased cell thickness. These characteristics indicated that they might have morphology suitable for micro-pelleted mutants, when grown in liquid cultures. A mutant strain, Mor-1, found to grow with short and thicker mycelium, produced higher concentration of gibberellic acid and was thought to be advantageous for the fermentation because the problem associated with poor mixing and oxygen transfer in fermenter could be minimized. During subsequent mutations using Mor-1 as a parent, a mutant Mor-25 was selected in similar way that had still shorter hyphal length and increased cell thickness.

The average terminal hyphal length had distinct differences between the three strains. The average apical hyphal length of Car-1 strain was 248 µm, that of Mor-1 mutant was 173 µm, while that of the Mor-25 strain was just 94 µm. These lengths were measured at 24 h in shake flask cultures when they were near the end of the logarithmic phase and therefore these lengths were of mature hyphae. The mutants have been deposited in National Collection of Industrial Micro-organisms (NCIM), National Chemical Laboratory, Pune, India under following culture collection numbers (Car-1, NCIM 1323; Mor-1, NCIM 1322; and Mor-25, NCIM 1321).

Production of GA3 in single strength medium

When the three selected strains Car-1, Mor-1 and Mor-25 were grown in 20 mL single strength medium in 250 mL flasks simultaneously, they exhibited different growth characteristics from each other. The mutant Car-1 had highly viscous growth while the mutant Mor-25 had minimal viscosity as appeared from the culture broth in shake flasks. Table 1 shows that there was no major change in the extent of growth of these three mutant strains (Car-1, Mor-1 and Mor-25) of G. fujikuroi in terms of dry cell mass. However, there were substantial differences in the sugar utilization, GA3 concentration and productivity of these strains. Table 1 also shows that the short filament strain Mor-25 resulted in substantially higher gibberellic acid content and had high yield, specific productivity and volumetric productivity. It is known that the oxygen availability causes a major change in proportion of the
metabolites produced by *G. fujikuroi*. In the present study, the decreased mycelial length presumably resulted in increased oxygen transfer in the submerged cultures of mutant *Mor-25*. This, in turn must have further channelled more carbon through the gibberellin pathway resulting in higher concentration of gibberellic acid. A considerable amount of sugar is normally utilized by cultures of *G. fujikuroi* in their stationary phase. The strains that have highest GA$_3$ production per gram sugar utilized, would be preferred because these are the mutants that have higher flux of carbon towards gibberellic acid through its metabolic mechanism.

### Production of GA$_3$ in larger volume media

The selection of mutants under investigation also depends on their growth characteristics and viscosity of the liquid cultures in shake flasks when grown in shake flasks at constant speed and temperature. In the first experiment, the medium volumes in 250 ml Erlenmeyer flask were varied. In the second experiment, the concentration of individual ingredients in the medium was increased keeping the volume constant. Increase in nutrient concentration as well as volume causes increase in oxygen demand in the shake flask culture because of increase in the cell mass. Figure 1 shows GA$_3$ concentration in the fermented broth of three different mutants in shake flasks with different medium volumes, after 5 days incubation at 28°C and 220 rev min$^{-1}$. It shows that there was a drastic decrease in GA$_3$ concentration in case of mycelial mutant *Car-1* when the volume of medium in 250 ml flask was increased from 30 to 60 ml. The change was proportional to the increase in the medium volume. On the contrary, in case of the strain with short hyphae, *Mor-25*, the production of gibberellic acid was least affected.

#### Production of GA$_3$ media with increased strength

The results presented in Fig. 2 show that in case of the mycelial mutant *Car-1*, the increase in strength of medium adversely affected the GA$_3$ production. Similar to the above results, this is likely to be due to the decreased oxygen availability for the growth and GA$_3$ production by mutant *Car-1*. This effect was considerably decreased in case of mutants *Mor-1* and *Mor-25* that had altered morphology. The GA$_3$ production by *Mor-1* and *Mor-25* did not decrease unlike in the case of mycelial mutant, *Car-1*. On the contrary, with increased strength of the medium, the GA$_3$ concentration increased in the case of *Mor-25* mutant and it could be advantageous to use higher strength media with such mutants for GA$_3$ production.

### Table 1 Growth and gibberellic acid production by three selected mutants of Gibberella fujikuroi

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Dry cell weight (g l$^{-1}$)</th>
<th>Sugar utilized (g l$^{-1}$)</th>
<th>GA$_3$ (mg l$^{-1}$)</th>
<th>Specific productivity GA$_3$/dry cell weight (mg g$^{-1}$)</th>
<th>Yield GA$_3$/sugar (mg g$^{-1}$)</th>
<th>Volumetric productivity (mg l$^{-1}$ d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Car-1</em></td>
<td>30</td>
<td>42</td>
<td>147</td>
<td>5</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td><em>Mor-1</em></td>
<td>27</td>
<td>49</td>
<td>184</td>
<td>6</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td><em>Mor-25</em></td>
<td>30</td>
<td>28</td>
<td>295</td>
<td>10</td>
<td>10</td>
<td>59</td>
</tr>
</tbody>
</table>

UV mutagenesis can generate mutants that result in higher product concentration because of alteration in metabolism. There was a possibility that Mor-25 mutant also had a modification in metabolism that would result in increased gibberellic acid as compared with the parent Mor-1 or Car-1. However, this effect would have been seen in all the flasks irrespective of the volume of the medium or concentrations of the medium ingredients. In the present case the liquid culture of the mutant Mor-25 was visibly less viscous as routinely observed during the shake flask experiments and produced more gibberellic acid presumably because of lower viscosity of the culture broth.

Production of $\text{GA}_3$ in 10 l fermenter

Excellent growth of the mutant Mor-25 with desired short-filament and micro-pellets could be achieved in 10 l fermenter. The agitation rate was found to be varied between 200 and 850 rev min$^{-1}$ depending upon the DO status. The DO remained around 50% air saturation during the run except some period of exponential growth. This was because of the fact that although the agitation rate reached 850 rev min$^{-1}$, the DO demand was even higher. Profiling the nitrogen source feeding can be optimised to avoid the decrease in DO tension. The volatile ester formation characterized by a typical smell during the normal fermentation (Gonzalez-Sepulveda and Agosin 2000) could be avoided by the DO control at 50% air saturation.

The results of fermentation batch using mutant Mor-25 are presented in Figs 3 and 4. The mutant Mor-25 grew rapidly during the logarithmic phase between 5 and 20 h reaching a high dry cell weight of about 20 g l$^{-1}$. The growth became linear later. Gibberellic acid is a secondary metabolite and its production began only when the logarithmic growth phase was over. The $\text{GA}_3$ accumulation continued throughout the batch time till 192 h to reach a concentration of 720 mg l$^{-1}$. There was a gradual decrease in pH, which remained stationary during the late stage of fermentation.

In the fermenter, during the logarithmic growth phase the sugar utilization was marginal and the sugar added to the medium did not contribute significantly to the vegetative cell mass. The rate of sugar uptake reached a maximal of 1.6 g l$^{-1}$ h$^{-1}$ at 48 h as could be seen in Fig. 4. The highest sugar uptake rate was observed during the changeover from logarithmic to stationary phase of the culture. The rate of $\text{GA}_3$ production although increased along with the sugar utilization rate during initial stationary phase, it did not coincide with sugar uptake rate between 72 to 96 h during which the $\text{GA}_3$ production rate remained above 6, irrespective of the rapid decrease in the sugar uptake rate.

Absence of mycotoxin

Figure 5 shows superimposed chromatograms of standard fusaric acid at 2 mg l$^{-1}$ and that of the fermentation broth of mutant Mor-25. The standard fusaric acid eluted at 3.86 min. In the sample, two peaks eluted at 3.64 and 4.13 min, respectively. On-line spectral scanning performed between 230 and 300 nm at a difference of 5 nm showed a peak purity index of 999.9 for the standard and had a maximal absorbance at 276 nm. On-line spectral scanning of chromatographic peaks is a very convenient technique for determination of peak purity. The limit of detection (LOD) of fusaric acid using the present HPLC method was 250 $\mu$g l$^{-1}$. The peaks near this retention time in the chromatogram of culture filtrate mutant

acid was 3-86 min. Two peaks at 3-54 and 4-13 min in sample had mutant line spectral scanning of the sample chromatogram. close to that of fusaric acid as could be confirmed by on-
were not fusaric acid although the retention time were M o r-25.

Figure 5 High performance liquid chromatography of standard fusaric acid at 2 mg L-1 and fermentation broth of Gibberella fujikuroi mutant Mor-25. Fusaric acid was absent. Retention time of fusaric acid was 3-86 min. Two peaks at 3-64 and 4-13 min in sample had different spectral pattern as describe in text.

Mor-25 had distinctly different spectral behaviour and were not fusaric acid although the retention time were close to that of fusaric acid as could be confirmed by on-line spectral scanning of the sample chromatogram.

Discussion

Gibberellic acid is commonly produced by fermentation using selected strains of G. fujikuroi. However, literature about such strains in terms of characteristics as well as fermentation conditions is rarely available. Although there are several excellent publications that deal with production of gibberellic acid and its control mechanism at cellular level, there are hardly any reports that describe use of mutants or procedure for selecting mutants for increasing the GA3 production. Several patents worldwide cover production of gibberellic acid. However, the description of strains and processes in patent documents is also very sketchy. In the present investigation, mutants that had altered morphology and resulted in higher GA3 production are described. The change in morphology in liquid medium was however, not always associated with increased production of gibberellic acid. Some of the small colonies that also had short filament length in liquid medium, resulted in very low gibberellic acid production. The pigmented colonies, presumably reverted to the parent form, generally led to decreased gibberellic acid concentration. Through careful selection it is possible to obtain the desired mutants that can affect productivity of expensive secondary metabolites. The mutant Mor-25 used in the present investigation did not produce soluble bikaverin or intracellular carotenoids to any considerable extent. The culture filtrate was clear and pale yellow. Such mutants that do not produce coloured by-products will be beneficial for the production of GA3 because the efforts to remove such undesired compounds during purification of the product can be saved.

Giordano and Domenech (1999) have described how the aeration affects the fate of acetate in G. fujikuroi during secondary metabolism. They also had achieved different levels of aeration using different volumes of medium in 125 ml Erlenmeyer flasks. They concluded that higher aeration increased the concentration of gibberellins and bikaverin while lower aeration resulted in increased accumulation of fatty acids and fusarin C. It is evident that the higher oxygen availability increases production of gibberellic acid during the fermentation using G. fujikuroi.

It is well known that supplying sufficient quantity of oxygen to an actively growing fungal culture in large-scale fermenters is often a challenge. A variety of efforts to increase gibberellic acid content in fermentation broth have been published however, use of morphological mutants for GA3 production has not been reported earlier. Selection and evaluations of mutants of G. fujikuroi that have altered morphology in terms of filament length and increase in the production of gibberellic acid in the present investigation clearly shows the benefits achieved in terms of production of gibberellic acid.

As seen during the evaluation of the selected mutant Mor-25 in a 10 l laboratory fermenter, the production of gibberellic acid began in the stationary phase. Its production was governed strongly by available nitrogen source and was initiated only when the rate of cell mass formation declined after 24 h as seen from the Fig. 3. A variety of nitrogen sources have been investigated for the production of gibberellic acid. Although several investigators have used glycine or ammonium salts as nitrogen source for investigation of GA3 production, organic nitrogen sources were found to enhance gibberellic acid production. In the present investigation, we used groundnut defatted cake as the main nitrogen source for the growth and was found to be suitable for the gibberellic acid production by the selected mutants of G. fujikuroi. The concentration of groundnut cake was selected on the basis of oxygen deliverability of the fermentation equipment used to avoid oxygen-depleted condition. The concentration of GA3 achieved in the 192 h fermentation batch is considerably high as compared with several earlier publications.

Although the dry cell weight accumulation rate was very high during first 24 h, the sugar utilization rate was very low indicating that most of the growth in the present medium was taking place at the cost of protein source provided. The sugar uptake rate increased rapidly after first 24 h and coincided with the increasing rate of GA3.
production. But soon the rate of sugar utilization decreased considerably although the rate of GA₃ production continued to be high till 96 h and this indicated that only a small amount of sugar was actually getting converted to GA₃. The information can be used for further fine-tuning of the gibberellic acid fermentation.

The mutant of *G. fujikuroi* studied in the present investigation (Mor-25) did not produce fusaric acid, which is another undesired and toxic metabolite of species of *Fusarium*. As detailed by Bacon et al. (1996) all the 78 strains of *Fusarium* and *Gibberella* they examined, produced fusaric acid and the strains that belonged to mating type 'C' had consistently moderate to high concentration of fusaric acid in the culture medium. The toxicity of fusaric acid for animals, plants and human is well documented in literature and it was therefore essential that the strain of *G. fujikuroi* to be used for gibberellic acid production does not produce fusaric acid. The present RP HPLC method of analysis of fusaric acid had some difficulty because of presence of compounds that eluted close to the standard fusaric acid peak. The technique of on-line spectral scanning could solve the problem and eliminate doubts about peak identification.

The present investigation was thus successful in obtaining a mutant of *G. fujikuroi* that did not produce undesired metabolites and had morphological characters that helped in enhancement of gibberellic acid production.

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References


Enhanced production of gibberellin A₄ (GA₄) 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 A₄ (GA₄) and gibberellin A₃ (GA₃) production, as well as its lack of pigmentation. Production of GA₄ 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 GA₄ and GA₃ was done by reversed-phase high-performance liquid chromatography and LC-MS. The mutants of G. fujikuroi produced more GA₄ when the pH of the medium was maintained above 5. During shake flask studies, the mutant Mor-189 produced 210 mg l⁻¹ GA₄ 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 GA₄. In 7-day fed-batch fermentation, 600 mg l⁻¹ GA₄ were obtained in the culture filtrate. The concentration of GA₄ and GA₃ combined was 713 mg l⁻¹, of which GA₄ 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 GA₄ production by the mutant Mor-189.

Keywords Gibberella fujikuroi · GA₄ · Mutant · Wheat gluten · Fermentation

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 GA₁, GA₃, GA₄ and GA₇, which belong to the group of “C₁₉ 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), Sphaeloma, Neurospora and Phaeosphaeria have been reported to produce gibberellins. These fungi produce GA₃ and/or GA₄ 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 GA₃ as the main product, the fungus also produces its precursors gibberellin A₄ (GA₄) and gibberellin A₇ (GA₇) [29]. During the terminal steps of GA₃ biosynthesis, 1,2-GA₄ desaturase converts GA₄ to GA₇ [30] and a nonspecific P₄₅₀ monoxygenase later oxidizes GA₇ to GA₃ [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 (GA3) 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 GA4 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].

GA4 and GA7 possess different bioactivities than GA3 and are immediate precursors of GA3. Commercially, GA2 and GA3 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 GA3, probably because of lower yields in commercial fermentations.

The GA4+7 mixture primarily stimulates the flowering and elongation of fruit cells. Growers of apples, pears and grapes use the mixture of GA2 and GA7 to produce larger fruits and an early harvest. The amount of GA4 and GA7 used varies depending on the crop, but it is generally applied at levels of 5–50 mg l⁻¹. GA4+7 is used with "Golden Delicious" apples to effectively prevent abnormal cell divisions in the epidermal layer that lead to undesirable "russetting" [6]. Using GA4+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 species. This enables better seed production of economically important forest trees. GA4 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 GA4 and GA7 has increased because of their horticultural uses.

A number of researchers have reported high GA4 and GA7-producing strains of *G. fujikuroi* that do not produce GA3. Gibberellin A4 (GA4) was initially isolated and identified in culture filtrate of *Phaeosphaeria* sp. L487 [27]. However, a biosynthetic gene study revealed that GA3 was the final gibberellin metabolite in *Phaeosphaeria* sp. L487 [15, 16]. Another fungus, *Sphaeloma manihotica*, which causes superelongation disease in cassava [11, 33], produces GA4 as the major gibberellin without any GA3 and GA7; however, the concentration of GA4 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 GA4 [17]. Another newly isolated fungus, *Fusarium proliferatum* KGL0401, from the root of *Physalis alkekengi* var. *frenchetti*, produced 17.3 µg l⁻¹ GA4 after 7 days of incubation in Hagem’s medium [26]. A US patent [9] describes the production of GA4 using *G. fujikuroi* LTB-1027 in which a mixture of equal quantities of GA4 and GA7 was obtained. This patent reports a total gibberellin concentration of 800 mg l⁻¹, where the ratio of GA4+7: GA3 was 4:1. The main aim of the present study was to investigate the production of GA4 by Mor-189, a mutant of *G. fujikuroi* selected on the basis of morphological differences and its high GA4 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) KH₂PO₄ 1.5, NaCl 0.5, MgSO₄ 7H₂O 0.2, Na₂MoO₄·2H₂O 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) H₂BO₃ 100, MnCl₂·4H₂O 100, ZnSO₄·7H₂O 100, FeCl₃·6H₂O 100, CaCl₂·2H₂O 1,000, and CuCl₂·2H₂O 50, to which a few drops of HCl were added until the solution became clear.

The basal medium used for GA4 production contained (values in g l⁻¹) KH₂PO₄ 1.5, NaCl 0.5, MgSO₄·7H₂O 0.2, Na₂MoO₄·2H₂O 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**

GA₃ (G 7645) and GA₄ (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 GA₄ and GA₇ 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 Materials and Methods...
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 PC10000 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 acid, 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 pigmentation 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 soybean meal as the nitrogen source. Separately autoclaved glucose, sucrose, soluble starch, insoluble corn starch, dextrin, maltodextrin, amylose, and amyllopectin 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 glucose 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$_3$ and GA$_4$ 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 GA4 plus GA3 (412 mg L−1), and that GA4 accounted for 17% of this mixture. The GA4 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 GA3 and 23-fold more GA4 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 GA4 production

The growths of all of the studied mutants were similar in the shake flasks with or without CaCO3 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 CaCO3 was added. It can be seen from Table 2 that maintaining the pH above 5 increased GA4 production in all of the mutants studied. Table 2 also shows that maintaining the pH around 5.5 exerted a positive effect on the GA4 production by almost all of the mutants without any increase in GA3 production. This increase in GA4 concentration was highest in the mutants Mor-25 and Mor-189. Although the ratio of GA4 to total gibberellin was almost the same for all studied mutants, more GA4 (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 CaCO3.

Effects of different carbon sources on GA4 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 GA3 production. The ratio of GA4 to total gibberellin varied from 2 to 30% depending upon the carbon source used. Mor-189 produced more GA4 when glucose, dextrin starch, and sucrose were used in combination with soyabean meal, but produced much less GA4 in media containing amylose, amylopectin and maltodextrin, although the growth remained relatively constant. There was no correlation between the nature (slowly utilisable or rapidly utilisable) 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 GA4 and GA3 production by selected mutants of G. fujikuroi in 7-day shake flask experiments

<table>
<thead>
<tr>
<th>Mutant</th>
<th>GA4 (mg L−1)</th>
<th>GA3 (mg L−1)</th>
<th>Total GA (mg L−1)</th>
<th>GA4/total GA (%)</th>
<th>Dry cell mass (g L−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. fujikuroi NCIM 1019</td>
<td>3 ± 1</td>
<td>56 ± 2</td>
<td>59</td>
<td>5</td>
<td>18.1 ± 0.6</td>
</tr>
<tr>
<td>Car-1</td>
<td>12 ± 2.6</td>
<td>194 ± 4.5</td>
<td>206</td>
<td>5</td>
<td>18.2 ± 0.4</td>
</tr>
<tr>
<td>Mor-1</td>
<td>2 ± 0.6</td>
<td>298 ± 5.2</td>
<td>300</td>
<td>0.6</td>
<td>17.7 ± 0.4</td>
</tr>
<tr>
<td>Mor-25</td>
<td>45 ± 4.5</td>
<td>275 ± 6.5</td>
<td>320</td>
<td>14</td>
<td>18.2 ± 0.8</td>
</tr>
<tr>
<td>Mor-189</td>
<td>71 ± 4.3</td>
<td>341 ± 5.0</td>
<td>412</td>
<td>17</td>
<td>17.8 ± 0.3</td>
</tr>
</tbody>
</table>

Total GA refers to GA3 + GA4. Concentrations are averages of three flasks each ±SD

Table 2 GA4 and GA3 production by mutants of G. fujikuroi in medium with CaCO3 for pH control in 7-day shake flask experiments

<table>
<thead>
<tr>
<th>Mutant</th>
<th>GA4 (mg L−1)</th>
<th>GA3 (mg L−1)</th>
<th>Total GA (mg L−1)</th>
<th>GA4/total GA (%)</th>
<th>Dry cell mass (g L−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. fujikuroi NCIM 1019</td>
<td>6 ± 1.0</td>
<td>71 ± 3.6</td>
<td>77</td>
<td>7.7</td>
<td>19.1 ± 0.4</td>
</tr>
<tr>
<td>Car-1</td>
<td>20 ± 2.6</td>
<td>200 ± 6.5</td>
<td>220</td>
<td>9</td>
<td>17.8 ± 0.3</td>
</tr>
<tr>
<td>Mor-1</td>
<td>10 ± 1.7</td>
<td>300 ± 6.0</td>
<td>310</td>
<td>3.2</td>
<td>17.7 ± 0.2</td>
</tr>
<tr>
<td>Mor-25</td>
<td>80 ± 2.6</td>
<td>300 ± 5.0</td>
<td>380</td>
<td>21</td>
<td>18.2 ± 0.2</td>
</tr>
<tr>
<td>Mor-189</td>
<td>94 ± 3.6</td>
<td>350 ± 8.5</td>
<td>444</td>
<td>21</td>
<td>18.1 ± 0.3</td>
</tr>
</tbody>
</table>

Total GA refers to GA3 + GA4. Concentrations are averages of three flasks each ±SD
Table 3 Effects of different carbon sources on GA₄ and GA₃ production by mutant Mor-189 in 7 day shake flask experiments

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>GA₄ (mg l⁻¹)</th>
<th>GA₃ (mg l⁻¹)</th>
<th>Total GA (mg l⁻¹)</th>
<th>GA₄/Total GA (%)</th>
<th>Dry cell mass (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose</td>
<td>10 ± 3</td>
<td>300 ± 10</td>
<td>310</td>
<td>3</td>
<td>17.5 ± 0.2</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>9 ± 2</td>
<td>247 ± 7.0</td>
<td>356</td>
<td>2</td>
<td>17.2 ± 0.2</td>
</tr>
<tr>
<td>Corn starch</td>
<td>70 ± 4.5</td>
<td>248 ± 9.1</td>
<td>318</td>
<td>22</td>
<td>18.0 ± 0.4</td>
</tr>
<tr>
<td>Dextrin type II</td>
<td>82 ± 3.4</td>
<td>211 ± 2.6</td>
<td>293</td>
<td>28</td>
<td>17.9 ± 0.1</td>
</tr>
<tr>
<td>Dextrin type III</td>
<td>89 ± 2</td>
<td>200 ± 4.5</td>
<td>289</td>
<td>30</td>
<td>16.9 ± 0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>92 ± 3</td>
<td>330 ± 6.0</td>
<td>422</td>
<td>22</td>
<td>17.1 ± 0.2</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>7 ± 1</td>
<td>240 ± 11.1</td>
<td>241</td>
<td>3</td>
<td>17.4 ± 0.3</td>
</tr>
<tr>
<td>Starch</td>
<td>80 ± 8.1</td>
<td>324 ± 10.4</td>
<td>404</td>
<td>19</td>
<td>18.1 ± 0.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>68 ± 1</td>
<td>290 ± 8.7</td>
<td>358</td>
<td>19</td>
<td>16.8 ± 0.3</td>
</tr>
</tbody>
</table>

Total GA refers to GA₃ + GA₄. Concentrations are averages of three flasks each ±SD

Table 4 Effect of different nitrogen sources on GA₄ and GA₃ production by the mutant Mor-189 in 7 day shake flask experiments

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>GA₄ (mg l⁻¹)</th>
<th>GA₃ (mg l⁻¹)</th>
<th>Total GA (mg l⁻¹)</th>
<th>GA₄/Total GA (%)</th>
<th>Dry cell mass (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>2 ± 1.0</td>
<td>240 ± 5.5</td>
<td>242</td>
<td>0.8</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>9 ± 1.0</td>
<td>205 ± 4.3</td>
<td>214</td>
<td>4</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>7 ± 1.0</td>
<td>200 ± 4.5</td>
<td>207</td>
<td>3</td>
<td>14.1 ± 0.3</td>
</tr>
<tr>
<td>Soya peptone</td>
<td>3 ± 1.0</td>
<td>61 ± 5.5</td>
<td>64</td>
<td>4.5</td>
<td>19.1 ± 0.4</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 ± 1.0</td>
<td>40 ± 3.4</td>
<td>45</td>
<td>11</td>
<td>18.2 ± 0.2</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>25 ± 2.0</td>
<td>297 ± 10.1</td>
<td>322</td>
<td>7.7</td>
<td>17.9 ± 0.3</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>26 ± 1.7</td>
<td>308 ± 10.6</td>
<td>334</td>
<td>7.8</td>
<td>18.1 ± 0.3</td>
</tr>
<tr>
<td>Soyabean meal</td>
<td>40 ± 3.4</td>
<td>331 ± 7.2</td>
<td>371</td>
<td>10.7</td>
<td>19.1 ± 0.4</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>210 ± 8.7</td>
<td>73 ± 4.6</td>
<td>283</td>
<td>74</td>
<td>20.0 ± 0.3</td>
</tr>
</tbody>
</table>

Total GA refers to GA₃ + GA₄. Concentrations are averages of three flasks each ±SD

considerably higher levels of total gibberellin (422 mg l⁻¹) and GA₄ (92 mg l⁻¹), although the ratio of GA₄ to total gibberellin was 22%.

Effect of nitrogen sources on GA₄ production by Mor-189

The growth of Mor-189 and the production of GA₄ 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 GA₄ production. It was observed that Mor-189 produced the highest level of GA₄ (210 mg l⁻¹) in wheat gluten medium, and the proportion of GA₄ 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 GA₄ was produced by Mor-189 using wheat gluten as the nitrogen source. This high GA₄ 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 GA₄ production.

Fed-batch fermentation for GA₄ 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 DO₂ 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 GA3 and GA4 by Mor-189 in a 14 l agitated fermenter. Filled squares, agitation speed in rpm; open diamonds, DO% in % air saturation; filled circles, dry cell weight in g l⁻¹; filled triangles, GA4 concentration in mg l⁻¹; unfilled triangles, GA3 concentration in mg l⁻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 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 GA4 production rate remained almost the same, and so there was no direct correlation between glucose utilization rate and GA4 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 GA4 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 GA4 production. The specific GA4 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 GA4 production started to decline after 5 days and reached 3.8 mg l⁻¹ h⁻¹, probably because of culture aging. The concentration of GA4 finally reached 600 mg l⁻¹ in 168 h. The combined concentration of the two gibberellins GA4 and GA3 finally reached 713 mg l⁻¹, with GA4 accounting for 84% of the total gibberellin. The production of GA4 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 GA3 and GA4. A 250 mm column was more suited to the analysis of GA4. Barendse et al. [2] and Gallazzo and Lee [9] have described the chromatographic separation of GA7 and GA4. These earlier investigators mentioned that, under the chromatographic conditions they used, GA7 eluted just before GA4, 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 GA7 just before the GA4 peak.

In the present investigation, GA4 eluted at 12.20 min (Fig. 3). The retention volume of GA4 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 GA4, 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 GA4 peak. 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 GA₄ by HPLC. The analysis was done on an RP C₁₈ Lichrospher 100, 250 x 4 mm column with a mobile phase consisting of 60% acetonitrile and 1% acetone in 5 mmol 1⁻¹ 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 GA₃ by HPLC. The analysis was done on an RP Lichrospher 100, 125 x 4 mm column with a mobile phase consisting of 20% acetonitrile in 5 mmol 1⁻¹ 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

Thus, the fermentation broth was substant­ially free of GA₇.

The analysis of GA₄ 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 (GA₃) and of the fermentation broth of mutant Mor-189. The GA₃ eluted at 6.12 min. The retention volume of the GA₃ peak was 3.6 ml under the present experimental conditions.

Discussion

GA₄ 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 GA₄ are well doc­umented, its commercial use in agriculture has remained rather limited compared to GA₃, presumably because of limited availability of the product and its very high cost in the market. Procedures for obtaining GA₄ and a mixture of GA₄ and GA₇ by fermentation using G. fujikuroi have been patented [9, 12, 32], but little information has been pub­lished regarding the production of GA₄ 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 GA₃. 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
In shake flasks, it is normally difficult to maintain the pH at any desirable value. The use of sterile CaCO₃ 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, GA₄ was the main product at a low pH, while the concentrations of GA₃ and GA₇ were higher at neutral pH. Although maintaining the pH at 5.6 is reported to be beneficial in GA₄ production by mutants selected specifically for its production [9], in the present investigation, maintaining the pH above 5 resulted in a marginal increase in the proportion of GA₄ produced by the parent and all the mutants studied. In our earlier investigation [20], we observed that the GA₃ 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 GA₃ 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 GA₄ 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 GA₄ 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 GA₃ and GA₄ 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 GA₄, and its proportion increased to 74%. Thus, wheat gluten was found to be suitable for GA₄ 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 GA₄ to GA₇ and then to GA₃ 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 GA₄ 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 GA₄ by G. fujikuroi. Transferring the process from the shake flask level to a 10-l agitated fermenter allowed better control over the growth and GA₄ production by the mutant Mor-189, and we successfully increased the GA₄ 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 (GA₄) production by mutagenesis and media optimization. The study indicated that, along with the pH, the nitrogen source used was a factor that significantly affects GA₄ production by the mutant Mor-189. 2.8-fold more GA₄ was produced in wheat gluten medium after 168 h of fermentation. Studies to define the exact role of wheat gluten in the enhanced GA₄ production exhibited by the Mor-189 mutant of G. fujikuroi are in progress.

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