Chapter 3

_Fusarium fujikuroi_ Cultivation in
Shake Flask
Abstract:

Five *Fusarium* sp. were obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune and maintained on potato dextrose agar slants. The cultures were inoculated in liquid medium for fermentation (LMF) in 250 ml Erlenmeyer flasks and cultivated for seven days for production of gibberellins. A selected strain, *F. fujikuroi* NCIM 1019, was subjected to mutagenesis for enhanced production of gibberellic acid. Several mutagenesis rounds were performed using intermittent mutants as parents for the next round. In all more than one thousand colonies were selected and cultivated for gibberellin production in test tubes and in Erlenmeyer flasks. Attempts of intergeneric protoplast fusion between *F. fujikuroi* and *Trichosporon cutaneum* and yielded a new culture that produced gibberellic acid and still had yeast-like morphology. A mutant, Mut189 was selected because of its short filament length, low viscosity in liquid fermentation medium and higher gibberellic acid production. Mutant Mut189 was a stable mutant and produced around 400 mg/l gibberellic acid under similar cultivation conditions.
Chapter 3

Flow Sheet of Work

Screening and Selection of Fusarium strain
for Production of GA₃

Growth and Maintenance of
F. fujikuroi Strains

Screening of Carbon and
Nitrogen sources

Strain Improvement

Mutagenesis of Selected Strain
of F. fujikuroi

Mutant Selection

Preliminary Screening

Shake Flask Screening

Comparison of Mutants

Selection of Improved GA₃
Producing Mutant Strain

Charaterization of Selected
Mutant

Intergeneric Protoplast
Fusion

Optimization of Protoplast
Formation

Separation and Regeneration of
Protoplasts

Protoplast Fusion and Selection
of Fusant

Production of GA₃ by Selected
Fusant
3.1 INTRODUCTION

The power of the microbial culture in the competitive world of commercial synthesis can be appreciated by the fact that not only structurally complicated bio-molecules but even simple molecules are currently made by fermentation more economically rather than by chemical synthesis. Although the chemical synthesis and technologies have evolved rapidly in the last few decades, many natural products are still so complex that they probably will never be made commercially by chemical synthesis.

Microbial metabolites can be largely grouped into primary and secondary metabolites. Microbial strains isolated from the nature normally produce tiny amounts of secondary metabolites. Although secondary metabolites are not essential for microbial growth, they are very important for the health, and nutrition of human and animals and therefore for sound economics of our societies (Berdy, 2005). The secondary metabolites are not essential for the growth of the producing cultures but the microbial cultures need small amounts of these compounds for their own competitive benefit (survival) in nature. The secondary metabolites are complex molecules synthesized through series of complex energy consuming steps and therefore the wild cultures do not overproduce these metabolites (Demain and Fang, 2000). Regulatory mechanisms have evolved in microorganisms that enable a strain to avoid excessive production of its metabolites. Thus, strain improvement programs are absolutely required for commercially viable production of very useful secondary metabolites. The strain improvement programmes normally initiate with an aim of isolating cultures from nature, exhibiting desired phenotypes (Adrio and Demain, 2006).

Commonly, the ability of a microbial strain to produce higher concentrations of a compound of interest is what is desired, although the spectrum of improvements can also include other traits. Several procedures are employed to improve microbial strains and all of them bring about changes in DNA sequence. These changes are achieved by mutation, genetic recombination, or the modern DNA splicing techniques of ‘genetic engineering’. The ease with which ‘permanent’ characteristics of microorganisms could be changed by mutation and the simplicity of the mutation techniques had tremendous appeal to microbiologists. The substantial increases in fermentation productivity and the resulting decreases in costs have come about
mainly by mutagenesis and screening for higher producing microbial strains (Demain and Adrio, 2008).

Mutation has been the major factor involved in the hundred to thousand-fold increased production of microbial metabolites. It has the ability to modify genetically a microbial culture to higher productivity, the most important factor in keeping the fermentation industry in its viable, healthy state (Vinci and Byng, 1999; Parekh et al., 2000). It has also been used to shift the proportion of metabolites produced in a fermentation broth to a more favourable distribution, elucidate the pathways of secondary metabolism, yield new compounds, and for other functions. Classical strain development has typically relied on mutation and systematic screening of improved strains. Application of strain improvement to new fermentation processes continued to be documented in the literature despite the age of the technology. Strain improvement has been the main factor involved in the achievement of impressive titers of industrial metabolites. *Penicillium chrysogenum* X-1612 was the first superior penicillin producing mutant, isolated after X-ray mutagenesis. This was the beginning of a successful relationship between mutational genetics and industrial microbiology (Hersbach et al., 1984). Through strain improvement program the production of tetracycline was reported to be increased over 20 g/l (Podojil et al., 1984) while production of penicillin 70 g/l and that of cephalosporin C over 30 g/l (Elander, 2003).

The most common method used to obtain high yielding mutants is to treat cells of a desired culture with a mutagenic agent until a desired kill rate is obtained, plate out the survivors on suitable, preferably selective media and test each resulting colony or a randomly selected group of colonies for product formation in shake flasks. The most functional mutagens comprise N-methyl N-nitro N-nitroso guanidine (NTG), methyl methane sulphonate (MMS), ethyl methyl sulphonate (EMS), hydroxylamine (HA) and ultraviolet light (UV) (Adrio and Demain, 2006; Parekh et al., 2000). Overall yield improvement has depended mainly on mutation and selection, combined with optimization of fermentation conditions.

Strain improvement may also be possible by the introduction of extra copies of genes of which the products are rate-limiting, or of genes conferring beneficial growth characteristics. Protoplasts are widely used for making genetically modified
organisms, since the cell wall would otherwise block the passage of DNA into the cell. The use of protoplast fusion has been reported to improve a wide range of industrial strains of bacteria and fungi including *Streptomyces*, *Nocardia*, *Penicillium*, *Aspergillus*, and *Saccharomyces*. This technique is frequently employed in the brewing industry for improving yield and incorporating traits are not easily achievable through simple mutation (Parekh, 2009).

In recent years, frequencies of recombination have increased and strain improvement programs now routinely include protoplast fusion between different mutant lines (Demain and Adrio, 2008). Although a considerable number of reports have appeared on the fusion of protoplasts of industrially useful microorganisms such as yeasts, filamentous fungi and bacteria, with regards to *F. fujikuroi*, intergeneric protoplast fusion has not been reported. Current literature did not reveal any use of protoplast fusion techniques to improve GA\textsubscript{3} production by *F. fujikuroi*.

*F. fujikuroi* is a mesophilic fungus with septate, branched mycelial morphology. The strains belonging to mating type ‘C’ produce less conidia and are prolific producers of gibberellins (Giordano *et al.*, 1999; Tudzynski *et al.*, 2005). Production of gibberellins by *F. fujikuroi* involves a long biochemical pathway and is a result of multi-gene activity; therefore the conventional mutagenesis is a preferred method for strain improvement in order to obtain enhanced gibberellins (GA\textsubscript{3}) producing mutants. It is a common observation that *F. fujikuroi* grows in viscous, filamentous form in liquid medium and the broth behaves in non-Newtonian manner. To increase the gibberellin productivity, it is necessary to increase the cell mass in the fermentation broth and in doing so; the culture broth becomes extremely viscous. This adversely affects the mixing and oxygen transfer rate. Viscosity of the culture broth and low solubility of oxygen in aqueous broth often leads to dissolved oxygen limitation during fermentation. Availability of dissolved oxygen governs the concentrations and ratio of secondary metabolites produced by *F. fujikuroi* during fermentation (Giordano and Domenech, 1999). Any change in morphology of the fungal strain that lowers the viscosity can result in improved oxygen transfer and in turn, increase the GA\textsubscript{3} production as they are oxidation products. Morphological mutants affected in mycelia formation, which produce colonies with a modified
appearance or new colours were found to be useful in strain improvement (Adrio and Demain, 2006).

*F. fujikuroi* produces two types of pigments, the polyketide bikaverin (Kajer et al., 1971) and the carotenoid neurosporaxanthin (Avalos and Cerda-Olmedo, 1987). Earlier, Candau et al., (1991) investigated mutants of *F. fujikuroi* for pigment accumulation. They had studied strain improvement of *F. fujikuroi* by screening of mutants with blocked carotenoid biosynthesis and reported that mutant strains with lower levels of neurosporaxanthin can produce more gibberellins. A decrease in production of pigments like bikaverin and carotenoids by *F. 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. Thus, in addition to improvement in gibberellin producing capabilities, undesirable properties, especially growth characteristic exhibiting viscosity to broth, undesirable other secondary metabolite formation can be eliminated by classical mutagenesis.

Interestingly, the compositions of fermentation products of *F. fujikuroi* also depend on the nature, composition and concentration of media constituents as well. The effect of a variety of carbon and nitrogen sources in nutrient medium on gibberellic acid (*GA*₃) production has been investigated by Gohlwar et al., (1984). All reported media yielding high amount of *GA*₃ contained low concentrations of nitrogen content. It is also reported that complex nutrients like peanut meal, soya meal and corn steep liquor positively affected *GA*₃ biosynthesis (Sanchez-Marroquin, 1963; Fuska et al., 1961; Podojil and Ricicoca, 1964). *GA*₃ production in complex media containing glucose as carbon source has been well documented. However, gibberellins biosynthesis was indicated to be suppressed by high amount of glucose (Borrow et al., 1964). There are a few reports available on *GA*₃ production with alternative carbon sources like sucrose, slowly utilisable carbon sources like starch, combination of fast and slowly utilisable carbon sources (Gonzalez et al., 1994) and also some oils such as sunflower oil, cooking oil, rapeseed oil (Gancheva et al., 1984).

An extensive search was carried out for the medium constituents for *GA*₃ production by fermentation and it was found high C: N ratio is essential parameter for its
production. Nitrogen limitation is the prerequisite for GA₃ formation because GA₃ production starts towards the end of the growth phase with the exhaustion of nitrogen source from the medium. Based on literature survey, a liquid fermentation medium was constituted to screen the cultures for GA₃ production.

Overall successful improvement in gibberellic acid production depends upon genetic makeup of the strain, along with physiological, nutritional and cultural parameters employed during fermentation as summarized below.
The objective of the present section of work was 1) selection of wild strains of *Fusarium fujikuroi* obtained from National collection of Industrial microorganism, NCL, Pune for their ability to produce GA₃, 2) to enhance GA₃ production and minimize unwanted by-products of *F. fujikuroi* by classical mutagenesis and 3) through primary and secondary screening study selection of morphologically altered mutant with enhanced GA₃ production.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Microorganisms

Five fungal strains namely *F. fujikuroi* NCIM 665, *F. fujikuroi* NCIM 850, *F. fujikuroi* NCIM 1035, *F. fujikuroi* NCIM 892, *F. fujikuroi* NCIM 1019 were obtained from National Collection of Industrial Microorganism (NCIM), CSIR-National Chemical Laboratory, Pune, India. These cultures were grown on Potato Dextrose Agar plates by spread plate technique using dilute suspensions and pure colonies were transferred to potato dextrose agar (PDA) slants (HiMedia Mumbai, India) supplemented with 2 g/l yeast extract for maintenance. The mutants generated during experiments of strain improvement were also maintained on PDA agar slants. For long-term storage of the selected mutants, soil culture and glycerol stocks were prepared and maintained at 4 °C and -80 °C, respectively. Agar slopes were incubated at 28 °C for 3–4 days and stored at 4 °C. The regeneration agar (RA) was used for growth of the survivors after mutagenesis.

#### 3.2.2 Chemicals

Ethyl methyl sulphonate (EMS), analytical grade sugars, Enzyme Novozyme 234, Polyethylene glycol (PEG, MW 3500) and gibberellins (GA₃ and GA₄) were purchased from Sigma-Aldrich, USA. All media ingredients and nystatin were purchased from HiMedia, Mumbai, India. Defatted soyabean meal and defatted cottonseed meal was from Chandrasekhar Exports Pvt. Ltd. (Kolhapur, India) while defatted peanut meal was from local cattle feed. Cane sugar was purchased from local sources. Pravastatin and Lovastatin were kind gifts from Lupin Pharmaceuticals Ltd. Mumbai, India.
3.2.3 Media Compositions

Media compositions used in the experiments were as follows

1) Potato Dextrose Agar (PDA)

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

<table>
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<td>NaCl</td>
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<td>MgSO₄.7H₂O</td>
<td>0.2</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
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<tr>
<td>Yeast extract</td>
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<tr>
<td>Glucose</td>
<td>30.0</td>
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<tr>
<td>Defatted peanut Meal</td>
<td>9.0</td>
</tr>
<tr>
<td>Trace mineral solution*</td>
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</tr>
<tr>
<td>pH</td>
<td>6.8 before autoclaving</td>
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</table>

3) Regeneration Agar (RA)

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<td>Na₂MoO₄.2H₂O</td>
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<tr>
<td>Yeast extract</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Soyapeptone</td>
<td>3.0</td>
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<tr>
<td>Bile salt</td>
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<tr>
<td>Trace mineral solution</td>
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Chapter 3

Agar 20.0
pH 6.8 before autoclaving

4) Liquid Medium for Fermentation (LMF)

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</thead>
<tbody>
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<tr>
<td>NaCl</td>
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<tr>
<td>MgSO$_4$.7H$_2$O</td>
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<td>Na$_2$MoO$_4$.2H$_2$O</td>
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<td>Glucose</td>
<td>30.0</td>
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<tr>
<td>Ammonium nitrate</td>
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<td>Trace mineral solution*</td>
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</tr>
<tr>
<td>pH</td>
<td>6.8 before autoclaving</td>
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</tbody>
</table>

5) Malt Extract Glucose Yeast extract Peptone agar (MGYP)

<table>
<thead>
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<th>Ingredient</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Malt Extract</td>
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<td>Glucose</td>
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<td>Yeast extract</td>
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<td>7.0 before autoclaving</td>
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</tbody>
</table>

6) Trace Mineral Solution*

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<th>g/l</th>
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<tbody>
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<tr>
<td>MnCl$_2$.7H$_2$O</td>
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</tr>
<tr>
<td>CuCl$_2$.7H$_2$O</td>
<td>0.05</td>
</tr>
<tr>
<td>FeCl$_2$</td>
<td>0.1</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.1</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in distilled water and a few drops of concentrated HCl were added till solution became clear.
3.2.4 Growth in Tube Culture

Small pieces of respective young cultures from PDA slants were suspended in 0.5 ml sterile physiological saline, mycelium was teased with sterile thick wire instead of a loop and the suspension was transferred to 5 ml liquid medium in 150 x 25 mm test tubes without rim and the tubes were incubated at 28 °C, 220 rpm, for 48 h.

3.2.5 Growth in Shake flasks

In case of shake flask cultures, the medium volume used was 45 ml in 250 ml Erlenmeyer flasks throughout the experiments and 5 ml inoculum was used to make the final volume 50 ml at the beginning of the experiments unless otherwise stated. All 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. Care was taken that the shake flask cultures do not remain stationary even for few minutes during sample withdrawal from the flasks.

Whenever other carbon sources were to be used, in place of glucose, they were used at 60 g/l and alternative nitrogen sources were used on equal nitrogen basis. Carbon sources were autoclaved separately for all the experiments and later mixed with other media components.

3.2.6 Screening of strains for GA₃ production

In an effort to select a suitable strain of the fungus for production of gibberellic acid, the cultures obtained from NCIM were screened for GA₃ production by inoculating them in 250 ml Erlenmeyer flasks with 50 ml LMF and incubating the flasks at 28 °C, 220 rpm for 168 h. During incubation samples were withdrawn aseptically at regular interval and analyzed for dry cell weight (DCW), pH, residual sugars and GA₃ concentration by high performance liquid chromatography (HPLC). The culture broth was filtered over qualitative filter paper circle under vacuum and the filtrate was used for analysis of residual sugar and gibberellic acid. The cell mass residue was washed with three volumes of distilled water to remove adhering soluble compounds from the medium and observed for conidia, cell and filament morphology, oil storage vacuoles and pigmentation. The parent strain were studied in
shake flask cultures for 1) repeated production of more GA3, 2) relatively less production of soluble as well as cell bound pigments 3) relatively few micro-conidia and macro-conidia and 4) and short mycelial length measured using a microscope and stage micrometer so that it would have lower viscosity in fermentation broth.

3.2.7 Choice of carbon source

Utilization of carbon sources namely glucose, sucrose and fructose was investigated for growth and GA3 production by *F. fujikuroi* NCIM 1019. The carbon sources (60 g/l) were autoclaved separately and later added to remaining constituents of LMF. Flasks were inoculated with respective seed cultures and incubated at 28 °C, 220 rpm. Samples were analysed after 168 h as described earlier.

3.2.8 Screening of nitrogen sources for selected GA3 producing cultures

The shake flask experiments were done to evaluate the nitrogen sources for GA3 production by *F. fujikuroi* NCIM 1019. The effect of nitrogen source was studied in LMF with different inorganic and organic nitrogen sources added at concentration equivalent to 0.7 g/l “N”. The nitrogen sources were used on equal “N” basis. Respective cultures were inoculated in 250 ml Erlenmeyer flasks with 45 ml medium and incubating at 28 °C, 220 rpm for 168 h. Samples were analyzed for dry cell weight, pH, and residual glucose and GA3 concentration.

3.2.9 Mutagenesis of selected strain

The parent strain *F. fujikuroi* (NCIM 1019) that was found to qualify most of the conditions laid down for strain selection (described in 3.2.6 above), was grown in 50 ml liquid medium (LMG) for growth, at 220 rpm and 28 °C for 72 h. The selected strain of the *F. fujikuroi* grows in a viscous mycelial form and produces very less or no conidia in plate cultures or submerged cultures. For mutagenesis it is necessary to have individual cells as colony forming units (CFU). The mycelial culture was filtered over sterile sintered glass funnel G-0, (Borosil, Mumbai, India) to get short fragments in the filtrate and then the filtrate was further filtered through sterile absorbent cotton layer of 1 cm thickness to get short fragments or individual cells for experiments with mutagenesis. The suspension of individual cells or of 1-2 cell fragments was collected and used for mutagenesis programme.
Three different approaches were employed for mutagenesis. Survival curves were drawn from the number of surviving cells with respect to time for each of the mutagen used and optimum time and concentrations were determined for the respective mutagens in order to get 90-95 % kill rate.

(1) UV irradiation: A small mechanical device fabricated using aluminum sheets with a UV lamp inside was used. This device allowed accurate exposure time of UV radiation without switching the UV lamp ON and OFF. Cells or fragments were counted under a microscope using Neubauer improved cell counting chamber (Marienfeld Germany). The cell number was adjusted to $1 \times 10^5$ cells/ml either by centrifugation followed by suspension in desired volume of sterile physiological saline or by dilution with sterile physiological saline. Twenty microlitre of this suspension were spread inoculated on regeneration agar plates. The plates were exposed to germicidal Ultra Violet (UV) radiation (Sankyo Denki Co. Ltd., Japan) for 0 to 15 seconds at a distance of 20 cm. The exposure time was controlled by changing the shutter position in the UV exposure device described above. The schematic representation of UV exposure chamber is given in Fig. 3.1.

![UV exposure chamber](image)

**Figure 3.1** Schematic representation of UV exposure chamber

(2) Ethyl Methyl Sulphonate (EMS) treatment: To 1 ml suspension of the cells/fragments ($1 \times 10^5$ cells/ml), 20 μl EMS was added, mixed thoroughly, and exposed
for different time intervals from 0 to 60 minutes. At fixed time interval, 0.5 ml of the treated cell suspension was withdrawn aseptically and transferred to 0.5 ml filter sterilized sodium thiosulfate solution (5%) to inactivate EMS.

(3) Combination of UV and EMS treatment: This treatment was carried out by irradiation of the cell suspension with UV for 5 seconds followed by the EMS treatment for 30 minutes as described above.

Mutagenesis programme was initiated with UV and the surviving colonies were screened for morphological variations and less pigmentation. Subsequently, UV mutagenesis was coupled to chemical mutagenesis. The sequence of mutagenesis procedures used in this study was UV → EMS → UV + EMS.

To exert higher selection pressure for selecting mutants, in some of the mutagenesis series, regeneration agar (RA) plates were incorporated with 250 mg/l Pravastatin or Lovastatin. Pravastatin inhibits synthesis of sterols and other secondary metabolites because it is a specific inhibitor of Hydroxy Methyl Glutaryl CoA (HMG-CoA) reductase. In some of the mutagenesis series, plates were incorporated with 4000 U/100 ml concentration Nystatin (polyene antibiotic) or Diphenylamine 25 mg/100 ml (an electron transport decoupler). Nystatin was dissolved in HPLC grade ethanol and then added to the molten agar before pouring the plates. The regeneration agar plates were incubated at 28 °C, for 5-6 days in dark, till colonies developed.

3.2.10 Mutant selection

After incubation of plates with treated cells at 28 °C for five to six days, the plates, which showed 5-10 % of survival, were selected. Colonies with different growth characteristics, appearance and pigmentation were marked, recorded, picked up and transferred to PDA agar slants for maintenance. Morphological observations of the colonies on plates and growth on slants were performed with respect to shape, size, pigmentation, surface appearance, roughness, margins and thickness.

3.2.10.1 Screening of the selected mutants in tubes

The preliminary screening was done in test tube cultures. For this, a small piece of the mycelium from freshly prepared slant was teased in sterile physiological saline as
described earlier and inoculated in 4.5 ml liquid medium for fermentation (LMF) in 25 X 150 mm test tubes. Cultures were incubated at 220 rpm 28 °C on rotary shaker for 5 days. The liquid cultures were observed microscopically for mycelium length and cell thickness. The mycelium was filtered using Whatman Grade 1 filter paper and filtrates were analyzed for pH, residual sugar and GA₃ concentration.

3.2.10.2 Shake flask screening of selected mutants for GA₃ production

The mutants that showed 15-20% more gibberellic acid production in preliminarily screening than their respective parent were studied in shake flask experiments. Five ml 48 h grown seed cultures in test tube were transferred to 45 ml LMF in 250 ml Erlenmeyer flasks and incubated for 5 days at 220 rpm 28 °C on rotary shaker as described in 3.2.4.2 above. The liquid cultures were observed microscopically for mycelial length and thickness. The apparent viscosity in shake flask cultures was noted in terms of "high medium and low". Samples were analyzed for dry cell weight (DCW), pH, residual glucose and GA₃ concentration. The mutants with enhanced GA₃ production were used as intermediate parent strains for mutagenesis for further desired strain improvement. Mutants which showed higher glucose utilization, enhanced GA₃ production, low or no pigmentation and morphological change that will suit the fermentation broth rheology, compared to parent, were selected. Intermediate mutants were also screened for enhanced GA₃ production by optimizing key medium components (carbon and nitrogen sources and their concentration) and the process parameters in shake flask.

3.2.10.3 Comparison of selected mutants

Viscosity of the fermentation broth is a major issue in all fungal fermentation because of the mycelial nature of the organism. The selection of the mutants in the present investigation was dependent upon growth characteristics and lower viscosity of the liquid cultures in shake flasks. Medium volume and strength of medium in Erlenmeyer flasks exerts effect on metabolism because of differences in the oxygen transfer rates and the dissolved oxygen levels in the fermenting mass. To investigate this, in one experiment, the medium volumes in 250 ml Erlenmeyer flasks were varied to have 15, 30, 45 and 60 ml LMF.
In another set of experiment, the concentrations of the ingredients in the medium were increased from 1 fold to 1.25, 1.5 and 1.75 fold keeping volume constant as shown in Fig. 3.2 below. These Erlenmeyer flasks with different medium volumes or medium strengths were inoculated with 48 h old 10% (v/v) inoculum of four selected mutant strains (with approximately 18 g/l dry cell mass).

All the experiments were performed in triplicates. The flasks were incubated on rotary shaker at 220 rpm 28 °C, for 5 days. The volumes of the cultures were corrected with distilled water. Samples were analysed for dry cell mass, pH, residual sugar and gibberellic acid concentration using HPLC as described earlier.
3.2.11 Characterization of selected mutant Mut189 of *F. fujikuroi*

3.2.11.1 Morphology

Mutant strain, Mut189 that showed improved gibberellic acid production in the screening studies was selected for optimization of fermentation medium and process parameters. Morphological and biochemical variations of the mutant compared to its parent were studied. Morphological variations of the mutant Mut189, compared to its parent *F. fujikuroi* NCIM1019 were examined by phase contrast and scanning electron microscopy (SEM) as detailed below.

Respective cultures were grown in fermentation medium for 48 h and washed twice with sterile physiological saline. Appropriately diluted cell suspensions were visualized under phase contrast microscope. For studies using SEM, a small sample was applied on the Polydimethrlsiloxane (PDMS) and dried under vacuum at room temperature for 30 min and visualized under Scanning electron microscopy (Quanta 200 3D, FEI). Multiple images were obtained. Optical images were taken with Nikon Eclipse, E600-POL (Japan).

3.2.11.2 Carbohydrate utilization

Carbohydrate utilization pattern of *F. fujikuroi* NCIM1019 and its mutant Mut189 was studied using various sugars such as arabinose, cellobiose, fructose, galactose, glucose, glycerol, maltose, mannose, raffinose, rhamnose, sucrose, and xylose. A small piece of the mycelium from freshly prepared slant was spot inoculated on the agar plates of media containing 25 g l⁻¹ of each of the above mentioned sugars. Plates were incubated at 28 °C and observed for growth. The growth on different sugars was compared with respect to colony size, shape, surface appearance, roughness, margins and pigmentation.

3.2.12 Protoplast fusion

Genetic manipulation in filamentous fungi can be successfully achieved through fusion of protoplast. Interspecific, intraspecific and intergeneric hybridation could be
done by this technique and strain improvement of industrial fungal strains is possible (Lalithakumari, 2000).

Intergeneric fusion of protoplasts is a means of acquiring desirable strain characteristic from other species. The present study was aimed to optimize the conditions for the isolation of viable protoplasts and to attempt intergeneric protoplast fusion between the selected mutant of *F. fujikuroi* and a yeast culture *Trichosporon cutaneum* (NCIM 3352) to obtain a yeast-like fusant producing GA<sub>3</sub>.

**Figure 3.4** Scheme of fusion for transference of genes for GA<sub>3</sub> production from fungi into the yeast culture
3.2.12.1 Microorganisms

Intergenic protoplast fusion was attempted between the selected mutant of *F. fujikuroi* and a yeast culture. Mut32, a mutant strain of *F. fujikuroi* selected for protoplast fusion was producer of carotenoid pigment and GA₃. The yeast, *Trichosporon cutaneum* (NCIM 3352) was selected on the basis of non-fermenting, non-pigmented and non-pseudo mycelium formation. The strains were maintained on PDA agar slants.

3.2.12.2 Optimization of protoplast formation

Protoplasts were prepared using commercial lysing enzyme (Novozyme 234, Sigma). To achieve maximum protoplast formation, age of the cultures, concentration of lysing enzyme and osmotic stabilizer were optimized as described below.

To optimize conditions for protoplasts formation preparation of fungal mycelium and yeast cells was performed. The Mut32, mutant strain of *F. fujikuroi* and *Trichosporon cutaneum* (NCIM 3352) were grown in 250 ml Erlenmeyer flasks containing 25 ml potato dextrose broth. The flasks were incubated on a rotary shaker at 220 rpm, 28 °C. The young mycelium of *F. fujikuroi* was separated by filtration while yeast cells were concentrated by centrifugation at 10,000 rpm for 10 min. Fresh mycelium and yeast biomass each were washed separately with sterile distilled water followed by washes with sterile osmotic stabilizer. About 100 mg fresh mycelium/yeast biomass with lytic enzyme in 100 ml shake flasks was incubated on rotary shaker at 100 rpm, 28 °C. The lysis of cell wall and the release of protoplasts were monitored at 30 min intervals under a light microscope. The protoplasts were counted using Neubauer improved cell counting chamber (Marienfeld Germany).

Culture age which is suitable for the isolation of maximum protoplasts was standardized by growing the test organisms, Mut32 and *T. cutaneum* NCIM3352 for 24 h as described above. The cultures were harvested at different time interval studied for the isolation of protoplasts. The commercial lysing enzyme Novozyme 234 tested with 2, 5, 10, 15 mg/ml concentration for both the cultures. While different osmotic stabilizers such as mannitol, potassium chloride, magnesium sulphate, sodium chloride were used and studied at a fixed concentration 0.6M and pH 6.8 for maximum protoplast formation.
3.2.12.3 Separation and Regeneration of protoplasts

Protoplasts of fungal culture Mut32, mutant strain of *F. fujikuroi* were separated by filtration over sterile cotton and a layer of tissue paper. While yeast *Trichosporon cutaneum* (NCIM 3352) protoplasts were isolated by centrifugation at 1000 rpm for 10 min. The resultant filtrates containing protoplasts of the both cultures were washed with osmotic stabilizer to remove the lytic enzyme remnants by centrifugation. The sediment protoplasts were re-suspended in known amount of osmotic stabiliser, their purity was checked under microscope and the number of protoplasts counted using Neubauer improved cell counting chamber (Marienfeld Germany). The protoplast suspension was spread inoculated on MGYP agar medium with and without 0.8 M KCl as osmotic stabilizer. Plates were incubated at 28 °C till colonies appeared. Growth only on MGYP agar plates with osmotic stabilizer confirms the absence of fungal or yeast cells in the suspension of protoplasts.

3.2.12.4 Intergeneric protoplast fusion and selection of fusant

Protoplasts were fused according to method of Ferenczy and Pesti (1982) with little modifications. One ml of the suspension containing $10^5$ protoplasts in phosphate buffer (0.6 M MgSO$_4$ at pH 6.8) was prepared and equal number of protoplasts from Mut32 and *T. cutaneum* were mixed. The mixture was centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in fusion mixture 30% polyethylene glycol (PEG, MW 3500) in 10 mM CaCl$_2$ solution and mixed gently by rolling the tube. The suspension was incubated for 10 minutes at room temperature. The fusion mixture was serially diluted with the osmotic stabilizer and plated on soft MGYP agar medium (Agar 1%) amended with 0.8M KCl osmotic stabilizer. Plates were incubated at 28 °C till colonies appeared. Fusants were selected based on change in their colony characteristics and morphology. Colony looking like yeast with pigmentation not normally present in the parent yeast was selected and studied further for GA$_3$ production.

3.2.12.5 Production of GA$_3$ by Selected Fusant

The selected yeast like hybrid strains were purified by three times plate streaking from very dilute suspensions and studied further for GA$_3$ production in shake flask cultures. Five ml 48 h grown seed cultures of parent fungus, parent yeast and
selected fusant were transferred to 50 ml LMF in 250 ml Erlenmeyer flasks and incubated for 5 days at 220 rpm, 28 °C on rotary shaker. The liquid cultures were observed microscopically for morphological differences. The apparent viscosity in shake flask cultures was noted as low, medium and high. Samples were analyzed for pH, residual glucose and GA$_3$ concentration by HPLC and further confirmed by LC-MS.

3.3 RESULTS AND DISCUSSION

3.3.1 Selection of strain for production of GA$_3$

Screening of microbial strains, their performance and suitable environment for GA$_3$ production is the most essential aspect to develop the technological solution for GA$_3$ production using biotechnological process. The effort was to identify GA$_3$ producing *Fusarium* strain, to study the strains using various culture conditions and consequently select the best strain suitable for mutagenesis for further desired improvements in GA$_3$ production. Newly isolated microbial strains and their mutants can be competitive to the current industrial production process, additionally productivity and yield could be further increased by optimizing different fermentation methods.

At present GA$_3$ is produced by submerged fermentation technique using fungus *F. fujikuroi*. It has been reported that *F. fujikuroi* is a complex of eight mating populations (MP-A to MP-C). All these *Fusarium* species are fungal pathogen of various crops such as maize, rice, barley and many more. So far, the ability to produce GAs and to cause bakanae disease has been confirmed only for rice isolate belonging to the species *F. fujikuroi* (sexual stage: *G. fujikuroi* MP-C) (Tudzynski, 1999). It is also reported that the strains belonging to mating type ‘C’ produce less conidia and are prolific producers of gibberellins (Giordano et al., 1999). Other fungi such as *Sphaceloma manihoticola*, *Neurospora crassa*, *Phaeoshaeria spp.* also produce some gibberellins but their yields are too low to be commercially available (MacMilan, 2002).

On the basis of these earlier reports in literature five *Fusarium* strains namely *F. fujikuroi* NCIM 665, *F. fujikuroi* NCIM 850, *F. fujikuroi* NCIM 1035, *F. fujikuroi*
NCIM 892, *F. fujikuroi* NCIM 1019 were selected from National Collection of Industrial Microorganisms (NCIM) for screening of their ability to produce GA3.

The five strains of *F. fujikuroi* obtained from National Collection of Industrial Microorganisms (NCIM) were screened for GA3 production and growth. When these strains were grown in 50 ml liquid fermentation medium containing ammonium nitrate as nitrogen source and glucose as carbon source, they showed differences in growth characteristics as well as product formed. Strains grew in a free, long mycelia form in liquid cultures making the broth viscous. The dry cell mass reached to about 10 g/l in all the cultures (Table 3.1). As compared to other four strains, *F. fujikuroi* NCIM1019 grew in a highly viscous filamentous form in shake flask. It produced only a few micro-conidia while the other four strains produced large number of micro and macro-conidia on solid medium as well as in liquid cultures. Along with less conidia production, *F. fujikuroi* NCIM1019 comparatively exhibited low production of cell bound and water-soluble pigment. Growth characteristics of cultures on RA medium plate are presented in Table 3.2 and Fig. 3.5.

The sugar uptake was similar in liquid cultures of the five strains studied. On screening, all five cultures were found to produce GA3 in LMF as presented in Table 3.1. However, there were differences in GA3 concentration and their specific GA3 productivity. The strains produced between 15 and 76 mg/l GA3 in shake flask cultures in 7 days. The GA3 concentration (76 mg/l) in the broth of *F. fujikuroi* 1019 was considerably higher than that in the other *F. fujikuroi* strains. The specific GA3 productivity (8.36 mg GA3/g dry cell weight) and GA3 yield (1.34 mg GA3 g/sugar utilized) was also highest.

*F. fujikuroi* NCIM 1019 was selected amongst the five *Fusarium* species obtained from NCIM on the basis of its growth characteristics, very low conidia formation, relatively higher GA3 production and its specific productivity. This strain was used for further strain improvement program.
Table 3.1 Comparison of GA$_3$ production by five *Fusarium* strains

<table>
<thead>
<tr>
<th>Culture</th>
<th>Dry cell mass g/l</th>
<th>Sugar utilized g/l</th>
<th>GA$_3$ mg/l</th>
<th>GA$_3$ mg/g DCW</th>
<th>GA$_3$ produced mg/g sugar utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM 665</td>
<td>10.94</td>
<td>57.06</td>
<td>17.0</td>
<td>1.55</td>
<td>0.29</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 850</td>
<td>10.71</td>
<td>56.77</td>
<td>15.5</td>
<td>1.44</td>
<td>0.27</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 1035</td>
<td>6.54</td>
<td>56.92</td>
<td>16.0</td>
<td>2.44</td>
<td>0.28</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 892</td>
<td>6.44</td>
<td>56.67</td>
<td>40.5</td>
<td>6.28</td>
<td>0.71</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 1019</td>
<td>9.15</td>
<td>56.92</td>
<td>76.0</td>
<td>8.36</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Table 3.2 Colony characteristics of *Fusarium* strains on RA media

<table>
<thead>
<tr>
<th><em>Fusarium</em> strains</th>
<th>Size</th>
<th>Shape</th>
<th>Margin</th>
<th>Surface Appearance</th>
<th>Roughness</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM 665</td>
<td>Larger</td>
<td>Circular</td>
<td>Regular</td>
<td>Fibrous</td>
<td>Soft</td>
<td>Reddish purple</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 850</td>
<td>Larger</td>
<td>Circular</td>
<td>Regular</td>
<td>Fibrous</td>
<td>Soft</td>
<td>Reddish purple</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 1035</td>
<td>5-4cm</td>
<td>Circular</td>
<td>Regular</td>
<td>Fibrous</td>
<td>Soft</td>
<td>Dark purple</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 892</td>
<td>6-5cm</td>
<td>Circular</td>
<td>Regular</td>
<td>Fibrous</td>
<td>Soft</td>
<td>Reddish purple</td>
</tr>
<tr>
<td><em>F. fujikuroi</em> NCIM 1019</td>
<td>4-3cm</td>
<td>Circular</td>
<td>Regular</td>
<td>Cottony</td>
<td>Soft</td>
<td>Faint reddish purple</td>
</tr>
</tbody>
</table>
Figure 3.5 Growth of *F. fujikuroi* strains obtained from NCIM on RA agar

### 3.3.2 HPLC analysis

The HPLC analysis method used in the current investigation well resolved GA₃ present in fermentation broth. Chromatograms of the reference compounds and a sample of fermentation broth showing GA₃ are presented in Fig. 3.6.
Figure 3.6 Reversed phase HPLC chromatogram of A) standard compound GA₃ B) a sample of fermentation broth

Fig. 3.6 shows chromatograms of standard gibberellic acid (GA₃) and that of the sample of fermentation broth. The GA₃ eluted at 6.12 min by using the reverse phase HPLC as described in Chapter 2. Total run time was 12 min with mobile phase flow rate of 0.6 ml min⁻¹. Excellent peak separation was achieved under isocratic mode of elution. Online scanning showed a peak purity index of 999 for the standard GA₃ peak and had maximal absorbance (λmax) at 205 nm. GA₃ peak in the chromatogram of the fermentation broth had identical spectral pattern and peak purity index to that of the standard GA₃.

3.3.3 Choice of carbon source

To select the suitable carbon source for GA₃ production, *F. fujikuroi* NCIM 1019 was grown in the liquid fermentation medium with various carbon sources. Results
presented in Table 3.3 show that irrespective of carbon source used, growth of \( F. fujikuroi \) NCIM 1019 was almost similar. The cell mass ranged between 7.3 and 9 g/l with different carbon sources used. Morphologically no distinct difference was observed. Except glycerol, filtrates of other sugars were faint purplish colored.

Table 3.3 also shows that varying carbon sources in the culture medium affected \( GA_3 \) production by \( F. fujikuroi \) NCIM 1019. When glucose was used as carbon source it produced higher amount of \( GA_3 \) (82 mg/l) as compared to other carbon sources. Also, specific gibberellic acid productivity (9.1 mg \( GA_3/g \) dry cell weight) of \( F. fujikuroi \) NCIM 1019 was relatively higher in glucose containing medium.

Glucose and sucrose have been regularly used carbon sources in \( GA_3 \) fermentation study (Bruckner and Blechschmidt, 1991). It is also easy to use because concentrated solutions can be prepared and sterilized on larger scale. Glucose is a better choice of carbon source and was used as carbon source for \( F. fujikuroi \) NCIM 1019 for successive experiments.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Dry cell weight g/l</th>
<th>( GA_3 ) mg/l</th>
<th>( GA_3 ) mg/dry cell weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>9</td>
<td>82</td>
<td>9.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>8.52</td>
<td>41</td>
<td>4.81</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.32</td>
<td>45</td>
<td>5.41</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>7.52</td>
<td>56</td>
<td>7.45</td>
</tr>
<tr>
<td>Glycerol</td>
<td>7.3</td>
<td>32</td>
<td>4.38</td>
</tr>
</tbody>
</table>

**Table 3.3** Effect of different carbon sources on \( GA_3 \) production
3.3.4 Screening of nitrogen sources for selected GA₃ producing culture

Nitrogen limitation is the precondition for gibberellins formation as production of gibberellins starts towards the exhaustion of nitrogen source from the medium (Borrow et al., 1964). Therefore in present investigation C: N of 100:1 was maintained in liquid fermentation medium. The nitrogen sources used during the present study can be grouped in three main categories. Ammonium salts or nitrates, digests prepared from vegetable or animal proteins and complex defatted plant meals.

Results of effect of different nitrogen sources on GA₃ production, the growth and GA₃ productivity by *F. fujikuroi* NCIM 1019 presented in Fig. 3.8 and Fig. 3.9 respectively.

**Figure 3.7** Growth of *F. fujikuroi* NCIM 1019 on RA agar containing different carbon sources

- Glucose
- Sucrose
- Fructose
- Glycerol
- Soluble starch
Figure 3.8 Comparison of nitrogen sources for GA$_3$ production by *F. fujikuroi* 1019

Figure 3.9 Effect of nitrogen sources on the growth of *F. fujikuroi* 1019 and its specific gibberellic acid productivity

From Fig. 3.9 it can be seen that *F. fujikuroi* NCIM 1019 could utilize a wide variety of inorganic and organic nitrogen sources for the growth. The growth of *F. fujikuroi*
NCIM 1019 in terms of dry cell mass ranged between 5.92-10.85 and 12.82-18.49 g/l in media containing inorganic and organic nitrogen sources respectively. Among the inorganic nitrogen source based media, ammonium compounds were more suitable for the growth as well as gibberellic acid production by *F. fujikuroi* 1019. Although the strain could utilize urea and potassium nitrate and grew to the same extent, it did not produce any gibberellic acid (Fig. 3.8 and Fig. 3.9). Thus inorganic nitrogen sources although helped growth, the production of gibberellic acid was rather low. This could be because of several different reasons like lowering of pH in the fermentation medium in the shake flask when chlorides and sulphates were used, failure of the cells to initiate secondary metabolism, unfavourable physiological stage for gibberellin biosynthesis, absence of key intermediates necessary for gibberellin production etc.

Fig. 3.8 and Fig 3.9 also illustrate that the digests prepared from plant and animal proteins (beef extract, meat extract, liver extract, yeast extract, casein enzyme digest, soya peptone) resulted in substantial increased cell mass of the *F. fujikuroi* NCIM 1019. The growth in terms of dry cell mass was between 12.82-18.49 g/l. As compared to inorganic nitrogen sources the growth was almost doubled. However increased growth did not result proportionally increased gibberellic acid production. The digested nitrogenous nutrients are a rich source of amino acids and polypeptides. This indicates that the strain prefered organic soluble nitrogen sources for growth. This is not uncommon because several secondary metabolites are produced by cells under severe nutritional stress.

It was reported that the source and concentration of the nitrogen in the fermentation media have the greatest effect on the growth of *F. fujikuroi* and its GA$_3$ production (Tadzynski, 1999). In general, complex organic nitrogen sources were found to be better for growth and GA$_3$ production by *F. fujikuroi* (Darken et al., 1959; Fiska et al., 1961). Complex nitrogen sources like plant extracts might contain precursors or inductors of the GA pathway and boosts gibberellins biosynthesis reported by Rademacher (1997). In present investigation also similar findings were obtained. Used different nitrogen sources influenced the growth and GA$_3$ production by *F. fujikuroi* NCIM 1019 (Fig. 3.8 and Fig. 3.9). Among studied different organic nitrogen sources, mainly three different defatted plant meals enhanced growth and
gibberellic acid production in *F. fujikuroi* NCIM 1019 (Fig. 3.8). These plant meals yielded almost similar quantities of GA3 under identical conditions. Difference in growth and specific gibberellic acid productivity (mg GA3/g dry cell weight) was also marginal as seen from Fig. 3.9.

In addition to gibberellic acid, *F. fujikuroi* 1019 produced cell bound or water soluble pigments like carotenoids or bikaverins in media with defatted peanut meal and cottonseed meal. *F. fujikuroi* is known to biosynthesize several polyketide pigments like bikaverin and nor-bikaverin (Kjaer *et al.*, 1971) as well as the carotenoids like neurosporaxanthin (Avalos and Cerda-Olmedo, 1987). The pigment production is undesirable because the pigments may interfere with extraction and purification of gibberellic acid (Shukla *et al.*, 2003). Therefore from present study amongst studied different nitrogen sources, defatted soyabean meal was elected as suitable nitrogen source for the growth and gibberellic acid production and used in all the subsequent experiments.

### 3.3.5 Mutagenesis

The parent strain *F. 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. Earlier Keller (1983) reported that although single cells or spores are preferred for mutagenesis, in case of non-spore-forming filamentous organisms, mycelia' protoplasts are mutagenized. Similarly in present study also the selected parent *F. fujikuroi* NCIM 1019 did not produce macro or microconidia on normal growth media and therefore small mycelial fragments having one or two cells were used for the mutagenesis.

General protocol followed during the strain improvement programme for *F. fujikuroi* NCIM 1019 is presented in flow diagram (Fig. 3.10) and the same protocol was also followed for the intermediate mutants until a desired mutant was obtained.

Survival curve of *F. fujikuroi* NCIM 1019 after the mutagen treatments are presented in Fig. 3.11a, b. The figure illustrate that 95% kill was obtained on treating the cells with UV for 7 seconds, whereas for the chemical mutagens (EMS) optimum time was found to be between 30 to 40 min.
The detailed summary of each mutagenesis series, selection of the mutants and the
selected mutant used as intermediate parents for next series of mutagenesis is given
in Table 3.4. Altogether 38 mutation series were performed from which 1056
mutants were selected and screened for GA₃ production.

*F. fujikuroi* 1019 was grown in LMG for 48h

- 

Fragment count was adjusted to 2000/20ul

- 

Mutagenesis by UV and/or Mutagenesis by EMS

- 

Incubation at 28°C for seven to eight days

- 

Selection of morphological variable mutants

- 

Preliminary screening of mutants in LMF in tube

- 

Screening and comparison of mutants in shake flask cultures

- 

Evaluation of mutants using HPLC for GA₃

- 

Further mutagenesis for desired strain improvement

**Figure 3.10** Technique used for strain improvement by random mutagenesis
Figure 3.11a UV survival curve for *F. fujikuroi* NCIM 1019

Figure 3.11b EMS survival curve for *F. fujikuroi* NCIM 1019
<table>
<thead>
<tr>
<th>Series</th>
<th>Parent used</th>
<th>Mutagen</th>
<th>No. of survivors selected</th>
<th>Mutant Selected</th>
<th>Inhibitors used in RA media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>F. fujikuroi</em> NCIM1019</td>
<td>UV</td>
<td>40</td>
<td>Car1</td>
<td>Pravastatin</td>
</tr>
<tr>
<td>2</td>
<td>Car1</td>
<td>UV</td>
<td>30</td>
<td>Mut4</td>
<td>Pravastatin</td>
</tr>
<tr>
<td>3-4</td>
<td>Mut4</td>
<td>UV</td>
<td>45</td>
<td></td>
<td>Pravastatin</td>
</tr>
<tr>
<td>5-10</td>
<td>Mut4</td>
<td>UV</td>
<td>35</td>
<td>Mut65</td>
<td>Nystatin</td>
</tr>
<tr>
<td>11-14</td>
<td>Mut65</td>
<td>UV</td>
<td>115</td>
<td>Mut189</td>
<td>Diphenylamine</td>
</tr>
<tr>
<td>15-22</td>
<td>Mut189</td>
<td>EMS</td>
<td>341</td>
<td></td>
<td>Diphenylamine</td>
</tr>
<tr>
<td>23-24</td>
<td>Mut189</td>
<td>UV+EMS</td>
<td>167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-27</td>
<td>Mut23</td>
<td>UV+EMS</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28-30</td>
<td>Mut189</td>
<td>UV</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31-35</td>
<td>Mut189</td>
<td>UV</td>
<td>78</td>
<td></td>
<td>Lovastatin</td>
</tr>
<tr>
<td>36-38</td>
<td>Mut189</td>
<td>UV</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td></td>
<td></td>
<td>1056</td>
<td>Car1</td>
<td>Mut4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Mut65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mut189</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.4 Summary of Mutagenesis Series**

The small mycelial fragments with one or two cells were reasonably suitable for the mutagenesis experiments. A variety of morphological mutants having different mycelial and soluble pigmentation as well as colony morphologies were generated from *F. fujikuroi* upon exposure to UV radiation and EMS. Various morphological differences in the generated mutants were noted at each mutagenesis stage. In general they were as follows:

- Small, compact dry colonies
- Orange, pale yellow or colourless colonies
Colonies with or without soluble dark red pigment

- Mycelial, cottony colonies to short surface mycelium with branching

To screen large number of survivors from plate culture, mainly two different approaches were employed for the selection of enhanced GA₃ producer. Approaches to the selection of mutants were 1) Mutants with modified colony characteristics

Usually in liquid media, *F. fujikuroi* strains grow with long filamentous mycelia that causes the fermentation broth to become very viscous. During fermentation either in shake flask or in fermenter, the viscosity of the broth causes limitation to the oxygen transfer rate and this declines the production of secondary metabolites like gibberellic acid that are oxidation products. Mutants with modified colony characteristics expected to grow with morphological change that will suit the fermentation broth rheology.

2) Mutants with low or no pigmentation

In *F. fujikuroi* culture the pathway leading to gibberellic acid synthesis, acetoacetyl CO-A is a branch point as several other metabolites are generated through this compound under different degrees of nitrogen limitation, oxygen availability, temperature and pH. One of them is the pigment either water-soluble (bikaverin) or cell bound (carotenoids). Mutants with low or no pigmentation was another key to the selection of mutants with possible increase in gibberellin production.

The parent strain *F. 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. Result presented in Table 3.4 show that after first mutagenesis 40 survivors producing white, cottony, circular colony on regeneration agar were selected. Strains were resistant to HMG CO-A reductase inhibitor (pravastatin). A mutant strain Carl was selected. Although morphologically similar to the parent, 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 also. Based on the loss of pigmentation and 25% increased GA₃ as compared to parent, Carl was selected and used as the parent in subsequent mutagenesis experiments.
The UV mutagenesis of Carl generated a wide variety of mutants. The survivors selected after mutagenesis of Carl strain exhibited various morphological changes. Amongst 30 screened survivors, non-pigmented mycelial mutant strain Mut4, exhibiting less apparent viscosity in liquid cultures as compared to parent (Carl) was selected.

During subsequent UV mutagenesis study of Mut4 survivors with different colony characteristics were generated. The colonies that grew rapidly and became large were presumably produced from the cells without any alteration. Those colonies that grew slowly, remained small, and compact showed variations in their phenotype, had genetic alteration presumably caused because of a mutation. The different mutants with different colony morphologies were expected to have modified morphology in liquid cultures also. The smaller colonies (0.5-1 cm) with crusty, uneven margins, rough surfaces and decreased/no pigmentation on regeneration agar plates were selected. These characteristics indicated that they might have morphology suitable for micro-pelleted mutants, when grown in liquid cultures. A mutant strain Mut65 grew with short length, thicker mycelium without pigmentation. It produced 10% increased GA$_3$ than immediate parent Mut4 while 3 fold increased as compared to first parent. Based on altered morphology and higher GA$_3$ production Mut65 was selected.

Later mutations were carried out using Mut65 as parent strain and a mutant strain Mut189 was selected in similar way. It produced small, compact, rough, dry and irregular colonies on RA media. It grew profusely in a free, short branched mycelial form with increased cell thickness in liquid cultures and the broth has lower apparent viscosity. Mut189 produced almost 4 fold increased GA$_3$ as compared to first parent and almost 70-80% increased GA$_3$ than Mut65.

UV, EMS and UV + EMS mutagenesis using Mut189 as parent were also performed. A wide range of mutants generated at each mutagenesis series. Although mutants were selected in similar way as discussed earlier but they produced either low or similar concentration of GA$_3$ as compared to Mut189.

The average terminal hyphal length had distinct differences between four strains. The average apical hyphal length of Carl strain was 248 μm. Mut4 mutant was 173 μm.
Mut65 strain was 94 µm while that of Mut189 was just 74 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 present invention indicate that use of morphologically changed mutant strains would be advantageous for the fermentation because the problem associated with poor mixing and oxygen transfer in fermenter could be minimized.

During screening of mutants, a mutant named Mut189 was selected because it produced considerably high amount of GA$_3$. The mutant Mut189 did not produce any pigment either cell bound or water soluble as appeared from filtered and washed cell mass and clear pale yellow filtrate respectively. The selected mutant showed morphological difference than the parent *F. fujikuroi*. It grew as short branched mycelial form with increased cell thickness and low apparent viscosity in liquid cultures.

Although almost nothing is known about the mechanisms causing higher production in superior random or morphological mutants, it is likely that many of these mutations involve regulatory genes, especially as regulatory mutants obtained in basic genetic studies and found to be altered in colonial morphology. So the morphological mutants have been important in strain improvement study (Adrio and Demain, 2006). Earlier researchers have reported several mutants of *F. fujikuroi* mainly deficient GA biosynthesis. They were studied specifically for the discovery of major steps of GA biosynthesis. Very few reports are available with respect to enhanced GA$_3$ production by strain improvement of *F. fujikuroi*. Avalos *et al.* (1995) isolated 12 mutants of the wild type *F. fujikuroi* IMI58289 by treatment with NTG. These mutants not produced GA$_3$ when grown in minimal liquid media. Along with 12 gibberellin defective mutants, they obtained SG22 super carotenoid producer. Avalos and Cerda-Olmeda (1987) studied strain improvement of *F. fujikuroi* by screening of mutants with blocked carotenoid biosynthesis. They reported that mutant strains with lower levels of neurosporaxanthin can produce more gibberellins when the competing biosynthetic pathway with the same precursor mevalonate is blocked. Khalaf *et al.* (2009) recorded best yield of GA$_3$ (2.40 g/l) from milk permeate by immobilized mycelia of mutant gamma-14 on loofa sponge discs under optimized cultural conditions (4 immobilized discs, 30°C and pH 5).
3.3.6 Preliminarily screening of the selected mutants

To study large number of survived mutant colonies for enhanced GA₃ production the tube level screening of *F. fujikuroi* was found to be suitable as a primary screening tool. The growth and GA₃ production by different mutants in test tubes differed considerably from the respective parents. The difference in GA₃ content was at times due to difference in growth and productivity. Some of the mutants could not grow well in liquid medium and resulted in poor GA₃ production. While some of the mutants grew well with modified morphology and produced higher GA₃ compared to the appropriate parent. Result presented in Table 3.5 show that in all 1056 survived mutant colonies were screened. More than 200 colonies showed better result in tube level screening as compared to their respective parent culture.

<table>
<thead>
<tr>
<th>Mutagenesis Series No</th>
<th>No of mutants screened in test tube</th>
<th>No of mutants selected for further study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>2-4</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>5-10</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td>11-14</td>
<td>115</td>
<td>28</td>
</tr>
<tr>
<td>15-22</td>
<td>341</td>
<td>39</td>
</tr>
<tr>
<td>23-24</td>
<td>167</td>
<td>24</td>
</tr>
<tr>
<td>25-27</td>
<td>53</td>
<td>12</td>
</tr>
<tr>
<td>28-30</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>31-35</td>
<td>78</td>
<td>40</td>
</tr>
<tr>
<td>36-38</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>38</td>
<td>1056</td>
<td>211</td>
</tr>
</tbody>
</table>

Table 3.5 *F. fujikuroi* mutants screened through test tube for enhanced production of gibberellic acid

3.3.7 Shake flask screening of selected mutants for GA₃ production

During preliminary screening, 1056 mutants of *F. fujikuroi* for enhanced GA₃ production were screened. Around 211 mutants that showed at least 15-20% higher GA₃ production compared to their respective parent were further studied in shake flask cultures. The oxygen transfer and mixing is far better in shake flasks and
several strains can be compared simultaneously. Twenty-three mutants as listed below in Table 3.6 were further selected through shake flask study and studied in detail with respect to growth characteristics on RA media, net gibberellic acid production, specific production (mg GA₃/g dry cell mass), yield (mg GA₃/g sugar utilized) as well as rate of production (GA₃ mg/l/day). They were evaluated in shake flask experiment simultaneously, under identical cultural conditions.

<table>
<thead>
<tr>
<th>Mutant names</th>
<th>Size</th>
<th>Shape</th>
<th>Surface appearance</th>
<th>Roughness</th>
<th>Margin</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM1019</td>
<td>2-3cm</td>
<td>Circular</td>
<td>Cottony</td>
<td>Soft</td>
<td>Regular</td>
<td>Red purple</td>
</tr>
<tr>
<td>Car1</td>
<td>1cm</td>
<td>Circular</td>
<td>Fibrous</td>
<td>Soft</td>
<td>Regular</td>
<td>White</td>
</tr>
<tr>
<td>Mut4</td>
<td>0.9cm</td>
<td>Circular</td>
<td>Cottony</td>
<td>Soft</td>
<td>Regular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut23</td>
<td>0.8cm</td>
<td>Circular</td>
<td>Cottony</td>
<td>Soft</td>
<td>Regular</td>
<td>White</td>
</tr>
<tr>
<td>Mut32</td>
<td>5mm</td>
<td>Circular</td>
<td>Cottony</td>
<td>Soft</td>
<td>Regular</td>
<td>Orange</td>
</tr>
<tr>
<td>Mut65</td>
<td>2mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut189</td>
<td>2mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut226</td>
<td>2mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Red</td>
</tr>
<tr>
<td>Mut362</td>
<td>3mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>White</td>
</tr>
<tr>
<td>Mut404</td>
<td>4mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut730</td>
<td>2mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut735</td>
<td>3mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Crusty</td>
<td>Regular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut736</td>
<td>3mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Regular</td>
<td>White</td>
</tr>
<tr>
<td>Mut749</td>
<td>4mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>White</td>
</tr>
<tr>
<td>Mut754</td>
<td>4mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>White</td>
</tr>
<tr>
<td>Mut757</td>
<td>5mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>White</td>
</tr>
<tr>
<td>Mut767</td>
<td>2mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>White</td>
</tr>
<tr>
<td>Mut768</td>
<td>2mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Regular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut772</td>
<td>3mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Regular</td>
<td>White</td>
</tr>
<tr>
<td>Mut775</td>
<td>3mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut780</td>
<td>4mm</td>
<td>Circular</td>
<td>Dry compact</td>
<td>Crusty</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut788</td>
<td>2mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Hard</td>
<td>Regular</td>
<td>White</td>
</tr>
<tr>
<td>Mut799</td>
<td>3mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Hard</td>
<td>Irregular</td>
<td>Pale orange</td>
</tr>
<tr>
<td>Mut801</td>
<td>3mm</td>
<td>Circular</td>
<td>Rough</td>
<td>Hard</td>
<td>Irregular</td>
<td>White</td>
</tr>
</tbody>
</table>

Table 3.6 *F. fujikuroi* mutants selected for shake flask culture study

Table 3.7 Growth characteristics of selected mutants of *F. fujikuroi* on RA media
<table>
<thead>
<tr>
<th>Mutant Names</th>
<th>Apparent viscosity</th>
<th>Dry cell weight g/l</th>
<th>Sugar Utilised g/l</th>
<th>GA4 mg/l</th>
<th>Specific productivity G A4 mg/g dry cell weight</th>
<th>Yield G A4 mg/g sugar utilised</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. fujikuroi</em> NCIM1019</td>
<td>Higher</td>
<td>18</td>
<td>60</td>
<td>118</td>
<td>6.56</td>
<td>1.97</td>
</tr>
<tr>
<td>Carl</td>
<td>High</td>
<td>19.5</td>
<td>60</td>
<td>295</td>
<td>15.13</td>
<td>4.92</td>
</tr>
<tr>
<td>Mut4</td>
<td>High</td>
<td>21.52</td>
<td>54.5</td>
<td>300</td>
<td>13.94</td>
<td>5.50</td>
</tr>
<tr>
<td>Mut23</td>
<td>High</td>
<td>19.81</td>
<td>58.2</td>
<td>153</td>
<td>7.72</td>
<td>2.63</td>
</tr>
<tr>
<td>Mut32</td>
<td>High</td>
<td>20.1</td>
<td>56.3</td>
<td>270</td>
<td>13.43</td>
<td>4.8</td>
</tr>
<tr>
<td>Mut65</td>
<td>Low</td>
<td>19.26</td>
<td>40.5</td>
<td>327</td>
<td>16.98</td>
<td>8.07</td>
</tr>
<tr>
<td>Mut189</td>
<td>Low</td>
<td>19.7</td>
<td>41.9</td>
<td>434</td>
<td>22.03</td>
<td>10.36</td>
</tr>
<tr>
<td>Mut226</td>
<td>Low</td>
<td>20.53</td>
<td>54.5</td>
<td>373</td>
<td>18.17</td>
<td>6.84</td>
</tr>
<tr>
<td>Mut362</td>
<td>Low</td>
<td>18.95</td>
<td>52.4</td>
<td>387</td>
<td>20.42</td>
<td>7.39</td>
</tr>
<tr>
<td>Mut404</td>
<td>Medium</td>
<td>18.29</td>
<td>57.7</td>
<td>375</td>
<td>6.50</td>
<td>6.50</td>
</tr>
<tr>
<td>Mut730</td>
<td>Low</td>
<td>21.8</td>
<td>58.1</td>
<td>343</td>
<td>15.73</td>
<td>5.90</td>
</tr>
<tr>
<td>Mut735</td>
<td>Medium</td>
<td>21.84</td>
<td>56.4</td>
<td>332</td>
<td>15.2</td>
<td>5.89</td>
</tr>
<tr>
<td>Mut736</td>
<td>Medium</td>
<td>20.53</td>
<td>57.3</td>
<td>322</td>
<td>15.6</td>
<td>5.62</td>
</tr>
<tr>
<td>Mut749</td>
<td>Low</td>
<td>21.3</td>
<td>58.9</td>
<td>381</td>
<td>17.89</td>
<td>6.64</td>
</tr>
<tr>
<td>Mut754</td>
<td>Low</td>
<td>22.4</td>
<td>60</td>
<td>391</td>
<td>17.46</td>
<td>6.52</td>
</tr>
<tr>
<td>Mut757</td>
<td>Medium</td>
<td>20.9</td>
<td>57.3</td>
<td>346</td>
<td>16.56</td>
<td>6.04</td>
</tr>
<tr>
<td>Mut767</td>
<td>Medium</td>
<td>21.12</td>
<td>56.9</td>
<td>346</td>
<td>16.38</td>
<td>6.08</td>
</tr>
<tr>
<td>Mut768</td>
<td>Medium</td>
<td>20.2</td>
<td>58.1</td>
<td>282</td>
<td>13.96</td>
<td>4.85</td>
</tr>
<tr>
<td>Mut772</td>
<td>Medium</td>
<td>19.9</td>
<td>59.1</td>
<td>304</td>
<td>15.28</td>
<td>5.14</td>
</tr>
<tr>
<td>Mut775</td>
<td>Medium</td>
<td>18.92</td>
<td>57.6</td>
<td>350</td>
<td>18.5</td>
<td>6.08</td>
</tr>
<tr>
<td>Mut780</td>
<td>Low</td>
<td>19.9</td>
<td>58.8</td>
<td>387</td>
<td>19.45</td>
<td>6.58</td>
</tr>
<tr>
<td>Mut788</td>
<td>Medium</td>
<td>20.7</td>
<td>56.9</td>
<td>338</td>
<td>16.33</td>
<td>5.94</td>
</tr>
<tr>
<td>Mut799</td>
<td>Medium</td>
<td>19.7</td>
<td>57.8</td>
<td>338</td>
<td>17.16</td>
<td>5.85</td>
</tr>
<tr>
<td>Mut801</td>
<td>Medium</td>
<td>18.42</td>
<td>58.7</td>
<td>352</td>
<td>19.11</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 3.8 Growth and gibberellic acid production by selected mutants of *F. fujikuroi*

When the selected 23 mutant strains were grown in 50 ml LMF medium they exhibited different growth characteristics from each other in liquid cultures. The
result of growth and gibberelllic acid production by selected mutants of *F. fujikuroi* is detailed in Table 3.8.

Table 3.8 shows that there was no major change in the extent of growth of mutant strains in terms of dry cell mass. The dry cell mass was ranged between 18-22.5 g/l. Parent strain *F. fujikuroi* NCIM 1019 grew profusely in a free, long mycelia form in liquid cultures making the broth highly viscous. While mutant strains Mut65, Mut189, Mut226, Mut730, Mut749, Mut754, Mut362 and Mut780 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.

Table 3.8 also shows that the short filament strains resulted in substantially higher gibberelllic acid content. It was reported that the oxygen availability causes a major change in proportion of the metabolites produced by *F. fujikuroi* (Giordano and Domenech, 1999). In the present study, the decreased mycelial length presumably resulted in increased oxygen transfer in the submerged cultures of these mutant strains. In turn they may have channelled more carbon through the gibberellin pathway resulting in higher concentration of gibberelllic acid. The total GA$_3$ produced by improved mutants was almost two-fold higher as compared to the parent strain.

A considerable amount of sugar was utilized by these cultures in their stationary phase. Except for mutant Mut65 and Mut189 the sugar uptake was nearly equal in all the liquid cultures of the strains studied. It can be also seen from Table 3.8 that mutant Mut189 produced 434 mg/l GA$_3$, which was considerably higher than that of other mutants and parent *Fusarium* strain. The growth of all the strains in the basal medium was almost similar and this showed that the specific gibberellin productivity of Mut189 was also highest (22.03 mg GA$_3$/g dry cell weight).

The actual rates of GA$_3$ production by these mutants at different time interval were also studied and are depicted in Fig. 3.12.
Chapter 3

Figure 3.12 Comparison of GA$_3$ production rates of selected mutants

The data presented in Fig. 3.12 show that the rate of gibberellic acid production by different mutants varied considerably. The rate of GA$_3$ production reached a peak between 48-72 h in selected mutants while in parent strain it was higher during 72-96 h. This showed that as compared to the parent, selected mutants grew faster and reached production phase earlier. However, GA$_3$ production rate declined later irrespective of the mutant and concentration of GA$_3$ in the fermentation broth. The production rate of gibberellic acid by mutant Mut189 was higher compared to others.

3.3.8 Comparison of selected mutants

The selection of mutants also depended on their growth characteristics and viscosity of the liquid cultures in shake flasks when they were grown in shake flasks at constant speed and temperature. Comparison of five strains namely *F. fujikuroi* NCIM 1019 (parent strain) and its four mutant strains Carl, Mut4, Mut65 and Mut189 with respect to viscosity and oxygen transfer were studied. To study this 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 medium volume in Erlenmeyer flasks causes increase in oxygen demand in the shake flask culture because of increase in the cell mass. The results of effect of
increased volume and increased strength of medium on GA₃ production illustrated in Fig. 3.13 and Fig. 3.14 respectively.

The data presented in Fig. 3.13 show that increase in medium volume in 250 ml Erlenmeyer flask adversely affected GA₃ production in the mycelial parent strain *F. fujikuroi* NCIM 1019 and intermediate parent Car1. There was drastic decrease in GA₃ concentration in case of mycelial parent and mutant Car1 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. This was likely to be because of decreased oxygen availability for their growth and GA₃ production. On the contrary, in case of the strains with altered morphology the GA₃ production were least affected. The mutant strains Mut65 and Mut189 are morphologically modified mutants and irrespective of medium volume their morphology remained same. They grew with short, thick and branched mycelial filamentous form in liquid cultures and exhibited lower apparent viscosity and better oxygen transfer rate as compared to mycelial strain. In these mutants GA₃ production did not decrease like mycelial mutants presumably because of higher availability of oxygen. This supports our choice of the mutants with short mycelial filament length. In general the oxygen availability has profound effect on gibberellie acid production because in all cases the increased volume in the shake flask decreased the GA₃ concentration.

![Figure 3.13 Effect of different medium volume in 250 ml Erlenmeyer flasks on GA₃ production by morphological mutants of *F. fujikuroi*](image-url)
To increase gibberellic acid concentration in fermentation broth, efforts were made to increase biomass in shake flasks. Selected mutants were studied in 50 ml LMF medium with different strengths, in 250 ml Erlenmeyer flasks. The result presented in Fig. 3.14 show that in case of the mycelial mutant increase in medium strength adversely affected the GA3 production. Increase in medium strength beyond 1.25 fold, decreased the GA3 content for mycelial mutants but not in the case of mutants with short filament length. Similar to the above results, this is mainly due to the decreased oxygen availability for the growth and GA3 production by mycelial strains. In the present investigation selection and evaluations of mutants of *F. fujikuroi* that have altered morphology in terms of filament length and increased production of gibberellic acid clearly indicated that the current objective of obtaining a morphological mutant for enhanced GA3 production was appropriate.

The rate of growth of *F. fujikuroi* and production of GA3 in fermenter is governed to a considerable extent by oxygen transfer in the fermenter. It is also reported that *F. fujikuroi* cultures enter a linear growth phase after initial logarithmic phase (Borrow et al. 1964) presumably because of the oxygen limitation. During stationary phase of *F. fujikuroi* culture several products like bikaverin, carotenoids, gibberellic acid, fusaric acid are produced from a common precursor, acetyl-CoA (Bruckner et al., 1964).
Chapter 3

1989: Avalos and Cerda-Olmedo, 1987; Kjaer et al., 1971; Avalos et al., 1994). Giordano and Domenech (1999) have described how the aeration affects the fate of acetate in F. fujikuroi during secondary metabolism. They also studied 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 fusarins C. Overall concentrations and ratio of secondary metabolites of F. fujikuroi are governed by the availability of oxygen in the liquid cultures.

Hydrodynamics, mass transfer and rheology of gibberellin acid production by F. fujikuroi in an airlift bioreactor were studied by Silva et al., (2007). They studied the growth kinetics of F. fujikuroi during fermentation and explained the culture medium rheological behaviour in terms of changes in the morphology of the fungus. In case of mycelial cultures, as the biomass concentration increased the broth became more viscous and non-Newtonian, leading to substantial decreased oxygen transfer rates. This effect is often important since for many aerobic processes involving oxygen supply is the limiting factor determining bioreactor productivity (Al-Masry and Dukkan, 1998).

3.3.9 Characterization of selected mutant Mut189 of F. fujikuroi

3.3.9.1 Morphology

Phase contrast and scanning electron micrographs (Fig. 3.15 and Fig. 3.16) illustrate that mutant Mut189 had morphological difference compared to its parent F. fujikuroi NCIM 1019. The parent mycelia were long filamentous with less thickness and branching. The mycelia of mutant were short, highly branched hyphae, curly at tips with thick, swollen cells as compared to parent as visualized under 5000 X magnification.
3.3.9.2 Carbohydrate utilisation by mutant Mut189

Mutant Mut189 showed carbohydrate utilization pattern similar to the parent *F. fujikuroi* NCIM 1019. It could utilize all studied carbohydrates for its growth. There was distinct difference in growth characteristics with the studied carbohydrate between parent and mutant strain as shown in Fig. 3.17a and 3.17b. The growth of parent *F. fujikuroi* NCIM 1019 was a large, circular, soft cottony colony with regular margin and reddish purple pigmentation. In comparison with fructose pigmentation was more intense observed visually on RA plate. The growth of mutant Mut189 was small, circular, compact and hard colony with irregular margin. There was no pigmentation with use of glucose and sucrose by mutant Mut189. Except glucose and sucrose although Mut189 showed pigment with other studied carbohydrates as compared to parent pigmentation was low.
Chapter 3

Arabinose

Xylose

Glucose

Fructose

Glycerol

Galactose

Maltose

Sucrose

Mannose

Raffinose

Rhamnose

Sorbitol

Cellobiose

Starch

Figure 3.17a  Carbohydrate utilization by *F. fujikuroi* NCIM1019
Figure 3.17b Carbohydrate utilization by Mutant Mut189
3.3.10 Protoplast fusion

3.3.10.1 Optimization of protoplast formation

The physiological age of the culture markedly influences protoplast yield (Peberdy, 1979). In present study the culture age suitable for the release of maximum protoplasts for two types of cells was standardized by growing the Mut32, a mutant strain of *F. fujikuroi* and a yeast strain *Trichosporon cutaneum* NCIM 3352 for 24 h in PDB broth. The result depicted in Fig. 3.18 illustrate that the number of protoplasts increased with increase in age of both the cultures. In case of *T. cutaneum* NCIM 3352 number of protoplasts release was maximum (10x10^5) in 16 h old culture. While for fungal mutant culture Mut32, number of protoplasts reached a high count (7x10^5) at 18 h old culture indicating the optimum age of the culture. Further yield of protoplasts in both the cultures were declined with increasing age of the cultures. This indicated that older cells are more resistant to enzymatic hydrolysis.

In general, young cells from exponential cultures are readily converted to protoplasts but cells from stationary phase cultures are resistant to lysis (Peberdy, 1980; Okanishi *et al.*, 1974) and resistance developed rapidly in the transitional period from exponential to stationary phase (Deutch and Parry, 1974). In present study similar findings were obtained. The optimum age of the culture suitable for the formation of protoplasts was 16 h for *T. cutaneum* NCIM 3352 and 18 h for Mut32.

![Figure 3.18 Effect of age of the mycelium on the release of protoplasts from *T. cutaneum* NCIM 3352 and Mut32](image)

**Figure 3.18** Effect of age of the mycelium on the release of protoplasts from *T. cutaneum* NCIM 3352 and Mut32
The isolation of protoplasts from fungi using lytic enzyme is now a well-established technique. Novozyme234 was reported to be the most effective enzyme for high yields of protoplasts in fungi as well as for yeast (Hamlyn et al., 1981). The protoplasts yield also significantly affected by the concentrations of lysing enzyme (Lalitkumari and Balasubramanian, 2008). In the present study to examine optimal concentration of lysing enzyme for the formation of protoplasts, 16 h old cells of \textit{T. cutaneum} NCIM 3352 and 18 h old Mut32 mycelium were treated with different concentrations (2, 5, 10, 15 mg/l) of Novozyme234 and results presented in Fig. 3.19.

![Figure 3.19](image)

**Figure 3.19** Effect of enzyme concentration on the release of protoplasts from \textit{T. cutaneum} NCIM 3352 and Mut32

As shown in Fig. 3.19, for both cells the rate of protoplast formation was increased with the increase of enzyme concentration indicating a better digestion of cell walls with more enzyme concentration. At high enzyme concentration of Novozyme234 (15 mg/ml) the fungal mycelium of Mut32 as well as \textit{T. cutaneum} NCIM 3352 yeast cells lysed effectively yielding large numbers of protoplasts ($11 \times 10^5$ and $8 \times 10^5$ respectively). But they get bursted immediately after release and disintegrated. Thus exposure to excessive Novozyme234 irreversibly destroyed the protoplasts. These results also suggest that the cell wall lysis depends on the concentration of used lytic enzyme Novozyme234.
Among different concentrations of lysing enzymes tested, Novozyme 234 at 10 mg ml⁻¹ was found to be suitable for preparation of protoplasts and was decided to use the optimal concentration for further protoplast fusion experimental study.

Osmotic stabilizer plays an important role in the release and maintenance of protoplasts (Hocart et al., 1987; Mukherjee and Sengupta, 1988). They protect the nascent protoplasts in different environments and support the protoplasts from being lysed. In present investigation four osmotic stabilizers were tested and result illustrated in Fig. 3.20.

![Bar Graph](image)

**Figure 3.20** Effect of osmotic stabilisers on release the of protoplasts from *T. cutaneum* NCIM 3352 and Mut32

It can be seen from Fig. 3.20 that among the studied four different stabilizers, yield of protoplasts in the presence of 0.6 M Mannitol was low while maximum with 0.6 M MgSO₄. Maximum protoplasts from yeast *T. cutaneum* NCIM 3352 (3x10⁶ ml⁻¹) and Mut32 mycelial cells (4x10⁷ ml⁻¹) was obtained in presence of 0.6 M MgSO₄.

An extensive range of inorganic salts, sugars, and sugar alcohols have been successfully used to stabilize released protoplasts by the various researchers. There is no one universal stabilizer suitable to all. The type and concentration of stabilizer can influence yield and stability of protoplasts (Gokhale, 1992). In general inorganic salts have proved more effective with filamentous fungi while sugars or sugar alcohols are
more effective with yeasts. The present study indicated that MgSO$_4$ followed by KCl was suitable to isolate more protoplasts, whereas NaCl and organic stabilizer mannitol were not suitable for both the cultures since proplast formation started after the end of 2 h and low numbers of protoplasts were released. Thus the inorganic stabilizers supported protoplast formation of both the cultures.

The use of MgSO$_4$ as osmotic stabiliser has also been found more effective for protoplast isolation in other filamentous fungi (Deshpande et al., 1987; Carragh et al., 1992). The beneficial effect of Mg$^{2+}$ for preventing lipid release from the plasmatic membrane has been speculated by Kavanagh and Whittaker (1991).

In the present study, it was also recorded that protoplasts released in the early hours (until 2-3 h) of incubation were smaller in size and moderately uniform and large size protoplasts after prolong incubation. The maximal yield of protoplasts from both the cultures was obtained after 3 h incubation at 28 °C with gentle shaking at 100-120 rpm. 3 h incubation was optimum for maximum release of protoplasts as the numbers of protoplasts get decreased after 3 h incubation due to bursting and prolonged incubation caused the protoplasts to lyse (autolysis) also reported by (Lalitkumari and Balasubramanian, 2008).

### 3.3.10.2 Protoplast Fusion and Selection of fusant

When the protoplasts were mixed with PI G solution, they stuck together and pairs of protoplasts were observed seen under microscope. The protoplasts were attracted each other fused together. Although aggregation of more than two protoplasts was seen, fusion was observed between only two protoplasts. The fused protoplasts became larger in size and later spherical in shape. Selection of fusant was based on colony morphology and pigmentation. Regeneration was started after 2-3 days and colony development was observed after 4 days on RA agar media. After 9-10 days of incubation a regenerated colony looking like yeast with pigmentation was selected. The growth of selected fusant strain and parent strains exhibited variation in growth, pigmentation and morphology on solid and liquid media.

As shown in Fig. 3.21 on RA media Mut32 (parent fungus) was grown as a large, circular and cottony colony with irregular margin and pink pigmentation. Parent yeast *T. cutaneum* NCIM 3352 was grown as medium large, circular, rubbery, moist...
colony with irregular margin and without pigmentation. While selected fusant was
grown comparatively small, circular, soft colony with regular margin and pink
pigmentation.

Figure 3.21 Growth of parent fungus, parent yeast and suspected fusant on
regeneration agar

Microscopic observations of these three cultures were given in Fig. 3.22. The
mycelia of parent fungal culture Mut32 were long filamentous, branched with less
thickness. Oval shaped yeast parent cells with few pseudomycelium were observed.
While selected yeast like hybrid was with mixed morphology. Morphologically it
was thin mycelia getting fragmented to oval shaped cells resembles similar
morphology like parent yeast strain.
3.3.10.3 Production of GA$_3$ by Selected Fusant

The fusant was repeatedly selected during transfer and incubation period. The stable fusant was obtained after about 5 transfers for two months and was not reverted to their primary forms. The selected fusant was studied for GA$_3$ production in shake flask cultures. When fusant, parent fungus Mut32 and yeast $T.\text{cutaneum}$ NCIM3352 were grown in 50 ml LMF in 250 ml flasks, they grew well and exhibited different growth characteristics from each other as discussed earlier. The liquid culture of Parent Mut32 was highly viscous. The apparent viscosity of liquid cultures of selected fusant was very low as compared to parent fungus Mut32. Parent fungus produced GA$_3$ (252 mg/l). Parent yeast did not produce GA$_3$. While selected yeast-like hybrid produced GA$_3$ (61 mg/l) and was confirmed by LC-MS. All fermentation tests were done with pure cultures. The cultures were periodically monitored for the presence of fungal contamination and none was found. Thus present invention successfully obtained yeast hybrid producing GA$_3$. 

Figure 3.22 Micrographs of parent fungus, parent yeast and selected fusant
<table>
<thead>
<tr>
<th>Parent Yeast</th>
<th>Yeast like Fusant</th>
<th>Parent Fungus</th>
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**Figure 3.23** Photograph of shake flask cultures of parent yeast, parent fungus and selected fusant
3.4 CONCLUSIONS

On screening *F. fujikuroi* strains from NCIM, *F. fujikuroi* 1019 that produced higher GA$_3$ concentration (76 mg/l) and showed highest specific GA$_3$ productivity (8.36 mg GA$_3$/g dry cell weight) was selected for further investigations. Studies on initial culture conditions illustrated that, glucose was the best carbon source for GA$_3$ production by *F. fujikuroi*. It could utilize a wide variety of inorganic and organic nitrogen sources studied for growth and GA$_3$ production. Among inorganic nitrogen source based media, ammonium compounds although helped growth, the production of gibberellic acid was rather low. In general, organic nitrogen sources were found to be superior for the growth and GA$_3$ production. Defatted soyabean meal was choice of nitrogen source for the growth and gibberellic acid production of *F. fujikuroi* strain. A suitable fermentation liquid medium was arrived at and used subsequently.

Filamentous nature of *F. fujikuroi* NCIM 1019 leads to excessive viscosity in the fermentation broth and demands higher agitation and aeration to maintain satisfactory levels of dissolved oxygen (DO$_2$). The expenditure on energy for aeration and agitation of such viscous broths is considerably high. GA$_3$ production was found to be mainly dependent on oxygen availability and also quality and quantity of nitrogen content in the medium.

The problems associated with *F. fujikuroi* filamentous fungus during fermentation such as viscosity, low oxygen availability can be overcome by using culture with modified morphology. From this point of view intergeneric protoplast fusion between protoplasts of *F. fujikuroi* mutant and yeast protoplasts was attempted.

Mutants of *F. fujikuroi* NCIM1019 were generated by UV irradiation and EMS treatment for desired improvement. In all, 1056 survived mutant colonies were developed through 38 mutagenesis series. Mutants were randomly selected based on their modified colony characteristics and with low or no pigmentation. Mutant colonies showed 10-15% improved GA$_3$ in tube level screening as compared to their respective parent culture were studied in shake flask cultures. Mut189 selected based on its distinct morphology. The colonies on regeneration agar plates were small, compact and dry. In liquid medium, mutant Mut189 grew in a micro-pelleted form. The mycelium was short, highly branched hyphae, curly at tips with thick, swollen cells. It grew rapidly in a medium containing defatted soyabean meal glucose and
salts. In media with higher nutrient concentrations as well as larger volumes it produced twofold more gibberellic acid than the parent. The mutant Mut189 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.

The yeast-like fusant obtained in this work was able to produce GA₃. The hybrid obtained could be exploited further for enhanced GA₃ production. Intergeneric protoplast fusion seems to be promising technique in strain improvement of *F. fujikuroi* for GA₃ production. This is the first report on an attempt for intergeneric protoplast fusion in *F. fujikuroi* for GA₃ production not reported earlier.

Thus present invention found that mutants with altered morphology resulted in higher GA₃ production. Growth of un-pigmented, morphological mutants of *F. fujikuroi* that led to lower viscosity in fermentation broth resulted in increased production of gibberellic acid. 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.
3.5 REFERENCES


Chapter 3


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