

Bioprocess Strategies and Recovery Processes in Gibberellic Acid Fermentation

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Abstract Gibberellic acid (GA_3) is a commercially important plant growth hormone, which is gaining much more attention all over the world due to its effective use in agriculture and brewing industry. Industrially it is produced by submerged fermentation technique using Ascomycetous fungus *Gibberella fujikuroi*. Solid state and immobilized cell fermentation techniques had also been developed as an alternative to obtain higher yield of GA_3 . This review summarizes the problems of GA_3 fermentation such as production of co-secondary metabolites along with GA_3 , substrate inhibition and degradation of GA_3 to biologically inert compound gibberellenic acid, which limits the yield of GA_3 in the fermentation medium. These problems can be overcome by various bioprocessing strategies e.g. two - stage and fed batch cultivation processes. Further research on bioreactor operation strategies such as continuous and / or extractive fermentation with or without cell recycle / retention system need to be investigated for improvement in yield and productivity. Down stream processing for GA_3 isolation is also a challenge and procedures available for the same have been critically evaluated.

Keywords: fermentation, gibberellic acid, *Gibberella fujikuroi*

INTRODUCTION

Gibberellins (GAs) are a large family of structurally related diterpenoid acids that occur in green plants and some microorganisms. To date a total of 121 gibberellins have been identified from these sources [1,2]. Gibberellic acid (GA_3) is an important member of gibberellin family and acts as a natural plant growth hormone, controlling many developmental processes such as the induction of hydrolytic enzyme activity during seed germination, stem elongation, induction of flowering, improvement of crop yield and overcoming dwarfism [3,4]. Due to these properties GA_3 has wide application in agriculture, nurseries, tissue culture, tea garden etc. In addition GA_3 is also used in a variety of activities related to research work and pharmacological applications [4]. At present GA_3 is produced throughout the world by fermentation technique using fungus *Gibberella fujikuroi* (recently named *Fusarium fujikuroi*). It has been found that *G. fujikuroi* is a species complex of at least eight mating populations (MP-A to MP-H). Only MP-C (*Fusarium fujikuroi*) produces Gibberellins, whereas *Fusarium moniliforme* (MP-A) does not produce [4-7]. Other fungi such as *Sphaceloma manihoticola*, *Neurospora crassa* and *Phaeosphaeria sp.* also produce some gibberellins but their yields are too low to be commercially available [8-10].

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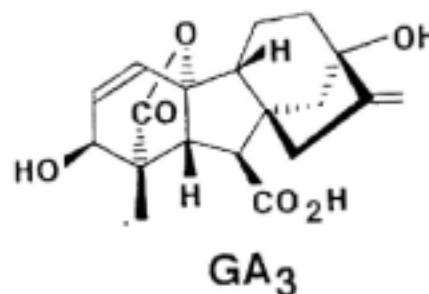


Fig. 1. Chemical structure of gibberellic acid (GA_3).

The annual world production of GA_3 exceeds about 25 tons with a market value of US\$ 100 million. To date, the cost of GA_3 has been great enough to preclude its extensive use for plant growth promotion, except for certain high value plants. Reduction in its production cost can lead to wider applications to a variety of crops [2]. Chemically, GA_3 is a tetracyclic dihydroxy γ -lactonic acid containing two ethylene bond and one free carboxylic acid group and having an empirical formula of $C_{19}H_{22}O_6$ [11]. The structure of GA_3 is shown in Fig. 1. The chemistry, biosynthesis, mode of action, relationship between structure and activity and the use of gibberellins have been thoroughly investigated [1,3,4,11]. The purpose of this review is to summarize recent progress on fermentation techniques, extraction processes and to focus on various problems associated with GA_3 fermentation.

FERMENTATION TECHNIQUES

Originally, GA₃ was produced by surface fermentation in Japan [12]. Only 40-60 mg/L GA₃ production was achieved in 15-30 days by using this technique [13]. Later on, submerged fermentation process was adapted throughout the world [1,4].

Submerged Fermentation

The submerged fermentation technique for the production of GA₃ is influenced to a great extent by a variety of physical (pH, temperature, light, aeration and agitation rate) and nutritional factors (carbon and nitrogen sources, C: N ratio *etc.*). The optimization of these factors is prerequisite for the development of commercial process [4,14]. Various researchers have optimized these factors for maximum production of GA₃ [15,16]. The production of GA₃ was found to be maximum at temperature 25°C in the pH range of 3.5-5.0. Some other intermediate compounds such as GA₄ and GA₇ of GA₃ pathway were also accumulated in the fermentation broth at temperature above 32°C in the pH range of 6-7 [15,16]. The kinetics of growth and GA₃ production in nitrogen-limited medium was established. After the period of exponential growth, when the uptake of glucose, nitrogen, and phosphate remained constant, no GA₃ was produced; GA₃ production began at or soon after the nitrogen exhaustion only [15]. The quality and quantity of nitrogen sources also played a significant role. All literature reported media yielding high amount of GA₃ contained low concentrations of nitrogen sources *e.g.* ammonium nitrate and ammonium chloride [17,18]. Besides media with low ammonium or nitrate concentrations, complex ingredients such as corn steep liquor [19,20], peanut meals [21] and soya meal [22] positively affected GA₃ biosynthesis. It was suggested that plant extracts might be containing precursors or inducers of the GA₃ pathway. Nitrogen repression is a well-known regulatory principle of secondary metabolite formation [23]. In a mutant strain of *Gibberella fujikuroi*, ammonium or nitrate ions was known to effect the production of GA₃ while phosphate does not influence the biosynthesis of GA₃ [17,24]. It was found that the negative effect of ammonium or nitrate ions was due to both the inhibition of activity and the repression of *de novo* synthesis of specific gibberellin producing enzyme [17]. Besides nitrogen control, the biosynthesis of gibberellins was indicated to be suppressed by high amount of glucose (> 20%), which had been the most commonly used carbon source for GA₃ production [4,25]. The mechanism of glucose repression is similar to nitrogen repression that has to be studied at the molecular level in the near future [2,8]. Alternative carbon sources, such as maltose, mannitol, glycerol, and galactose increased the GA₃ production rate [20,26]. The mixture of sucrose-starch was identified as the best carbon source for the GA₃ production [27]. Some oils such as sunflower oil, cooking oil and rapeseed oil have also been successfully used to enhance GA₃ production [14, 28]. The biosynthesis of GA₃ is based on acetate and fol-

lows the isoprenoid pathway [2,8]. Therefore, plant oil, as a carbon source is not only inert for carbon catabolite repression but also makes available a pool of acetyl CoA and additionally might contain natural precursors for GA₃ biosynthesis. Several industrial residues such as milk whey, molasses, sugar beet pulp and hydrol had also been used as carbon sources. These residues give low but economically useful yield [29-32]. Highest yield of GA₃ (*i.e.* 3.0 g/L) was obtained from mussel processing waste by submerged fermentation [33,34]. Reports on the continuous fermentation for the production of GA₃ are also available [35,36] but are not used by the industry probably because of economic reasons. Recently, some techniques have also been developed for the production of mixture of gibberellins containing GA₄, GA₇, GA₃ [37, 38]. A very few patents are available in the literature which reported that the addition of intermediate compound of gibberellin pathway (*e.g.* mevalonic acid, *ent*-kaurene) as precursors in the fermentation medium have greatly improved the yield of GA₃ [39,40].

Solid-state Fermentation

Solid-state fermentation has been used successfully for the production of secondary metabolites and also been indicated to produce product at high concentration, requiring less energy, in smaller fermenter and easier downstream processing measures [41-43]. This technique has also been applied for the production of GA₃ with substrates such as corn grains, wheat bran and rice bran, cassava bagasse and pretreated coffee bean pods in the medium [44-46] and gives 1.6 times higher production of GA₃ than in submerged culture, based on equivalent carbohydrate content of the medium [44]. Effect of physical factors on GA₃ production has been studied extensively and maximum GA₃ production was achieved at temperature 30°C, pH 3.5 and 50% moisture content [47]. Many solid-state cultivation studies using *G. fujikuroi* have been reported to study the influence of substrate feeding policies [48], environmental conditions [45,49], nutritional factors [45] and different solid supports [50] on the culture. Few have also included the modeling of biomass growth and metabolite production as well [51]. Bandelier *et al.* [52] developed an aseptic pilot scale reactor (50 L) for GA₃ production, which yielded GA₃ at about 3.0 g/kg of substrate. A comparative study has also been done for the production of GA₃ in liquid culture and solid substrate cultivation [53]. Product yield for organic support was found to be 6.7 times higher as compared to liquid culture medium. Recently, some inert carriers (such as amberlite) have also been tried for growth of *Gibberella fujikuroi* and GA₃ production and it was found that maximum biomass concentration and GA₃ production were 40 mg/g inert support and 0.73 mg/g inert support, respectively [54]. Indeed, the solid-state fermentation technique has shown a number of economic advantages over submerged fermentation process in the production GA₃ and in the utilization of agro industrial byproduct. The main drawbacks of this is the difficulty in controlling important parameters (temperature, water

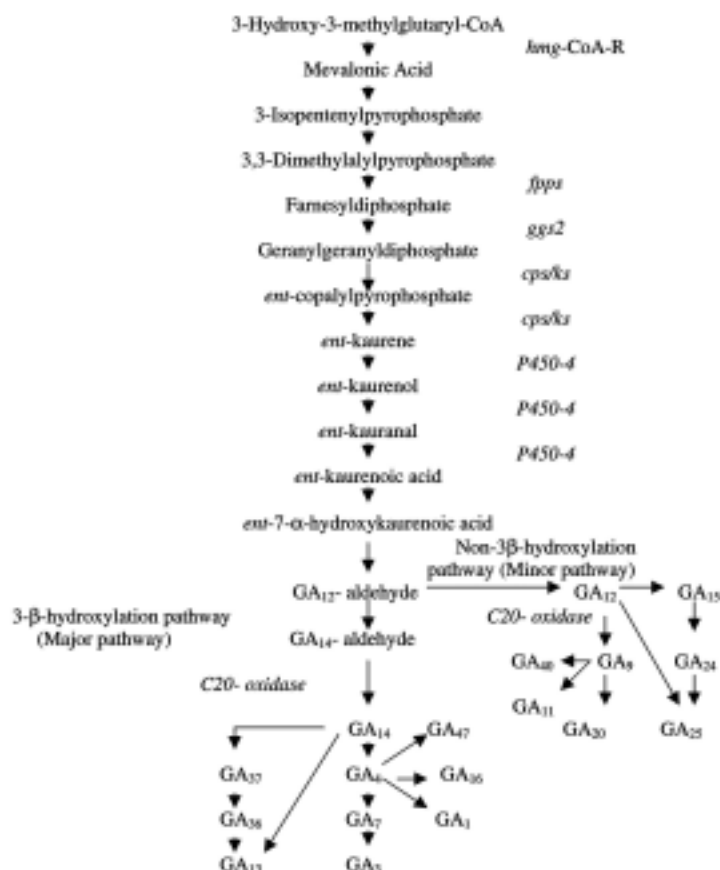


Fig. 2. Gibberellin biosynthesis pathway in *Gibberella fujikuroi*. Genes with known functions are indicated.

content of the medium), scale up of the production to an industrial level and the difficulty in maintaining aseptic culture conditions during the process [42,43].

Immobilized Cell Fermentation

In recent times, a number of advantages on the use of immobilized cells of *Gibberella fujikuroi* have been reported [55,56]. It has been speculated that these techniques will be applied extensively in future due to their economic nature compared to conventional fermentation techniques [57,58]. Several polymeric matrices such as polyurethane, carrageenan and calcium alginate have been reported for the immobilization of *G. fujikuroi* cells [57,59,60]. Adhesion of *G. fujikuroi* cells on the fibrous carriers covered by copolymers of hydrophilic hydroxyethyl acrylate and hydrophobic trimethylpropane triacrylate have also been investigated to obtain more stable cells [61,62]. It was found that the GA₃ production in fluidized bioreactor with immobilized mycelia of *G. fujikuroi* in Ca-polygalacturonate could reach 3.9 g/L, which is three times greater than previously reported values for submerged and solid state fermentation [56]. Several difficulties have been encountered in immobilized cell technology for large-scale production of GA₃. These were risk of strain mutation, the problem of sterility maintenance and the process control requirement. The devel-

opment of appropriate immobilization method and scale up should be able to overcome all of these problems [55, 56,58].

BIOSYNTHETIC PATHWAY OF GIBBERELLINS (GAs)

The biosynthesis of gibberellins (GAs) has been investigated for many years in *Gibberella fujikuroi* and reviewed by various researchers [1-4]. The terpenoid nature of GAs was established by the incorporation of [2-¹⁴C] mevalonic acid into GA₃, which is the end product of the fungal pathway [4]. The biosynthesis follows isoprenoid pathway to geranyl geranyl diphosphate (GGPP), which undergoes two-step cyclization reaction in which GGPP is converted to *ent*-kaurene via *ent*-copalyl diphosphate (CPP). The formation of CPP is the first rate-limiting step in GA pathway [2,3,63]. *ent*-kaurene is then oxidized to *ent*-kaurenoic acid via *ent*-kaurenol and *ent*-kauranal, which on hydroxylation and ring contraction gives GA₁₂-aldehyde. GA₁₂-aldehyde represents the branch point for two parallel pathways producing the 3-β non-hydroxylated and 3-β hydroxylated GAs. The major pathway begins by 3-β-hydroxylation of GA₁₂-aldehyde to GA₁₄-aldehyde and leads to the formation of GA₄, GA₇ and GA₃ [2]. The whole biosynthetic pathway is shown in Fig. 2 [63-65].

Molecular studies of the GAs pathway started few years back. Most of the genes of the early isoprenoid pathway have been cloned from *Gibberella fujikuroi* including HMG-CoA reductase [66], farnesyl diphosphate [67] and a general geranylgeranyl diphosphate (GGPP) synthase (ggs1) [68]. Genes of gibberellin biosynthesis comprising GA-specific geranylgeranyl diphosphate (GGPP) synthase (ggs2), bifunctional *ent*-copalyl diphosphate / *ent*-kaurene synthase (cps/ks) and four cytochrome P450 monooxygenase designated as P450-1, P450-2, P450-3 and P450-4, have been cloned recently and shown to be organized in a gene cluster in the fungus *Gibberella fujikuroi* [8,63,69]. Most of the genes have been characterized by gene replacement and gene expression in a GA-deficient mutant in which the entire GA-gene cluster had been deleted [64,65]. The identification of these genes has provided the opportunity to study the fungal enzymes and metabolic steps they catalyze, which had previously proved difficult at the protein level [64]. Considerable progress has also been made in isolating and characterizing the genes of GA biosynthesis in plants. These include genes encoding the membrane-associated cytochrome P450-monooxygenases and soluble dioxygenases that catalyze, intermediate and late steps of the pathway, respectively [65,70,71]. Comparison of the products of fungal and plant genes indicates important biochemical differences in GA biosynthesis between these kingdoms. For example, formation of the early intermediate *ent*-kaurene from geranylgeranyl diphosphate requires two enzymes in plants but is catalyzed by a single bifunctional cps/ks in *G. fujikuroi*. Although the *ent*-kaurene oxidase, which converts *ent*-kaurene to *ent*-kaurenoic acid, is a cytochrome P450 monooxygenase in plants and *G. fujikuroi*, the enzymes encoded by *GA5* in plants and *P450-4* in *G. fujikuroi* are highly diverged and are placed in different P450 families [65,70,71]. Recently it was found that four oxidation steps from *ent*-kaurenoic acid to GA₁₄ via GA₁₂-aldehyde are catalyzed by a single multifunctional cytochrome P450, encoded by gene *P450-1* in *G. fujikuroi* [64,72].

In both 3- β -hydroxylation and non 3- β -hydroxylation branches of the pathway the C₂₀-gibberellins, still possessing 20 carbon atoms, have to be transferred into the biologically active C₁₉ gibberellins by successive oxidation of C-20 from a methyl group in GA₁₂ or GA₁₄ through the alcohol and aldehyde. The carbon atom is lost as CO₂ producing C₁₉ lactones. In plants these steps are catalyzed by a multifunctional 2-oxoglutarate-dependent dioxygenase whereas in the *G. fujikuroi* 20-oxidase enzyme (encoded by *P450-1*) catalyzes the oxidation of GA₁₄ to GA₄ in the main pathway and its non-3 β -hydroxylated analog, GA₁₂ to GA₉ [2,65]. The fact that the GA20-oxidase is a cytochrome P450 monooxygenase in *G. fujikuroi* and not a 2-oxoglutarate dependent dioxygenase as in plants, together with the significant differences in regulation of gene expression, are further evidence for independent evolution of the GA biosynthetic pathway in plants and fungi [65,71].

Although fungal cultures convert GA₁₄ to GA₄ (major pathway) and GA₁₂ to GA₉ (minor pathway), it has not

been possible to demonstrate the involvement of intermediates in these conversions. Potential intermediates such as GA₃₇, GA₃₆ and GA₁₃, are present in cultures, but these are not formed from GA₁₄ when they are applied to the fungal mutant strain B1-41a. Similar results are also found for GA₁₅, GA₂₄ and GA₂₅, the non-hydroxylated analogs of these possible intermediates [63,65,73].

PROBLEMS ASSOCIATED WITH GIBBERELIC ACID FERMENTATION

As discussed earlier, high nitrogen concentration in production medium represses the gibberellins biosynthesis. It was suggested that dehydrogenation of GA₄ to GA₇, catalyzed by 1,2- GA₄ dehydrogenase is reduced by excess nitrogen [2,3]. When the mutant strain of *Gibberella fujikuroi* 14\141 blocked at the last step in GA₅ biosynthesis was grown under high concentration of ammonium ions, the ratio of GA₄: GA₅ rose very slightly. This clearly indicates that 1,2- GA₄ dehydrogenase is not the major site of ammonium regulation [2]. Like *Aspergillus nidulans* and *Neurospora crassa*, nitrogen repression has been studied in a molecular level in *Gibberella fujikuroi* and it was found that positive DNA-binding protein AREA effects the regulation of *areA-GF* gene involved in utilizing nitrogen sources [2]. Expression studies on the recently cloned *areA-GF* gene from *Gibberella fujikuroi* clearly demonstrated that the transcript level drastically increased under condition of ammonium limitations. It has been experimentally proved that disruption of the *areA-GF* gene in *Gibberella fujikuroi* led to the total loss or significant reduction of gibberellin production capacity in production medium. These results strongly suggest that gibberellin biosynthesis is under the control of AREA-GF factor [2,8]. Besides this nitrogen regulation some other specific regulator must be identified and studied on molecular level.

During the fermentation of GA₃, the *Gibberella fujikuroi* produces other secondary metabolites such as carotenoids, anthraquinone, bikaverin and fusarin C along with GA₃ [8,60,74,75]. Among these secondary metabolites bikaverin is an undesirable byproduct of GA₃ fermentation and complicates the purification of gibberellins from fermentation media. It has been difficult to reduce bikaverin production during GA₃ fermentation because both compounds are produced under conditions of nitrogen limitation, which coincides with the cessation of the active cell growth [60]. Some studies have been conducted in the mutant strain of *G. fujikuroi* to minimize the production of other secondary metabolites [76]. It was found that high aeration could result in decreased production of fatty acids and fusarin C but it stimulated the growth and production of both GA₃ and bikaverin simultaneously. Recently, a polyketide synthase gene *pk4* has cloned from *Gibberella fujikuroi*, which is responsible for the first step of bikaverin biosynthesis. Disruption of *pk4* gene resulted in the total loss of both *pk4* transcripts and bikaverin biosynthesis in *G. fujikuroi* cultures [8]. However, *pk4* disruption in *Gibberella fujikuroi* mu-

tant did not improve gibberellin production. Both *pks4* and *GA* gene transcripts were almost undetectable in the wild type *G. fujikuroi* grown in high nitrogen medium. This suggests that *pks4* expression, like GA biosynthetic genes, is under the control of the global nitrogen regulator AREA [2,8]. The molecular mechanism by which AREA differentially modulates expression of gibberellin and bikaverin biosynthetic genes is not yet clear. Although some research has been carried out in this direction but some experimental evidence has still to be identified. It was also observed that immobilized *Gibberella fujikuroi* cells on calcium alginate produced the red pigment bikaverin, while free cells did not produce [77]. Immobilization could modify the physiological behavior of *Gibberella fujikuroi* cells and stimulated bikaverin production [78]. Thus immobilization techniques have potential for the redirection of secondary metabolism.

In addition to the nitrogen regulation, optimal bikaverin production requires a low pH [8,74]. Northern blot analysis of RNA from mycelia grown at low to high pH (3.0-8.0) confirmed that *pks4* expression is induced by low pH (< 5) and repressed by high pH [8]. Further studies should also be carried out on environmental factors that affect the bikaverin synthesis from *G. fujikuroi*. Under the fermentation conditions high concentration of glucose (> 20%) inhibits the growth of *G. fujikuroi*, but low (< 4%) concentration of glucose is necessary for the production of GA₃ and for the maintenance of biomass in the production phase [3,4,26]. Thus some operational strategy such as fed batch culture must be employed to simulate the typical nutrient availability for optimum product accumulation. In literature only few reports are available which reported the high yield of GA₃ by employing the constant feeding of glucose [15,20].

It has been observed that GA₃ is an unstable compound in an aqueous solution and is hydrolyzed to a biologically inert, stable product gibberellic acid [79,80]. Thus the chemical decomposition is also one of the important factors that limit the yield of GA₃ in fermentation medium. Till date no attempt has been made to control the rate of decomposition of GA₃ to gibberellic acid.

It was suggested that like most of the organic acid fermentation, production of GA₃ is inhibited by itself [81, 82]. There is only one report, which indicated the two-fold increase in the yield of GA₃ as compared to conventional batch fermentation by employing extractive fermentation using solvent polyalkoxylate [81]. This increase in the yield of GA₃ indirectly shows that the elimination of product inhibition enhances the GA₃ biosynthesis. However no direct experimental evidence is available to support product inhibition in GA₃ fermentation.

BIOPROCESS STRATEGIES TO ELIMINATE ABOVE LIMITATIONS

Fed Batch Process

Fed batch culture also called semi batch or extended batch culture, involves a fermenter operation strategy

based on constant or intermittent nutrient feeding strategy and confinement of the product in the fermenter until the end of the experiment [4,48]. Fed batch culture acts as a powerful tool to overcome several restrictions on fermentation efficiency such as substrate inhibition, catabolic repression and high cell concentration. It also provides an efficient mode for the extension of the production phase, replacement of water loss by evaporation and for decreasing the viscosity of broth. In addition, it also allows the voluntary control of the concentration of nutrients fed into the fermenter by changing the feed rate, which is not possible in batch culture. As discussed earlier, the production of GA₃ in batch submerged fermentation process is controlled by catabolic regulation and involves catabolic repression and substrate inhibition. These problems could be overcome by employing nutrient feed policy to achieve higher yield of GA₃ [4,15,20]. The fed batch culture technique has also been applied to the solid-state fermentation for GA₃ production. This process was able to overcome substrate inhibition by soluble starch thereby leading to an improvement in the yield of GA₃ by 18.2% as compared to the conventional solid-state batch fermentation [41].

Continuous Culture with Cell Recycle / Cell Retention

Continuous fermentation with cell recycle and cell retention system has distinct advantage with respect to achieving high cell density in continuous fermentation. It has been found to be successful in improving the productivity of product inhibited fermentations, mainly because the reactor can be operated at high dilution rate to flush out the inhibitory products and at the same time the filter device can retain the cells. In addition, this system also offers several other advantages such as it is economical, simple, and is known to feature high productivity [83]. Because of the secondary metabolite nature of GA₃ there is no report of GA₃ production with *in situ* cell retention system however other primary metabolites such as ethanol, lactic acid and acetone-butanol have been successfully produced by this technique [84-86]. Nevertheless the possibility of using cell retention culture to remove product inhibition cannot be ignored, as it has been possible to eliminate the inhibition in acetone-butanol ethanol fermentation [85].

Extractive Fermentation Process

Extractive bioconversion is one of the techniques for *in situ* product recovery, which involves separation of products from their biocatalyst immediately after they are formed [87]. In this technique two immiscible liquid phases are employed, the objective being to run a bioconversion in one liquid phase, while the product is extracted into the other phase. The benefits of *in situ* product recovery technique are found mainly in their potential for improving yield and productivity of existing processes. Yield improvements may be obtained by the reduction of product inhibition, through immediate product removal, or by prevention of product degradation, through mini-

Table 1. Down stream processing of cell free broth adopted by various researchers

Neumann <i>et al.</i> [104]	Koellner <i>et al.</i> [105]	Li <i>et al.</i> [106]	Rachev <i>et al.</i> [107]	Heropolitanski <i>et al.</i> [108]
Acidification of culture filtrate to 2-4	Precipitation of culture filtrate with CaCl ₂ -Kaoline mixture at weak acid pH	Acidification of culture filtrate to 2-3	Acidification of culture filtrate to 2.0-2.5 with 18% HCl	Adsorption of culture filtrate on Amberlite XAD-16
↓	↓	↓	↓	↓
Adsorption of filtrate on ion exchange resin column	Microfiltration / Reverse osmosis with cellulase acetate membrane (Flow rate - 0.2-2.0 L/min.; Pressure- 0.3-1.0 mPa)	Counter current extraction with organic phase (contains sulfonated kerosene, organophosphorus compound, amines and octanol) and saturated NaHCO ₃	Extraction with ethyl acetate	Desorption with aq. Solution of sodium or potassium hydroxide (pH > 10)
↓	↓	↓	↓	↓
Elution with ethyl acetate	Crystallization	Concentration	Concentration	Acidification of eluate to pH- 2 - 3.
↓	↓	↓	↓	↓
Concentration under reduced pressure	GA ₃	GA ₃	Reextraction with 1N NH ₄ OH	Extraction with ethyl acetate
↓			↓	↓
GA ₃			Acidification and Reextraction with ethyl acetate	Concentration under reduced pressure
			↓	↓
			Organic phase was dried over anhydrous Na ₂ SO ₄ and concentrated	GA ₃
			↓	
			Crystallization	
			↓	
			GA ₃	

mization of the product residence time in the vicinity of the biocatalyst [88]. Hollmann *et al* [81] reported two fold increase in the yield of GA₃ as result of on line extraction of the product by polyalkoxylate solvent. Various other acids like citric acid [89], propionic acid [90], butyric acid [91] and lactic acid [92] have been produced in high concentration by this technique.

Extractive fermentation in aqueous two-phase systems (ATPS) is a meaningful approach to overcome the limitations of product inhibition and low product yield in a conventional fermentation process. In ATPS the product has to be preferentially partitioned into the phase opposite to the one in which biocatalyst is located and by proper design of the two phase system it is possible to obtain the product in a cell free stream [93]. Examples of extractive fermentation in such system include ethanol [94], acetone butanol [95] and lactic acid production [96]. Phase system using polyelectrolytes in the presence of salts can provide useful bioconversion media [97]. The major disadvantages of this technique, which have hampered widespread industrial application of extractive bioconversions with ATPS, are the cost of the polymers and the difficulties in predicting the biocatalyst and product partitioning in ATPS due to the lack of adequate mathematical models [88].

Two Stage Process

In GA₃ fermentation, the high (> 0.5%) nitrogen me-

dium is needed in the first phase to achieve rapid mycelial growth. On the other hand, low growth rate and maintenance of optimum conditions for maximum production of GA₃ are essential in the second phase. These requirements were met satisfactorily in the two-stage fermentation technique [4,98]. Multi-stage fermentation technique was also developed to achieve higher yield than those attainable in the single and two-stage technique [4]. Recently, two-stage technique has also been successfully used for other products also such as xylitol, alcohol, ganoderic acid, propionic acid and 1-3 propanediol [99-103]. Although all the techniques described above have a great potential to overcome the problems of GA₃ fermentation, a detailed study on the application to GA₃ fermentation has not been reported so far.

RECOVERY PROCESSES

A great deal of work with GA₃ is focused on its extraction and analysis from plant source. From the fermentation broth, GA₃ could be recovered either by adsorption or by solvent extraction and then purified by repeated liquid-liquid partition and concentrated under a vacuum. Finally an amorphous powder or crystalline product is obtained [3]. Although several reports are available on the isolation of GA₃ from the broth (Table 1), its isolation in a pure state remains a most challenging task for the researchers.

In solid-state fermentation some other methods have also been developed for GA₃ extraction such as supercritical fluid extraction and multiple counter current leaching [109,110]. Supercritical fluid extraction (SCFE), a technique based on the exploitation of the enhanced power of supercritical fluids at temperature and pressure near the critical point. It offers an efficient and powerful alternative to conventional distillation and solvent extraction processes for separation of heat labile substances of low volatility at moderate temperature. Thus there is a potential use of SCFE in biotechnology for recovery of GA₃ from dry moldy bran produced under solid-state fermentation [109].

Multiple contact countercurrent leaching led to a higher concentration of product in the extract as compared to other techniques. Important advantage of this technique is the requirement of lesser quantity of solvent. Only one report is available in the literature on extraction of GA₃ from dry moldy bran by multiple-contact counter current leaching. A gradual increase in the concentration of GA₃ in the extract and in the percentage recovery of GA₃ was related to an increase in the number of contact stages. The use of four stages leads to about 87% extraction efficiency and 0.9 mg/L of GA₃ in the extract [110].

CONCLUSION AND FUTURE SCOPE

Gibberellic acid (GA₃), an important plant growth regulator, is used extensively in agriculture, nurseries, viticulture and tea garden for a variety of economic benefits. Its use, at present, is limited to high premium crops mainly because of its cost. Reduction in cost will lead to its wider application to a variety of crops and also to the harvest of innumerable industrial and economic benefits. It is traditionally produced by submerged fermentation using *G. fujikuroi*. The solid-state fermentation technique has shown a number of economic advantages over the submerged fermentation process in production of GA₃. In recent time, immobilized cells of *G. fujikuroi* have also been used for GA₃ production but there are very few reports available in the literature. It might be expected that GA₃ fermentation involve many problems like substrate inhibition, product inhibition, production of co-secondary metabolites along with GA₃ and degradation of GA₃ to biologically inert compound gibberellic acid. Only some researchers have experimentally proved the occurrence of above problems in GA₃ fermentation that may lead to low yield of GA₃. Some of the techniques like two-stage, fed batch and extractive fermentation using solvent have already been developed for GA₃ production. In future, further research on the development of efficient bioprocess strategies such as continuous fermentation with cell retention / cell recycle and extractive fermentation using ATPS system should be carried out to overcome above problems that may lead to higher yield and productivity of gibberellic acid. Other complicated processes of carbon and nitrogen regulation in the GA pathway as well as the involvement of a pathway-specific regulator must be studied in great detail on molecular

level in near future. This is one of the great challenges in gibberellic acid production process.

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