13 Gibberellic Acid Production

Cristina Maria Monteiro Machado &
Carlos Ricardo Soccol

13.1. INTRODUCTION

Gibberellins (GAs) are a group of diterpenoid acids that function as plant growth regulators influencing a range of developmental processes in higher plants. One of them, gibberellic acid (GA_3), has received the greatest attention. It affects stem elongation, germination, elimination of dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence. GA_3 is a high-valued plant growth regulator with various applications in agriculture (Arteca 1995).

The industrial process currently used for the production of GA_3 is based on submerged fermentation (SmF) techniques, using *Gibberella fujikuroi,* or *Fusarium moniliforme*. It is a typical secondary metabolite. Upon exhaustion of nitrogen sources, exponential ceases and secondary metabolism is triggered, with concomitant biosynthesis of GA_3 (Borrow et al., 1955; Brückner & Blechschmidt 1991).

In spite of the use of the best process technology, the yield of GA_3 is low. In fact, as early as 1979, it was stressed that the SmF process used for its production was approaching a saturation point beyond which cost reduction would be impossible. The presence of product in dilute form in SmF was recognized as a major obstacle in economic manufacture of the product, mainly due to the consequent higher costs of downstream processing and disposal of wastewater. Moreover, the cost of separation of the microbial cells from fermentation broth using centrifugation or microfiltration has been reported to involve between 48 and 76% of the total production cost of the microbial metabolite by submerged fermentation. (Jefferys 1970; Kumar & Lonsane 1989).

Recognizing that it is essential to look beyond the conventional submerged technique and explore other alternatives to achieve a more economical processes, different studies have been carried out to decrease the production costs, using

several approaches such as screening of the fungi, optimization of the nutrients and culture conditions, development of alternative processes (immobilized cells, fed-batch culture) and minimization of the cost of extraction procedure (Bandelier et al., 1997). Another technique, solid-state fermentation (SSF), has been investigated to increase the yields of GA_3 and also to decrease production costs (Kumar & Lonsane 1987a; Pastrana et al., 1995; Tomasini et al., 1997; Bandelier et al., 1997; Gelmi, et al., 2000). Indeed, the SSF technique has shown a number of economic advantages over SmF process in the production of microbial biomass and metabolites and the valorization of agro-industrial by-products (Pandey 1992; Soccol 1996; Pandey et al., 2001; Soccol & Vandenberghe 2003).

The present chapter will firstly briefly describe gibberellic acid physical, chemical and biological properties. Then, it will treat about this metabolite production by fermentation, considering its biosynthesis pathway, fermentation techniques, GA_3 formation physiology in fermentative process, factors influencing fermentation and newest advances carried out to decrease the production costs, with special focus on SSF. Finally, a case study of GA_3 production by *G. fujikuroi*, using mixed substrate of important Brazilian agro-industrial wastes as a practicable alternative for the production of GA_3 by solid-state fermentation will be presented.

13.2. PROPERTIES AND USES OF GIBBERELLIC ACID

The gibberellins (GA_s) are all tetracyclic diterpenoid acids strictly related, representing an important group of plant growth hormones. Known gibberellins are identified by subscribed numbers Ga_n were "n" corresponds, approximately, the order of discovery. Gibberellic acid, which was the first gibberellin to be structurally characterized, is GA_3 (Hill 1977; Arteca 1995). All gibberellins have an *ent*-gibberellane ring system and are divided in two main types based on the number of carbon atoms, the C_{20}GAs which have a full complement of 20 carbon atoms and C_{19}GAs in which the twentieth carbon atom has been lost by metabolism. Many structural modifications can be made to the *ent*-gibberellane ring system. This diversity accounts the large number of known GAs (Kumar & Lonsane 1989; Sponsel & Hedden 2004).

Gibberellins are distributed widely through the plant Kingdom where they play an important role in plant growth and development. They have also been isolated from fungi and bacteria. Of the 131 presently known naturally occurring GAs, 105 have been found exclusively in higher plants (including angiosperms, gymnosperms and ferns), 11 in the fungi only, and the rest from both the sources. Of them, gibberellic acid (GA_3) has received the greatest attention. GA_3 regulates the growth tax in plants being extensively used to produce a series of benefic effects useful in agriculture (Mander 2003; Leitch et al., 2003; Crow et al., 2006).

Gibberellic acid ($C_{19}H_{22}O_6$), chemically characterized as a tetracarbocyclic dihydroxy-g-lactonic acid containing two ethylene bonds and one free carboxylic acid group (Cross, 1954). It is a white crystalline powder, with a melting point of 233 –235 °C, soluble in alcohols, acetone, ethyl acetate, butyl acetate, while is soluble with difficulty in petroleum ether, benzene and chloroform. The product can't be decomposed in dry condition and it is rapidly decomposed in hot condition and in aqueous solutions. It's half live in diluted aqueous solutions is about 14 days at 20 °C and 2 hours at 50 °C (O'Neil, 2001).

Plant tissues usually contain only about 0.001 to 1.0 mg of gibberellic acid equivalent per kilogram of fresh weight. Different gibberellins are usually found in the same plants, and their pattern can change depending on stage of development. Sensitivity to various effects caused by different gibberellins can differ depending on the plant genera, organs and development stage (Takahashi 1986; Brückner & Blechschmidt 1991). GAs appear to be involved in every aspect of plant growth and development, but their most typical (and spectacular) property is the enhancement of stem growth. Active gibberellins show many physiological effects, each depending on the type of gibberellin present as well as the species of plant (Martin 1983; Mander 2003).

Considering the numerous effects of gibberellins, it seems logical that they would be used in commercial applications. Their major uses are (Martin 1983; Taiz & Zeiger 1991; Mander 2003):

- Management of fruit crops – most seedless table grapes are now grown with the application of GA_3, inhibiting senescence of citrus fruit maintaining the rind in better condition; controlling skin disorder in "golden delicious" apples;
- Production of ornamental plants – inducing to ûower either earlier than usual, or in off-seasons. Sporadic ûowering in some plants is often a problem with plant breeders, but may be ameliorated with GA applications;
- Malting of barley: 2–3 days may be saved by the addition of 25–500 μg of GA_3 for each kg of barley.
- Extension of sugarcane: increase in grown and sugar yield.

13.3. GIBBERELLIC ACID PRODUCTION

13.3.1. The gibberellin biosynthesis pathway

The biosynthesis of GAs in plants and in fungi has received a great deal of attention and has been the subject of several reviews in the last ten years (Mac Millan, 1997; Hedden 1999; Tudzynski 1999, 2005; Sponsel & Hedden 2004; Kawaide 2006). Gibberellins are diterpenes synthesized from acetyl CoA

via the mevalonic acid pathway. They all have either 19 or 20 carbon units grouped into either four or five ring systems (Sponsel 1995).

GAs, like other diterpenoids, are produced from hydroxymethylglutaryl (HMG) coenzyme A (CoA) via mevalonic acid, isopentenyl diphosphate, geranyldiphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP), which is a precursor not only for GAs, but also for the carotenoid neurosporaxanthin and ubiquinones. Ent-kaurene, the first GA-specific intermediate, is produced in two cyclisation steps from GGDP via ent-copalyl diphosphate (CPP). Sequential oxidation of ent-kaurene at C-19 via ent-kaurenol and ent-kaurenal yields entkaurenoic acid, which is further oxidised to ent-7α-hydroxykaurenoic acid. A final oxidation at C-6β, resulting in contraction of ring B, leads to formation of GA12-aldehyde. These steps of the pathway are identical in the fungus and in higher plants (Tudzynski, 2005). The subsequent steps in plants will not be described in this work. In *G. fujikuroi*, GA_{12}-aldehyde is first 3β-hydroxylated to GA_{14}-aldehyde, which is then oxidized at C-7 to form GA_{14}. GA_{14} is then converted to the 19-carbon gibberellin GA_4 by 20-oxidation. GA_4, the first biologically active GA, is desaturated to GA_7, which is then converted to GA_3 by late 13-hydroxylation. GA_1 is formed in a minor side reaction by 13-hydroxylation of GA_4 (Tudzynski, 2005).

13.3.2. Fermentation techniques

Fermentation is the industrial method practiced for the manufacture of GA_3 preferentially with *G fujikuroi* or its imperfect stage *F moniliforme* (Borrow et al., 1955). Is possible produce it by chemical synthesis (Corey et al., 1978; Hook et al., 1980) or extraction from plants (Kende 1967) but these methods are not economically feasible. Liquid surface fermentation (LSF) was employed in earlier years for the production of GAs and its use was continued until 1955. It offers advantages such as no foam formation and no mechanical damage to mycelial cells, as compared to the submerged fermentation process. Although, due to disadvantages inherently present in LSF as production of a wide range of by-products, very low yield (40 to 60 mg GAs.$L_{substrate}^{-1}$), prolonged incubation time (10 – 30 days) and prone to contamination this technique was abandoned for GA_3 production being substituted by submerged fermentation (SmF) (Kumar & Lonsane 1989; Brückner & Blechschimidt, 1991).

The presence of product in dilute form in SmF was recognized as a major obstacle in economic manufacture of the product, mainly due to the consequent higher costs of downstream processing and disposal of wastewater. Moreover, the cost of separation of the microbial cells from fermentation broth using centrifugation or microfiltration has been reported to involve between 48 and 76% of the total production cost of the microbial metabolite by submerged

fermentation (Vass & Jefferies 1979; Kumar & Lonsane 1987b; Bandelier et al., 1997).

Recently different studies have been carried out to decrease GA_3 production costs using several approaches as screening of fungi, optimization of the nutrients and culture conditions, use of agro-industrial residues as substrate, development of new processes (immobilizes cells, fed-batch culture) and minimization of the cost of the extraction procedure. Some of these listed in Table 1.

Solid-state fermentation (SSF) has also been investigated to increase yields of GA_3 and also minimize production and extraction costs (Kumar & Lonsane 1986, 1987a, 1987b, 1987c; 1988, 1990; Bandelier et al., 1997; Tomasini et al., 1997; Gelmi et al., 2000, 2002; Machado et al., 2001, 2002, 2004; Escamilla et al., 2000; Corona et al., 2005). Indeed, The SSF technique has shown a number of economic advantages over submerged fermentation processes in the production of microbial biomass and metabolites and in the valorization of agro-industrial by products (Pandey 1992; Pandey & Soccol 1998; Padey et al., 2000a,b; Soccol & Vandenberghe, 2003).

13.3.3. GA_3 formation physiology in fermentative process

Fermentative production of gibberellins is a classic example of secondary metabolite production as the phases of growth can be clearly distinguished and related to nutritional and environmental states operating in the fermentor. Borrow et al., (1961; 1964a,b) have exhaustively studied this process and established producing and non-producing phases of the gibberellin fermentation process.

The conventional *lag phase* in nitrogen-limited medium is undetectable as the strain requires little or no adaptation and growth in the fermentor starts quickly due to the use of vigorous mycelial cells as inoculum. Growth during the *balanced phase* is initially exponential and subsequently becoming linear. The uptake of glucose, nitrogen and other nutrients remains almost constant per unit increase in dry weight. This phase extends until exhaustion of one of the nutrients occurs subjecting the cells to a deceleration stage. No GA is produced in this phase. The following *storage phase* occurs with the presence of excess glucose and the exhaustion of nitrogen, causing an increase of dry weight due to accumulation of lipids (45%), carbohydrates (32%) and polyols. In this phase the production of gibberellins and other secondary metabolites begins and is continued in the presence of available glucose. The next *maintenance phase*, is operative between the maximum mycelial formation and the onset of terminal breakdown of mycelial components. Because it is the main gibberellin-producing phase, its continuation, even for several hundred hours, if glucose is present in excess, is of industrial importance. Except for the continued

Table 1: Studies with different strategies for GA_3 production

Substrate[1]	*Strategy used/fermentation technique*[2]	*Reference*
Olive bagasse (after oil extraction)	Use of agro-industrial wastes/SmF	Hernandez & Mendoza 1976
Diary waste	Use of agro-industrial wastes/SmF	Maddox & Richert 1977
Cheese whey in sodium alginate beads	Use agro-industrial wastes and immobilized cells /SmF	Kahlon & Malhotra 1986
Wheat bran	Screening of fungi/SSF	Kumar & Lonsane 1986
Synthetic medium in carrageenan	Use of immobilized cells /SmF	Jones & Pharis 1987
Wheat bran	Fed-batch culture/SSF	Kumar & Lonsane 1987a; 1988
Wheat bran	Minimization of cost of extraction procedure/SSF	Kumar & Lonsane 1987b
Synthetic medium in calcium alginate beads	Use of immobilized cells /SmF	Nava-Saucedo et al., 1989
Wheat bran	Optimization of the nutrients and culture condition/SSF	Kumar & Lonsane 1990
Synthetic medium in polymeric fibrous carriers	Use of immobilized cells /SmF	Lu et al., 1995
Mussel processing wastes	Fed-batch culture & use of agro-industrial wastes/SmF	Pastrana et al., 1995
Synthetic medium	Use of extractive fermentation/SmF	Hollmann et al., 1995
Vinasse	Use of agro-industrial wastes/SmF	Cihangir & Aksöza 1997
Fruit pomasse	Use of agro-industrial wastes/SmF	Cihangir & Aksöza 1997
Beet pulp	Use of agro-industrial wastes/SmF	Cihangir & Aksöza 1997
Whey	Use of agro-industrial wastes/SmF	Cihangir & Aksöza 1997
Wheat bran	Fed-batch culture/SSF	Bandelier et al., 1997
Cassava flour	Optimization of the nutrients and culture condition/SSF	Tomasini et al., 1997

Substrate[1]	*Strategy used/fermentation technique*[2]	*Reference*
Synthetic medium	Use of inert support of amberlite/SSF	Gelmi et al., 2000
Coffee husk	Screening of fungi/SSF	Machado et al., 2001
Coffee husk and cassava bagasse	Optimization of the nutrients and culture condition/SSF	Machado et al., 2002
Synthetic medium	Immobilized fungi in fluidized bioreactors/SSF	Escamilla et al., 2000
Synthetic medium	Minimization of cost of extraction procedure/SmF	Tang et al., 2000
Synthetic medium in amberlite support	Modelling *G. fujikuroi* growth and GA_3 production/SSF	Gelmi et al., 2002
Synthetic medium	Use of precursors and other fungus (*A.niger*)/SmF	Cihangir 2002
Coffee husk and cassava bagasse	Modelling *G. fujikuroi* growth and GA_3 production	Machado et al., 2004
Wheat bran and soluble starch	Optimization of the nutrients and culture condition/SSF	Corona et al., 2005
Synthetic medium	Developing and selecting morphological mutants/SmF	Lale 2006
Synthetic medium	Modelling *G. fujikuroi* growth and GA_3 production /SmF	Shukla et al., 2005

[1]Substrate as the main C and N source;

[2]Fermentation techniques: SmF – submerged fermentation; SSF – solid-substrate fermentation

uptake of glucose, the cells take up no other nutrients and their dry weight remains constant. Finally, in the *terminal phase* the mycelial cells undergo many changes due to no availability of sources of utilizable carbon. This phase is not allowable to occur in fermentations, as the fermentor run for production of GAs is terminated just prior to the onset of this phase (Borrow et al., 1961, 1964 a,b; Kumar & Lonsane 1989; Brückner & Blechschmidt 1991).

13.3.4. Nutritional factors influencing GA_3 production

Glucose and sucrose have often been used as carbon source, but concentrations above 20% of glucose at the beginning of the fermentation should be avoided, as it causes catabolic repression (Borrow et al., 1964a). Many workers have used alternative carbon sources, such as maltose, mannitol, starch and plant meals or mixtures of fast and slowly utilized carbon sources, e.g. glycerol, glucose and galactose (Darken et al., 1959; Sanchez-Marrokin, 1963; Hernandez & Mendoza 1976; Maddox & Richert 1977; Kahlon & Malhotra 1986; Kumar & Lonsane 1989; Pastrana et al., 1995; Cihangir & Aksöza 1997; Tomasini et al., 1997; Machado et al., 2001).

The quality and quantity of nitrogen are very important for gibberellin fermentation because of the ammonium regulation of this process. All described media that guarantee high yields of GAs are low nitrogen media, as gibberellic acid production begins at, or soon after, nitrogen exhaustion (Borrow 1964a; Brückner & Blechschmidt 1991; Tudzynski 1999). Favorable nitrogen sources are ammonium sulfate, ammonium chloride and slowly assimilable sources as glycine, ammonium tartarate, plant meals and corn step liquor (Kumar & Lonsane, 1989).

The influence of C:N ratio is directly related with GA_3 production metabolism. So, their values must provide initial active mycelial growth in a nitrogen-limited balanced medium, initiation of GA_3 production after nitrogen exhaustion and extended metabolite production in the presence of sufficiently available carbon substrate. Therefore, the ratio normally used is high, between 6:1 and 45:1. In SmF is common the utilization of two stage processes, were the initial stage has C:N ratios between 10:1 and 25:1 and final stage varies from 25:1 and 200:1 (Borrow 1964a; Jefferies 1970; Kumar & Lonsane, 1989; Brückner & Blechschmidt 1991; Tudzynski 1999).

Besides carbon and nitrogen, magnesium, potassium, phosphate and sulfate are all needed in the biosynthesis of secondary metabolites. In spite of that, negligible information is available on these aspects in the microbial production of GAs. Normally the requirements are met efficiently using the salts combinations from Czapek-Dox and Raulin-Thom media. Trace element requirements are often met by impurities in commercial media (Borrow 1964a; Kumar & Lonsane, 1989; Brückner & Blechschmidt 1991).

13.3.5. Environmental factors influencing GA_3 production

pH variation is one of the most influent factors in the composition of the produced gibberellin mixture. For GA_3 production, pH generally employed is between 3.5-5.8. The pH increase will lead a bigger production of $GA_{4,7}$ and more acid media (pH < 3.5) will increase GA_1 production (Kumar & Lonsane, 1989). In solid-state fermentation pH measurement is complex. Therefore are few works describing values used for GA_3 production using this technique. Gelmi et al., 2000, using amberlite inert support established for studied system initial pH of 4.5, Tomasini et al., 1997 in a fermentation in cassava flour used initial pH 6.0 and Machado et al., (2001) with a mix substrate of coffee husk and cassava bagasse had better GA_3 production with initial pH between 5.0-5.4.

The effect of temperature on the GA_3 production is dependent on the strain employed. Values between 25-34 °C are reported (Hernandez & Mendoza 1976; Maddox & Richert 1977; Kahlon & Malhotra 1986; Jones & Pharis 1987; Nava-Saucedo et al., 1989; Kumar & Lonsane 1990; Pastrana et al., 1995; Cihangir & Aksöza 1997; Tomasini et al., 1997; Escamilla et al., 2000; Machado et al., 2002; Corona et al., 2005). Jeferys (1970), reported that the optimum temperature for growth of the strain is between 31-32°C while the production of GA_3 was maximized at 29°C.

Since the biosynthesis of gibberellins involves many oxidative steps, a good aeration of fermentors is critical for an optimal production process. In fact, since the value of oxygen consumption for a growing mycelium in the exponential phase of growth remains constant, the demand of oxygen increases more or less exponentially (Tudzynski 1999). Jefferys 1970 suggests that the aeration must be as vigorous as possible, being reported, for SmF rotations between 150-1400 rpm. In in a feed-batch reactor with SSF Bandelier et al., 1997 used an air flow of 0.9 $L_{air}.(h.kg_{dry\ matter})^{-1}$, Tomasini et al., 1997 of 0.42 $L_{air}.(h.kg_{dry\ matter})^{-1}$, and Gelmi et al., 2000 of 0.46 $L_{air}.(h.kg_{dry\ matter})^{-1}$ in a glass column reactor. In this kind of reactor, Machado et al., 2004 determined that better GA_3 production should be achieved with a low aeration (0.24 $L_{air}.h^{-1}.g_{dry\ matter}^{-1}$) in the first 72 h of fermentation and, after that, with a much more vigorous aeration (0.72 $L_{air}.h^{-1}.g_{dry\ matter}^{-1}$).

13.4. GIBBERELLIC ACID PRODUCTION BY SOLID-STATE FERMENTATION ON MIXED SUBSTRATE OF COFFEE HUSK AND CASSAVA BAGASSE

Although GA_3 has been conventionally produced by submerged fermentation, it can also be produced by SSF. Production through SSF route could be relative cheaper and may involve lower costs, if several approaches such as application of cheaper substrate viz. agro-industrial residues, optimization of the nutrients

and culture conditions, minimization of the cost of extraction procedure, etc. are applied.

The residues of coffee industry represent about 50% of total mass and practically do not find any industrial or useful application. Instead, their disposal is a major environmental concern. These products are rich in organic matter, which could make them suitable for bioconversions processes. The principal difficulty for its utilization in fermentations is the presence of inhibitory metabolites, as phenolic compounds (tannins, chlorogenic acid and caffeic acid) and caffeine, which represents about 6-14% of coffee pulp dry weight. But if these difficulties are overcome useful products can be produced from these residues by cultivating microbes in submerged fermentation or solid-state fermentation (Elias 1978; Perraud-Gaime et al., 2001).

Laboratory studies were carried out in 250 mL erlenmeyer flasks and in aerated columns using statistical experimental designs. The strains of *G fujikuroi* or *F moniliforme* used were maintained in potato dextrose agar (PDA) and were inoculated in Czapek Dox medium in rotatory shaker at 30°C for 4 days for developing the inoculum (Bandelier et al., 1997). The GA_3 isolation was done with phosphate buffer, pH 8 and metabolite quantification by high performance liquid chromatography (Varian system composed of a pump, a diode array detector and a column C18. Mobile phase methanol:water - 40:60). Aqueous layer was separated and quantification was made by high performance liquid chromatography.

13.4.1. Selection of microorganism and treatment of substrate

The first part of the work aimed at exploring the possibility of using coffee husk as substrate for the production of GA_3 (Machado et al., 2001). Solid-state fermentation was compared with liquid fermentation (done in the aqueous extract of the substrate) with five strains of *G fujikuroi* and one of its imperfect state, *F moniliforme*. The conditions of fermentation were similar for both systems. The results showed that SSF resulted higher quantities of GA_3 and that the strain *G fujikuroi* LPB-06 was best adapted in the chosen substrate. Hence, further works were conducted with the strain LPB-06 in solid-state fermentation.

Because coffee husk has phenolic compounds that could interfere in GA_3 production, pre-treatment of the substrate was studied. For this, different conditions of pre-treatment (alkaline hydrolysis), utilizing different concentrations of KOH and time of treatment were tested. With this study, a production of about 100 mg $GA_3.kg._{dry\ matter}^{-1}$ were reached in the best conditions: 45 minutes hydrolysis in aqueous solution of 5 g KOH/L.

13.4.2. Studies in Erlenmeyer flasks – optimization of fermentation conditions

Studies were carried out on optimization of nutritional and physical factors, such as supplementation of minerals and nitrogen to the substrate, inital pH and moisture of substrate and incubations temperature, for improved yields of GA_3 (Soccol et al., 2000; Machado et al., 2002). To change the original coffee husk C:N relation that was about 14:1, cassava bagasse was used. This residue contains a large amount of amide, resulting in a very high C:N relation (110:1). The cassava bagasse was added in six different concentrations, 15, 30, 45, 60, 75 and 90%. The best results were obtained in substrate enriched with 30% of cassava bagasse, resulting in a C:N relation of 43:1, and reaching a production of 230.6 mg $GA_3.kg._{dry\ matter}^{-1}$ an increase of almost 100% compared with a substrate 100% coffee husk.

To test the influence of addition of certain salts, two statistical experimental designs were performed. In the first one, the parameters studied with $2^{(7-4)}$ experimental design were presence or absence of seven salts reported as useful in gibberellins production (KH_2PO_4; K_2SO_4; $MgSO_4$; $ZnSO_4$; $CuSO_4$; $FeSO_4$ and $(NH_4)_2SO_4$), in the concentrations suggested by Kumar & Lonsane (1989). The results demonstrated that there several salts, in spite of being indicated as useful in the production of GA_3, had a negative effect. That is probably explained by their natural presence in coffee husk, and an addition becoming inhibitory for the growth and metabolism of the microorganism.

The second optimization was done varying the concentration of the salts that had the best positive influence in the production of GA_3: FeSO4 and $(NH_4)_2SO_4$. The concentrations chosen varied between the highest and lowest values suggested by Kumar and Lonsane (1989). With this optimization, a production of 389 mg $GA_3.kg._{dry\ matter}^{-1}$ was achieved with the saline solution, consisting of 30 mg of $FeSO_4$ and 10 mg of $(NH_4)_2SO_4$.100 mL^{-1}, as shown in the contour response (Figure 1).

The physical parameters studied were incubation temperature, initial moisture and pH of substrate. To test the influence of these parameters, two experimental designs were performed. The first was a $2^{(3-1)}$ and under the best conditions of initials pH and moisture of substrate as 5.0 and 70%, respectively, and incubation temperature of 30°C, 325 mg $GA_3.kg._{dry\ matter}^{-1}$ was produced. Observing the Pareto chart of effects (Figure 2), it is noticed that the variable of larger influence in the production of GA_3 is the pH, followed by the initial moisture of the substrate, both suggesting that larger values would take to an increasing of the answer variable.

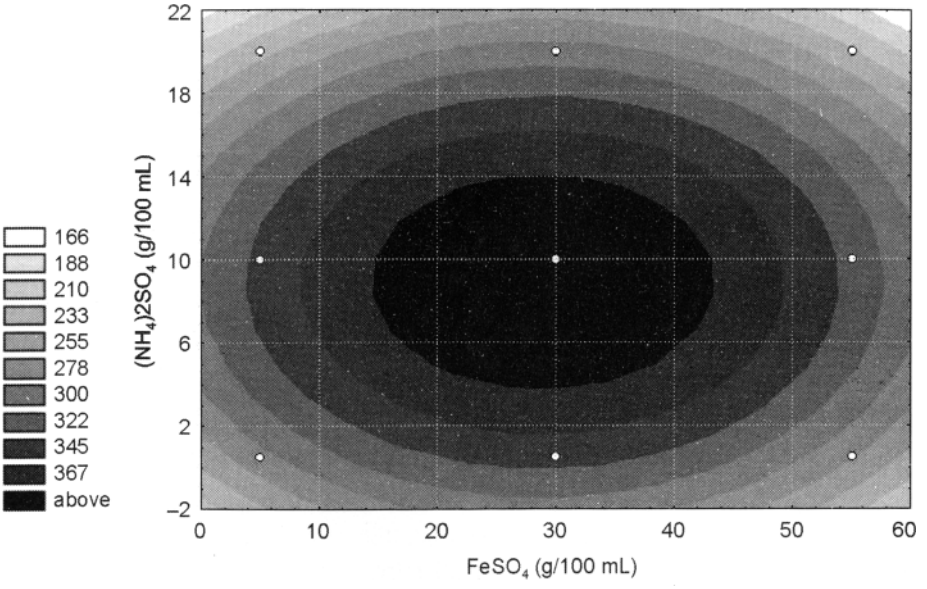

Figure 1: Contour response to GA_3 production as a function of $(NH_4)_2SO_4$ and $FeSO_4$ concentrations (levels given in mg $GA_3.kg_{dry\ matter}^{-1}$).

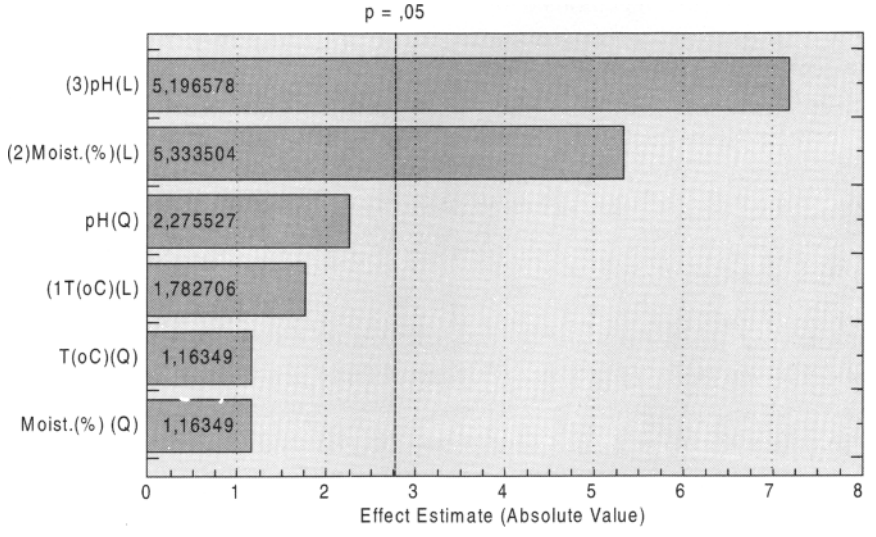

Figure 2: Pareto chart of effects of first optimization of physical parameters in erlenmeyer flasks (response variable mg $GA_3.kg_{dry\ matter}^{-1}$)

According to Kumar & Lonsane (1989), the pH is one of the most important factors for gibberellins production, mainly for definition of produced gibberellin, suggesting concentrations among 3.5-5.8 for increase the GA_3 production. The levels used in this experiment were 4.0, 4.5 and 5.0. It is well known that in SSF initial moisture of substrate is one of the most important parameters. According to Raimbault (1998), in most of SSF processes, values between 35 and 80% are used, depending on the microorganism and substrate. Among the used levels, temperature did not significantly influent in the GA_3 production. Thus, in subsequent experiments, temperature was maintained at 29°C and higher values for initial pH and moisture were chosen; moisture and pH were 78% and 5.5, respectively. The results are shown in the contour response (Figure 3).

A maximum of 492.5 mg $GA_3.kg._{dry\ matter}^{-1}$ was produced using a mixed substrate comprising coffee husk and cassava bagasse (7:3, dry wt), supplemented with saline solutions containing (%) 0.03 $FeSO_4$ and 0.01 $(NH_4)_2SO_4$, pH and moisture as 5.3 and 75%, respectively, and incubation temperature as 29°C; the GA_3 yield was almost 5 times more than the results without optimization.

13.4.3. Studies in packed-bed column bioreactor

13.4.3.1. Optimization of fermentation conditions

Studies were carried out on optimization of gibberellic acid production in 14 glass columns placed in a temperature controlled water bath (29°C), which were packed using pre-inoculated substrate with forced aeration as described by Raimbault & Alazard (1980). For this, two statistical experimental designs were performed, both varying the airflow rate and initial moisture of the substrate. The other parameters used were the ones determined in Erlenmeyer flasks: 7 days fermentation, mixed substrate comprising coffee husk and cassava bagasse (7:3, dry wt), supplemented with saline solutions containing (%) 0.03 $FeSO_4$ and 0.01 $(NH_4)_2SO_4$, pH 5.3.

With aeration levels of 0.24, 0.48 and 0.72 $L_{air}.h^{-1}.g_{drymatter}^{-1}$ and initial moisture of 68, 73 and 78 %, the best result obtained was of 373 mg $GA_3.kg_{dry\ matter}^{-1}$ with 78% of initial moisture and aeration of 0.2 $L_{air}.h^{-1}.g_{dry\ matter}^{-1}$. Comparing the results obtained in Erlenmeyer flasks with the same initial moisture content, a value 25% lower was obtained. Observing Pareto chart of effects (Figure 4), it could be assumed that the aeration has a negative influence on fermentation. Nevertheless, this observation opposes with the theory, for as much the metabolite biosynthesis progress over growing oxidation levels. In fact, according to Tudsynski (1999) considering that the oxygen consumed for mycelium production might remain constant, the oxygen demand increases approximately in exponential way.

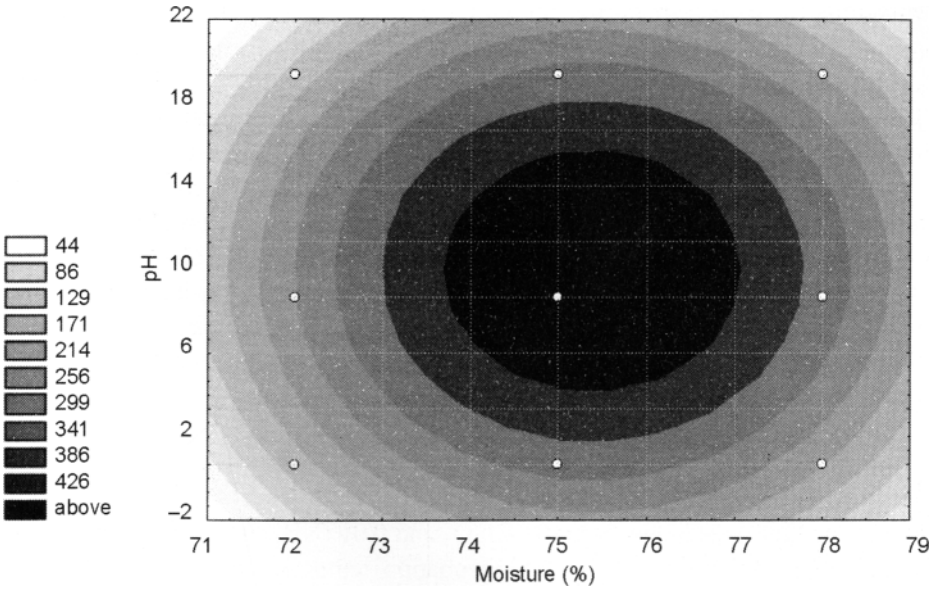

Figure 3: Contour response to GA_3 production as a function of pH and initial moisture of substrate (levels given in mg $GA_3.kg_{dry\ matter}^{-1}$).

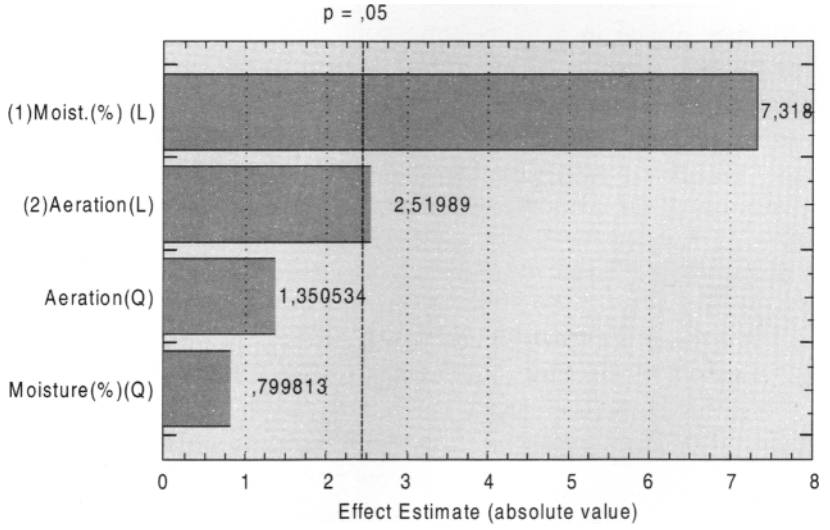

Figure 4: Pareto chart of effects of first optimization of moisture (%) and aeration ($L_{air}.h^{-1}.g_{dry\ matter}^{-1}$) in packed-bed columns bioreactor (response variable mg $GA_3.kg_{dry\ matter}^{-1}$)

Therefore, in the second optimization was tested the hypothesis that a vigorous aeration in the beginning of fermentation should be affecting the microorganisms growth and, as a result, the gibberellic acid production. Hence, a low aeration (0.24 $L_{air}.h^{-1}.g_{substrate}^{-1}$) was used in the first 72 h of fermentation and then the levels of 0.24, 0.72 and 1.20 $L_{air}.h^{-1}.g_{substrate}^{-1}$ were tested. As shown in Pareto chart of effects for first optimization (Figure 4), the initial moisture had a positive influence on gibberellic acid production, hence, higher levels were experimented (78, 80 and 82%). With this, a better production of GA_3 was achieved, reaching 625 mg $GA_3.kg_{dry\ matter}^{-1}$ with moisture and aeration of 78% and 0.72 $L_{air}.h^{-1}.g_{substrate}^{-1}$ respectively (Figure 5). It was an improvement of 27% compared to Erlenmeyer flasks fermentation and of 68% compared with columns fermentation with constant aeration.

13.4.3.2. Kinetics of G fujikuroi growth and GA_3 production

The present work was undertaken to study the kinetics of growth and production of GA_3 by *G. fujikuroi*, using coffee husk and cassava bagasse as substrate in aerated columns bioreactor (Machado et al., 2004). SSF was carried out in 14 glass columns placed in a temperature controlled water bath (29°C). The air flow was set at 0.24 $L_{air}.h^{-1}.g_{substrate}^{-1}$ for the first 72 h of SSF and was increased to 0.72 $L_{air}.h^{-1}.g_{substrate}^{-1}$ after that. After each 24 h, two columns were withdrawn as samples for analysis. A tendency of increasing moisture and water activity was observed during the course of fermentation. This behavior could be expected due to the use of saturated air for aeration during the process, resulting in significant mass transfer during the experiment. In Erlenmeyer flasks without aeration, with the same substrate the moisture decreased gradually (Soccol et al., 2000; Machado et al., 2002).

As carbon compounds in the substrate are metabolized, they are converted into biomass and carbon dioxide. Production of carbon dioxide causes the weight loss of fermenting substrate, and the amount of weight lost can be correlated to the amount of growth that takes place (Raimbault, 1997). In this experiment, loss of weight reached 9% at 7th day of fermentation and followed the Monod model: a lag phase of 48 h, exponential phase 48-120 h and stabilization until 144 h.

In Figure 6 the behavior of the system for biomass and GA_3 in the 7 days of fermentation is shown. Table 2 was made considering all data for the kinetic study. For all kinetic calculations, the 7th day of fermentation was not considered since GA_3 production decreased after the 6th day. Although the substrate was of complex nature, the main carbon source for this fermentation was considered the non-reducing sugars, which came mainly from cassava bagasse starch. Therefore, in Table 2, these were the values considered in column "S". Biomass

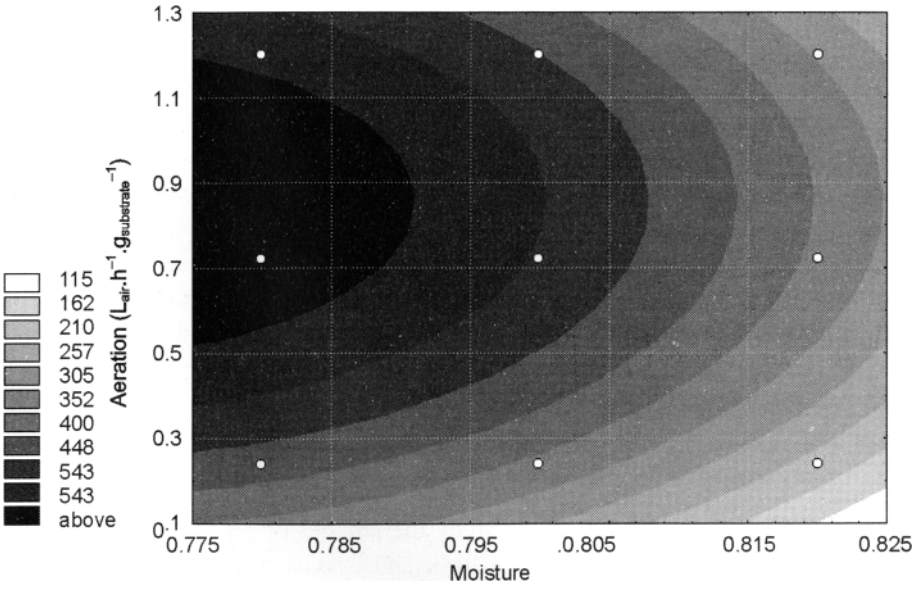

Figure 5: Contour response to GA_3 production as a function of aeration and initial moisture of substrate in packed-bed columns bioreactor (levels given in mg $GA_3.kg_{dry\ matter}^{-1}$)

Table 2: Kinetic parameters in SSF for gibberellic acid production

Time (h)	*Xg.(kg DM)$^{-1}$*	*Sg.(kg DM)$^{-1}$*	*Pg.(kg DM)$^{-1}$*	*μ (h^{-1})*	*r_pg.(kg DM.h)$^{-1}$*
0	1.122	76.665	0.000	-	-
24	1.677	64.226	0.153	0.0167	0.006
48	5.872	38.742	0.206	0.0522	0.002
72	10.791	37.504	0.315	0.0254	0.005
96	15.764	36.886	0.417	0.0158	0.004
120	19.276	36.443	0.711	0.0084	0.012
144	21.743	35.519	0.925	0.0050	0.009
168	24.185	28.664	0.740	0.0044	-0.008

Where: DM – dry matter (dry fermented substrate); X – biomass; S – substrate (non-reducing sugars); P – product (gibberellic acid); μ – specific growth rate; r_p – GA_3 production per hour

was calculated as twice the increasing protein assuming that 50% of fungal biomass was protein, and considering that the protein consumed in the substrate was proportional to the loss of weight of the substrate.

The phases of biomass evolution can be analyzed observing Figure 6. Evidently it followed a Monod model having a lag phase (days 0 and 1), where the specific growth rate was practically null, and represented a stage in which microbial biomass did not start to multiply; logarithmic or exponential growth phase (day 1 to 3), reaccelerated growth phase (after the 3rd day) when the biomass synthesis started to demit in the process. Stationary phase was not observed as that probably might reach after the 7th day.

The maximum specific growth rate (μ_{max}) were determinate considering Monod Model between days 1 and 3 (logarithmic growth phase) and it was 0.041 h^{-1}. This value was similar to those reported for other SSF-grown *G. fujikuroi* that varied between 0.12 and 0.57 (Gelmi et al., 2000), between 0.06 and 0.08 (Pastrana et al., 1995) and 0.018 (Bandelier et al., 1997).

The main product of this fermentation was gibberellic acid. After 6 days concentration of 0.940 g GA_3.kg dry $matter^{-1}$ was achieved. Table 2 showed that the best production of the metabolite was between 4 and 6 days of fermentation, just after the logarithmic growth phase. This behavior is characteristic for secondary metabolites kinetics as gibberellic acid. In secondary metabolism, the desired product is usually not derived from the primary growth substrate but a product formed from the primary growth substrate acts as a substrate for the production of a secondary metabolite. The secondary metabolites are not essential for the organisms' own growth and reproduction. In fact, normally, they are produced in "stress" situations for the microorganism (Pandey et al., 2001). Because the metabolic rout of gibberellic acid production is known and it happens after several oxidation reactions, high yields of oxygen are required. Hence, the change of aeration rate in 4th day contributed for the raise in GA_3 production rate too.

The kinetics parameters calculated were the maximum and the total rate of GA_3 production: r_{Smax} = 0.254 $g.kg^{-1}.day^{-1}$ r_{Total} = 0.154 $g.kg^{-1}.day^{-1}$. Kumar and Lonsane (1987) obtained a yield of 1.2 g GA_3.kg dry $matter^{-1}$ utilizing wheat bran in a pilot scale bioreactor after seven days of fermentation (r_{Total} = 0.171 $g.kg^{-1}.day^{-1}$) and Bandelier et al., (1997) in a similar system 3 g GA_3.kg dry $matter^{-1}$ after 10 days (r_{Total} = 0.3 $g.kg^{-1}.day^{-1}$). Gelmi et al., (2000) achieved 0.760 g GA_3.kg dry $matter^{-1}$ with vermiculite inert support after 5 days of fermentation (r_{Total} = 0.152 $g.kg^{-1}.day^{-1}$) and Tomasini et al., (1997) reported 0.24 g GA_3.kg dry $matter^{-1}$ after 3 days of fermentation with cassava flour as substrate (r_{Total} = 0,08 $g.kg^{-1}.day^{-1}$). Considering the same system, the concentration achieved was the best, having the advantage of the utilization of agro-industrial residues as substrates.

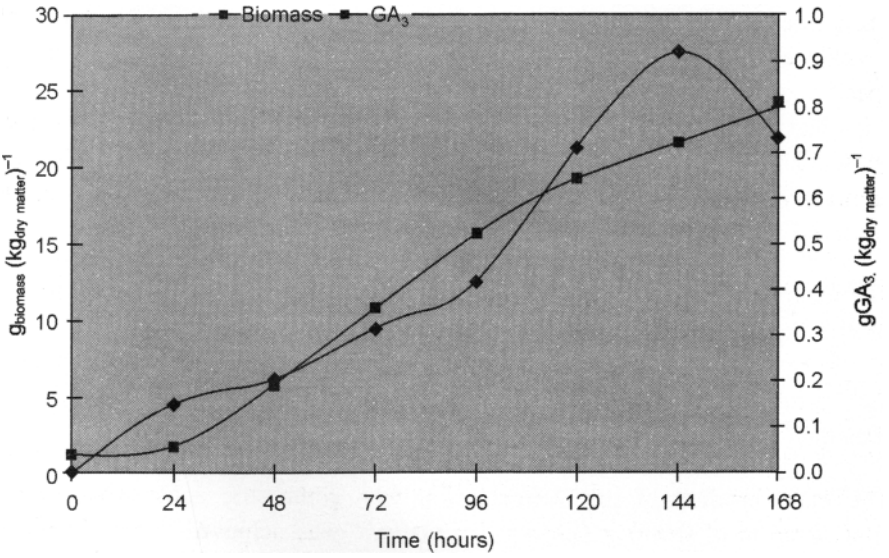

Figure 6: Biomass and gibberellic acid production in SSF

It has been observed that after some time, the GA_3 decays in substrate (Tomasini et al., 1997; Bandelier et al., 1997; Machado et al., 2001). This phenomenon was probably due to GA_3 decomposition in production system (Perrez et al., 1996; Gelmi et al., 2002).

13.4.3.3 Matematical modelling of G fujikuroi growth and GA_3 production

The packed-bed columns bioreactor was connected to a gas chromatograph to make the exit gas analysis. With the respirometric data, a logarithmic correlation between accumulated CO_2 and biomass production was determined and the mathematical model were compared with experimental data, leading to a better knowledge about the behavior of the system for future works in scaling up, and comparison with submerged fermentation (Machado 2004). On-line gas monitoring system measured the flow rate and gas composition of the outlet air stream from four columns. These columns were connected to a gas chromatograph linked to a personal computer, as described by Saucedo-Castañeda et al., (1994). The respirometric data were processed in Excel®, in witch instantaneous curves of CO_2 and O_2 calculations were determined using the equations developed by Pandey et al., 2001.

To determine the correlation between biomass and CO_2, the logistic law (Ebune et al., 1995) was applied. This model (equations 1 and 2) describes the mycelial growth in logarithmic and stationary phases in solid-culture of substrate.

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_m}\right) \tag{1}$$

$$X = \frac{X_m}{1 + \beta e^{-\mu_m t}} \quad \rightarrow \quad \beta = \frac{X_m}{X_0} - 1 \tag{2}$$

Where X is the biomass ($g_{biomass}.kg_{dry\ matter}^{-1}$) at time t (h); X_m the maximum biomass concentration, μ_m the maximum specific growth rate (h^{-1}) and X_0 is the initial biomass concentration.

From the experimental data of biomass using the *Statistica* program by *StatSoft*, X_m = 24.38 $g_{biomass}.kg_{dry\ matter}^{-1}$; X_0 = 1.171 $g_{biomass}.kg_{dry\ matter}^{-1}$; μ_m = 0.037 h^{-1} were determined. The high regression coefficient (0.996) obtained and the good correspondence with the experimental values X_m = 24.18 $g_{biomass}.kg_{dry\ matter}^{-1}$; $X0$ = 1.122 g $g_{biomass}.kg_{dry\ matter}^{-1}$; μ_{max} = 0.0522 h^{-1} proved the adequacy of the model.

In SSF processes, the amount of biomass produced at a particular period of time can be related to the O_2 consume pattern, if the fraction employed in metabolites synthesis is known or irrelevant. Gibberellic acid synthesis has several oxidative stages. In this work, agro-industrial residues with different nature of carbon sources were used as substrate. Hence, its mathematical modeling relating to O_2 consumed with biomass production was considered as of complex nature. Considering CO_2 was only produced by microbial respiration, this parameter seemed to be more directly related to biomass.

Hence, using the model achieved a theoretical value of biomass was determined for every time that had a CO_2 analysis. Finally, a regression of the curve accumulated CO_2 *vs.* biomass was made, leading to a logarithmic correlation between these two variables (equation 3):

$$X = 3.9872 \ln (CO_{2\,acum}) + 23.824 \tag{3}$$

The kinetic parameters of the microorganism growth were established with the values of experimental and estimated biomass. As shown in Figure 7, the two growth curves were very similar and followed a Monod model having the same *lag phase* (0 to 24 h) and *exponential growth phase* (24 to 48 h). The *desacelerated growth phase* started in both the cases after 48 h but the *stationary phase* appeared in experimental data after 120 h and was not found in estimated data. The maximum specific growth rates (m_{max}) were determined between 24 and 48 h (exponential growth phase) and were almost the same for experimental and estimated biomass (0.0522 h^{-1} and 0.0520 h^{-1}, respectively). For the global

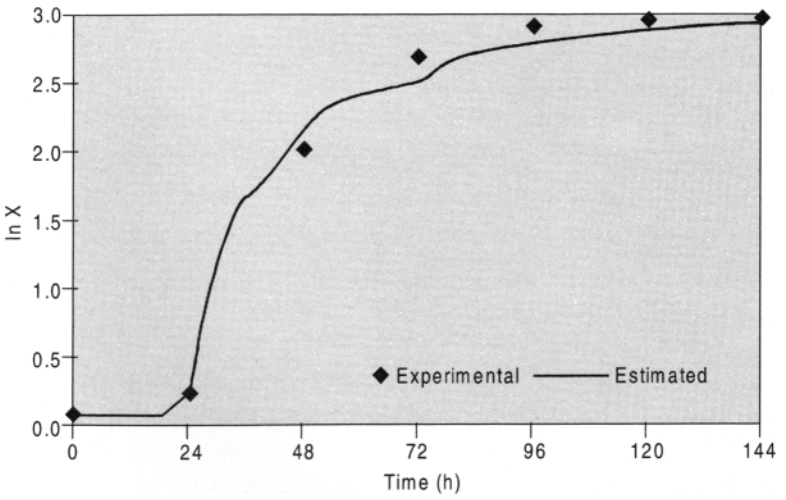

Figure 7: Growth curve for experimental and estimated biomass

specific growth rate ($\overline{\mu}$), calculated between 0 and 144 h, the same value of 0.0206 h^{-1} was found.

13.5. CONCLUSIONS

This work proved that with judicious selection of nutritional and physical factors, the yield of GA_3 can be much enhanced. In the presented case, the increase was almost 10 times. Also, it confirmed that a mixture of coffee husk and cassava bagasse could be a good substrate for the production of GA_3 by SSF. The biomass is a key variable in fermentation studies, since typically metabolic activities such as substrate consumption and product formation are strongly related to either or both of the growth rate and the actual biomass present. However, it is very often necessary to use indirect methods of determining biomass in SSF, such as the measurement of cell components, this being especially true for those processes involving fungi. Even with the difficulties that are encountered in SSF process, kinetic procedure can not be substituted by goodwill, subjectivism or even by the simple and overall process description. Mathematical models can be tools in the development of rational strategies for the design and optimization of operation of large-scale bioreactors.

References

Arteca RN, 1995, Plant growth substances – principles and applications, Chapman & Hall, New York, 332 p.

Bandelier S, Renaud R & Durand A, 1997, Production of gibberellic acid by fed-batch solid state fermentation in as aseptic pilot-scale reactor, *Process Biochemistry*, 32, 141-145.

Borrow A, Brian PW, Chester VE, Curtis PJ,Hemming HG, Henehan C, Jefferys EG, Lloyd PB, Nixon IS, Norris GLF & Radley M, 1955, Microbial production of gibberellins, *Journal of the Science of Food and Agriculture*, 6, 340-348.

Borrow A, Jefferys EG, Kessel RHJ, Lloyd EC, Lloyd PB & Nixon IS, 1961, Metabolism of *Gibberella fujikuroi* in stirred culture, *Canadian Journal of Microbiology*, 7, 227-276.

Borrow A, Brown S, Jeferrys EG, Kessel RJH, Lloyd EC, Lloyd PB, Rothwell A, Rothwell B & Swait JC, 1964a, The kinetics of metabolism of *Gibberella fujikuroi* in stirred culture, *Canadian Journal of Microbiology*, 10, 407-444.

Borrow A, Brown S, Jeferrys EG, Kessel RJH, Lloyd EC, Lloyd PB, Rothwell A, Rothwell B & Swait JC, 1964b, The effect of varied temperature on the kinetics of metabolism of kinetics of *Gibberella fujikuroi* in stirred culture, *Canadian Journal of Microbiology*, 10, 445-466.

Brückner B & Blechschmidt D, 1991, The gibberellin fermentation, *Critical Reviews in Biotechnology*, 11, 163-192.

Cihangir N & Aksöza N, 1997, Evaluation of some food industry wastes for production of gibberellic acid by fungal source, *Environmental Technology*, 18, 533-537.

Cihangir N, 2002, Stimulation of the gibberellic acid synthesis by Aspergillus niger in submerged culture using a precursor, *World Journal of Microbiology and Biotechnology*, 3, 727-729.

Corona A, Sanchez D, Agosin E, 2005, Effect of water activity on gibberellic acid production by *Gibberella fujikuroi* under solid-state fermentation conditions, *Process Biochemistry*, 40, 2655-2658.

Cross BE, 1954, Gibberellic acid Part I, *Journal of Chemical Society*, 4670-4676.

Crow JR, Thomson RJ & Mander LN, 2006, Synthesis and confirmation of structure for the gibberellin GA_{131} (18-hydroxy-GA_4), *Organic Biomolecular Chemistry*, 4, 2532-2544.

Darken MA, Jensen AL & Shu P, 1959, *Applied Microbiology*, 7, 301-303.

Ebune A, Al-Asheh S & Duvnjak Z, 1995, Effects of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid state fermentation, *Bioresources Technology*, 54, 241-247.

Elías LG, 1978, Composición química de la pulpa de café y otros subproductos. In- Pulpa de Café – composición tecnología y utilización, JE Brahan & R Bressani (eds), Instituto de Nutrición de Centro América Y Panama INCAP, Bogota, pp 19-27.

Escamilla EM, Dendooven L, Magaña IP, Parra R & De La Torre M, 2000, Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors, *Journal of Biotechnology*, 76, 147-155.

Gelmi C, Perez-Correa R, Gonzalez M & Agosin E, 2000, Solid substrate cultivation of *Gibberella fujikuroi* on an inert support, *Process Biochemistry*, 35, 1227-1233.

Gelmi C, Perez-Correa R & Agosin E, 2002, Modeling *Gibberella fujikuroi* growth and GA_3 production in solid-state fermentation, *Process Biochemistry*, 37, 1033-1040.

Hedden P, 1999, Recent advances in gibberellin biosynthesis, *Journal of Experimental Botany*, 50, 553-563

Hernández E, Mendoza MD, 1976, Producción de ácido giberélico por *Gibberella fujikuroi* en substratos que contienen pulpa de aceituna aceite de oliva o subproductos de la extracción de este último, *Revista de Agroquimica y Tecnologia de Alimientos*, 16, 357-366.

Hill TA, 1977, Hormonas Reguladoras del Crescimento Vegetal, Ediciones Omega S.A., Barcelona, 74 p.

Holbrook AA, Edge WJW & Baily F, 1961, Spectrophotometric method for determination of gibberellic acid, *Advances in Chemical Series*, 28, 159-167.

Hollmann D, Switalski J, Giepel S & Onken U, 1995, Extractive fermentation of gibberellic acid by *Gibberella fujikuroi*, *Journal of Fermentation and Bioengineering*, 79, 594-600.

Hook JM Mander LN & Rudolf U, 1980, *Journal of American Chemistry Society*, 102, 6628-6629.

Jefferys EG, 1970, The gibberellin fermentation, *Advances in Applied Microbiology*, 13, 283-316.

Jones A, Pharis RP, 1987, Production of gibberellins and bikaverin by cells of *Gibberella fujikuroi* immobilized in carrageeanan, *Journal of Fermentation Technology*, 65, 717-722.

Kahlon SS, Malhotra S, 1986, Production of gibberellic acid by fungal mycelium immobilized in sodium alginate, *Enzyme and Microbial Technology*, 8, 613-616.

Kawaide H, 2006, Biochemical and molecular analyses of gibberellin biosynthesis in fungi, *Bioscience, Biotechnology and Biochemistry*, 70, 583-590.

Kende H, 1967, Preparation of radioactive gibberellin A_1 and its metabolism in dwarf peas, *Plant Physiology*, 42, 1612-1618.

Kumar PKR & Lonsane BK, 1986, Spectrofluorodensitometric estimation in thin-layer chromatography of gibberellic acid produced by solid-state fermentation, Journal of Chromatography, 369, 222-226.

Kumar PKR & Lonsane BK, 1987a, Potential of fed-batch culture in solid-state fermentation for production of gibberellic acid, *Biotechnology Letters*, 9, 179-182.

Kumar PKR & Lonsane BK, 1987b, Extraction of gibberellic acid from dry mouldy bran produced under solid-state fermentation, *Process Biochemistry*, 10, 138-143.

Kumar PKR & Lonsane BK, 1987c, Gibberellic acid by solid state fermentation: consistent and improvement yields, *Biotechnology and Bioengineering*, 30, 267-271.

Kumar PKR & Lonsane BK, 1988, Batch and fed-batch solid-state fermentations: kinetics of cell growth hydrolytic enzymes production and gibberellic acid production, *Process Biochemistry,* April, 43-47.

Kumar PKR & Lonsane BK, 1989, Microbial Production of Gibberellins: state of the art. *Advances in Applied Microbiology*, 34, 29-139.

Kumar PKR & Lonsane BK, 1990, Solid-state fermentation: physical and nutritional factors influencing gibberellic acid production, *Applied Microbiology and Biotechnology*, 34, 145-148.

Lale G, Jogdand VV & Gadre RV, 2006, Morphological mutants of *Gibberella fujikuroi* for enhanced production of gibberellic acid, *Journal of Applied Microbiology*, 100, 65-72.

Leitch SK, Blake PS & Mander LN, 2003, Synthesis and structure determination of three new 12α-hydroxy C_{20} gibberellins (GA_{127} GA_{128} and GA_{129}), *ARKIVOC Journal*, 7, 145-160.

Lu ZX, Xie ZC & Kumakura M, 1995, Production of gibberellic acid in *Gibberella fujikuroi* adhered onto polymeric fibrous carriers, *Process Biochemistry*, 30, 661-665.

Machado CMM, Oliveira BH, Pandey A & Soccol CR, Coffee husk as substrate for the production of gibberellic acid by fermentation, 2001, In- Book on Coffee Biotechnology and Quality, T Sera, CR Soccol, A Pandey & S Roussos (eds), Kluwer Academic Publishers, Dorcrecht, pp 401-408.

Machado CMM, Soccol CR & Pandey A, 2002, Gibberellic acid production by solid sate fermentation in coffee husk, *Applied Biochemistry and Biotechnology,* 102, 179-192.

Machado CMM, Oishi B, Pandey A & Soccol CR, 2004, Kinetics of *Gibberella fujikuroi* growth and gibberellic acid production by solid-state fermentation in a packed-bed column bioreactor, *Biotechnology Progress*, 20, 1449-1453.

MacMillan J, 1997, Biosynthesis of the gibberellin plant hormones, *Natural Product Research*, 14, 221-243.

Maddox IS & Richert SH, 1977, Production of gibberellic acid using dairy waste as the basal medium, *Applied and Environmental Microbiology,* 1977, 201-202.

Mander LN, 2003, Twenty years of gibberellin research, Natural Product Reports, 20, 49-69.

Martin GC, 1983, Commercial uses of gibberellins, In- The Biochemistry and Physiology of Gibberellins, A Crozier (ed), Praeger, New York, pp 395-444.

O'Neil MJ (ed), 2001, The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, Whitehouse Station, New Jersey, 2564 p.

Nava-Saucedo JE, Barbotin JN & Nava-Saucedo DT, 1989, Physiological and morphological modifications in immobilized *Gibberella fujikuroi* mycelia, *Applied Environmental Microbiology*, 55, 2377-2384.

Pandey A, 1992, Recent developments in solid-state fermentation, *Process Biochemistry*, 27, 109-117.

Pandey A, Soccol CR, Nigan P, Soccol VT, Vandenberghe LPS & Mohan R, 2000a, Biotechnological potential of agro-industrial residues. I: sugarcane bagasse *Bioresource Technology*, 74, 69-80.

Pandey A, Soccol CR, Nigan P, Soccol VT, Vandenberghe LPS & Mohan R, 2000b, Biotechnological potential of agro-industrial residues. II: cassava bagasse *Bioresource Technology*, 74, 81-87.

Pandey A, Soccol CR, Rodriguez-Leon JÁ & Nigan P (eds), 2001, Solid State Fermentation in Biotechnology – Fundamentals and Applications, Asiatech Publishers Inc., New Delhi, 221 pp.

Pastrana LM, González MP, Torrado A & Murado MA, 1995, A fed-batch culture model for improved production of gibberellic acid from a waste medium, *Biotechnology Letters*, 17, 263-268.

Perraud-Gaime I, Saucedo-Castañeda G, Augur a & Roussos S, Adding value to coffee solid by-products through biotechnology, 2001, In- Coffee Biotechnology and Quality, T Sera, CR Soccol, A Pandey & S Roussos (eds), Kluwer Academic Publishers, Dorcrecht, pp 437-446.

Perrez FJ, Vechiolla A, Pinto M & Agosin E, 1996, Gibberellic acid decomposition and its loss of biological activity in aqueous solutions, *Phytochemistry*, 41, 675-679.

Raimbault M, 1998, General and microbiological aspects of solid substrate fermentation, *Electronic Journal of Biotechnology*, 1, 26-27.

Raimbault M, Alazard D, 1980, Culture method to study fungal growth in solid fermentation *European Journal of Applied Microbiology and Biotechnology*, 9, 199-209.

Sanchez-Marroquin A, 1963, Microbiological production of gibberellic acid in glucose media applied microbiology, *Applied Environmental Microbiology*, 11, 523-528.

Saucedo-Castañeda G, Trejo-Hernández MR, Lonsane BK, Navarro JM, Roussos S, Dufour D & Raimbault M, 1994, On-line automated monitoring and control systems for CO_2 and O_2 in aerobic and anaerobic solid-state fermentations, *Process Biochemistry*, 29, 13-24.

Shukla R, Chand S & Srivastava AK, 2005, Batch kinetics and modeling of gibberellic acid production by *Gibberella fujikuroi*, *Enzyme and Microbial Technology*, 36, 492-497.

Soccol CR, Machado CMM & Oliveira BH, 2000, Produção de ácido giberélico por fermentação no estado sólido em substrato misto, Brasil, Industrial Patent n. 525-8.

Soccol CR & Vandenberghe LPS, 2003, Overview of applied solid-state fermentation in Brazil, *Biochemical Engineering Journal*, 13, 205-218.

Sponsel VM & Hedden P, 2004, Gibberellin biosynthesis and metabolism, In-Plant Hormones: Biosynthesis Signal Transduction, Action! PJ Davies (ed), Kluwer Academic Publishers, Dordrecht, pp 62-98.

Taiz L & Zeiger E, 1991, Plant Physiology, The Benjamin / Cummings Publishing Company, California, 559 p.

Takahashi Y, 1986, Chemistry of plant hormones, CRC Press, Boca Raton, 277 p.

Tang Z, Zhou R & Duan Z, 2000, Separation of gibberellic acid (GA_3) by macroporous adsorption resins, *Journal of Chemical Technology and Biotechnology*, 75, 695-700.

Tomasini A, Fajardo C & Barrios-Gonzáles J, 1997, Gibberellic acid production using different solid-state fermentation systems, *Word Journal of Microbiology and Biotechnology*, 13, 203-206.

Tudzynski B, 1999, Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects, *Applied Microbiology and Biotechnology,* 52, 298-310.

Tudzynski B, 2005, Gibberellin biosynthesis in fungi: genes enzymes evolution and impact on biotechnology, *Applied Microbiology and Biotechnology*, 66, 597-611.

Vass RC & Jefferys EG, 1979, Gibberellic Acid, In- Economic microbiology: secondary products of metabolism,. vol 3, AH Rose (ed), Academic Press, London, pp 421-433.