SOLID STATE FERMENTATION OF GIBBERELLIC ACID BY *PENICILLIUM VARIABLE*: SCREENING, OPTIMIZATION AND KINETIC STUDIES

by

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LIST OF SYMBOLS

A	Input variable – incubation times	Day
B	Input variable – inoculum sizes	%
C	Input variable – Precursor concentration (olive oil)	%
dX/dt	Growth rate	mg/kg h
K_S	Substrate concentration at one – half the maximum specific growth rate	mg/kg
m_S	Maintenance coefficient	mg/mg h
n	Average cell count per square	Dimensionless
P_c	Product form at the critical time	mg/kg
P_o	Product concentration at initial time	mg/kg
P_t	Product concentration at any time	mg/kg
q_{p1}	Specific product formation rate	mg/mg h
q_{p2}	Specific product formation rate	mg/mg h
S	Limited substrate concentration	mg/kg
S_o	Substrate concentration at initial time	mg/kg
S_t	Substrate concentration gas at any time	mg/kg
t	Time	H
t_c	Time at which the cell reach critical cell concentration	H
t_c	Time to reach 50% survival	H
t_o	Initial time	H
X	Biomass concentration	mg/kg
X_c	Critical cell concentration	mg/kg
x_i	The level of independent variable	Dimensionless

x_j	Coded independent variable	Dimensionless
X_m	Maximum biomass	mg/kg
X_o	Initial biomass	mg/kg
$Y_{X/S}$	Biomass yield on the utilized substrate	mg X/mg S
$Y_{P/S}$	Product yield on the utilized substrate	mg P/mg S

Greek Letters

α	Growth associated constant	mg P/mg X
β	Non-growth associated	mg P/mg X h
β_i	Coefficient of the linear parameters	Dimensionless
β_{ii}	Coefficient of the quadratic parameters	Dimensionless
β_{ij}	Coefficient of the interaction parameters	Dimensionless
β_o	Model intercept or constant term	Dimensionless
γ	Coefficient for inverse yield	mg S/mg X
δ	Coefficient for yield	mg S/mg X h
ε	The residual associated to the experiment	Dimensionless
μ	Specific growth rate	h^{-1}
μ_m	Maximum specific growth rate	h^{-1}
μ_F	Decline rate constant	h^{-1}
μ_L	Growth rate constant	h^{-1}

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BBD	Box Benhken Design
BP	Banana Peel
CB	Corn Bran
CCD	Central Composite Design
CCLF	Combined Continuous Logistic and Fermi
CCLFLP	Combined Continuous Logistic and Fermi incorporated Ludeking-Piret
CP	Citric Pulp
CV	Coefficient of Variances
DNS	Dinitrosalicylic Asid
DOE	Design of Experiment
FDP	Farnesyl diphosphate
GA_3	Gibberellic Acid
GDP	Geranyldiphosphate
GGDP	Gerabylgeranyl diphosphate
HMG	Hydroxymethylglutaryl
KA	Kono and Asai
LLP	Logistic-incorporated Luedeking-Piret
LLPL	Logistic-incorporated Luedeking-Piret like
MEA	Malt Extract Agar
MLP	Monod-incorporated Luedeking-Piret
MMLP	Monod-incorporated modified Luedeking-Piret
MP	Mango Peel

MW	Mango Waste
OFAT	One-Factor-At-A-Time
PBD	Plackett Burman Design
PDA	Potato Dextrose Agar
PP	Papaya Peel
RMSE	Root Mean Square Error
RSM	Response Surface Methodology
SmF	Submerged Fermentation
SSF	Solid State Fermentation
SW	Soy Waste

FERMENTASI PEPEJAL ASID GIBERELIK OLEH *PENICILLIUM VARIABLE*: SARINGAN, PENGOPTIMAAN DAN KAJIAN KINETIK

ABSTRAK

Asid giberelik (GA_3) adalah salah satu daripada ahli keluarga giberelina yang mempunyai aplikasi yang lebih luas ke arah peningkatan pertumbuhan tumbuhan. Tumbuhan yang normal hanya boleh menghasilkan hormon pertumbuhan dalam kuantiti yang sangat kecil, tetapi dengan penambahan GA_3 yang mencukupi, boleh mempengaruhi peningkatan pertumbuhan organ-organ tumbuhan itu.

Beberapa sumber substrat telah diuji bagi mengetahui keupayaan mereka sebagai medium pertumbuhan bagi penghasilan GA_3 di dalam fermentasi pepejal. Kulit pisang (BP) didapati sesuai digunakan sebagai substrat kerana ia mengandungi jumlah karbon yang lebih tinggi dan jumlah nitrogen yang lebin rendah iaitu 44.85% ± 0.15 dan 12.16% ± 0.16. Dalam kajian ini, enam belas kulat makro dan mikro dari keluarga yang berbeza (Polyporaceae, Coriolaceae, Lentinaceae, Schizophyllaceae, Trichocomaceae, Hypocreaceae, dan Moniliaceae) telah disaring bagi penghasilan GA_3 dengan menggunakan BP sebagai substrat. Didapati *P. variable* menghasilkan GA_3 yang tertinggi diikuti oleh *A. niger*, *P. simplicissimum* dan *P. spinulosum* iaitu masing-masing sebanyak 16.25 ± 0.06 mg/kg substrat, 12.45 ± 0.11 mg/kg substrat, 5.56 ± 0.06 mg/kg substrat dan 3.90 ± 0.06 mg/kg substrat.

Analisa melalui reka bentuk Plackett-Burman telah digunakan bagi memilih komponen media penting yang mempengaruhi pertumbuhan dan meningkatkan penghasilan GA_3. Antara semua komponen yang telah diuji, sukrosa, ekstrak yis, minyak zaitun, dan unsur surih telah menunjukkan kesan positif terhadap penghasilan GA_3. Pengoptimaan keadaan kultur juga telah dikaji dengan

menggunakan kaedah "satu-faktor-dalam-satu-masa" (OFAT) dan kaedah tindak balas permukaan (RSM) menggunakan reka bentuk Box-Behnken. Keadaan kultur yang optimum telah dicapai pada masa fermentasi 7 hari, saiz inokulum 21%, dan kandungan minyak zaitun 2% dengan penghasilan GA_3 pada 31.97 mg/kg substrat. Penghasilan GA_3 telah meningkat 1 kali ganda dengan menggunakan RSM berbanding kaedah OFAT.

Model kinetik tidak berstruktur telah dicadangkan untuk pertumbuhan, penghasilan GA_3 dan penggunaan substrat. Keputusan menunjukkan bahawa model Logistik berpadanan dengan data experiment bagi pertumbuhan *P. variable* pada fasa eksponen dan statik dengan nilai $R^2 > 0.98$ dan RMSE < 1.1 untuk semua kepekatan sukrosa. Untuk penghasilan GA_3 dan penggunaan substrat, model Logistik yang digabungkan dengan Luedeking-Piret (LLP) dan model Logistik yang digabungkan dengan Luedeking-Piret yang serupa (LLPL) menepati dengan baik dengan data eksperimen iaitu R^2 lebih tinggi daripada 0.98 dan RMSE lebih kurang daripada 17. Ini menunjukkan bahawa GA_3 boleh dihasil oleh *P.variable*, dan menggunakan kulit pisang iaitu sisa dari industri makanan sebagai substrat di dalam fermentasi pepejal.

SOLID STATE FERMENTATION OF GIBBERELLIC ACID BY *PENICILLIUM VARIABLE*: SCREENING, OPTIMIZATION AND KINETIC STUDIES

ABSTRACT

Gibberellic acid (GA_3) is one of the family members of gibberellins that have wider applications toward plant growth acceleration. A normal plant can only produce growth hormone in a very small quantity, but with sufficient addition of GA_3 could affect the growth development of plant organs.

A few sources of substrates were evaluated for their capabilities as a growth medium for the production of GA_3 in solid state fermentation (SSF). Banana peel (BP) was found as a suitable substrate as it contain higher amount of carbon and lower amount of nitrogen source at 44.85 % ± 0.15 and 12.16 % ± 0.16, respectively. In the present study, sixteen macro- and micro-fungi from different families (Polyporaceae, Coriolaceae, Lentinaceae, Schizophyllaceae, Trichocomaceae, Hypocreaceae, and Moniliaceae) were screened for the production of GA_3 using BP as a substrate. It is found that *P. variable* produced the highest GA_3 followed by *A. niger*, *P. simplicissimum* and *P. spinulosum* at 16.25 ± 0.06 mg/kg substrate, 12.45 ± 0.11 mg/kg substrate, 5.56 ± 0.06 mg/kg substrate and 3.90 ± 0.06 mg/kg substrate, respectively.

The Plackett-Burman design was used to select the critical media components that influenced the growth and enhancing GA_3 production. Among all the components tested, sucrose, yeast extract, olive oil, and trace elements showed positive effect towards the production of GA_3. Optimization of culture conditions were also studied using "one-factor-at-a-time" (OFAT) method and response surface methodology (RSM) couple with Box Behnken design. The optimum culture conditions were attained at incubation time 7 days, inoculum size 21% and amount

of olive oil 2%, with GA_3 production at 31.97 mg/kg substrate. The GA_3 production increased 1 fold using RSM as compared to the OFAT method.

A few unstructured kinetic models were selected for the growth, GA_3 production and substrate utilization. Results showed that the Logistic model fitted well with the experimental data for growth of *P. variable* at the exponential and stationary phase with $R^2 > 0.98$ and RMSE < 1.1 for all sucrose concentrations. For GA_3 production and substrate utilization, the Logistic-incorporated with Luedeking-Piret (LLP) and Logistic-incorporated Luedeking-Piret like (LLPL) models agreed well with the experimental data with R^2 higher than 0.98 and RMSE less than 17. Thus showing that GA_3 can be produced by *P. variable*, and banana peel waste from the food industry as a substrate in solid state fermentation.

CHAPTER 1

INTRODUCTION

1.1 Background

Under the 10th Malaysia Plan, Malaysia has targeted to increase about 3.4 percent in agricultural sector towards producing higher value added product ("The Edge," 2009). The development of this sector involved large-scale farming that prioritized on the production of higher quality food crops. Many efforts have been done by the government such as giving an incentive for new investor in agricultural sector and supplied fertilizer and plant hormone to the small-scale farming ("The Edge," 2009).

Plant hormone is one of the important formulae used for increasing the yield and quality of food crops, since it could regulate the growth of plant. There are five known plant hormones such as auxin, cytokonins, gibberellins, abscisic acid and ethylene that controlled the development of growth by affecting the division, elongation, and differentiation of the cells and they are also able to mediate short term physiological responses plants to the environmental stimuli (Srivastava, 2002). One of the well-known plant hormone that involved in agricultural sector is gibberellic acid (GA_3), which belonged to the family of gibberellins (Brückner and Blechschmidt, 1991; Machado et al., 2002).

A normal plant is able to produce GA_3 by itself with only a small quantity, ranges 10^{-11} to 10^{-9} g/g fresh weight (Cerdá-Olmedo et al., 1994; Sponsel et al., 2010). An addition of this hormone could help to controll many developmental processes of plant growth such as induction of hydrolytic enzyme activities during seed germination, stem elongation, induction of flowering, improvement of crop

yield and overcome dwarfism (Brückner and Blechschmidt, 1991; Tudzynski, 1999). Table 1.1 shows the capabilities of gibberellins produced by some plants and also other microorganisms that affected on plant development (Baca and Elmerich, 2007). Gibberellins hormone was firstly isolated from rice pathogen *Gibberella fujikuroi* which caused a disease known as 'bakanae effect' (silly plant), an extensive overgrowth of infected rice plants. Later, investigation leads to identification of important plant growth promoter namely GA_3 which was one of the gibberellins family (Cross, 1954; Borrow et al., 1955; Sponsel et al., 2010).

Table 1.1: Gibberellins, family of GA_3 produce by plants and microorganisms, and their effect on plant morphology and development (Baca and Elmerich, 2007)

Endogenous production or causative agent	Observed effect on plant
Plant	
Arabidopsis thaliana *Oryza sativa* *Zea mays* *Pisum sativum*	Seed germination, development and reproduction of plants, floral development
Fungus	
Gibberella fujikuroi	"bakanae" effect in maize, rice, and other plants
Bacterium	
Azospirillum brasilense *Azospirillum lipoferum* *Azospirillum brasilense*	Reversion of dwarfism in maize and rice Promotion of shoot elongation, growth, and root-hair density

GA_3 was firstly produced on surface cultivation, and at present it is commercially produced throughout the world using submerged fermentation of *Gibberella fujikuroi* or *Fusarium moniliforme* due to its capability of producing large amount of GA_3 as the main product (Cross et al., 1963; Cerdá-Olmedo et al., 1994; Tudzynski, 1999). Production of this hormone was reported to exceed 12 tonnes annually in a country such as United State, Japan, European Union and Israel

(Rademacher, 1994), and its global usage was more or less 50 tons per annum excluding China (Sponsel, 2010). Commercial usage of GA_3 in most countries is to promote the growth of a variety of fruit crops like seedless grape, to increase the sugar yield in sugarcane, to enhance yield of variety fruit crops like tart cherries, to delay or prevent rind-aging like citrus crops and to stimulate the barley-malting process in the beer-brewing industries (Sponsel, 2010).

Recently, optimization of GA_3 production was carried out via solid state fermentation (SSF) process due to its uniqueness characteristics that microbes are grown on solid substrate from agricultural residues with low moisture content (Kumar and Lonsane, 1990; Machado et al., 2002). In fact, SSF has been reported one of most favourable method for the production of microorganism metabolite which the fermentation condition is similar to the microbe's natural habitat (Manpreet et al., 2005; Wang and Yang, 2007). As a simple process, SSF facilitate for cost reduction and also could help diminish pollution potential.

Up to now, GA_3 production in SSF was carried out using microbial culture of *Gibberella fujikuroi* and only some studies attempt to assess others species especially from Trichocomaceace family. Ates et al. (2006) reported that *Aspergillus niger* from Trichocomaceae family was capable producing GA_3. *Penicillium variable* is a species belongs to the Trichocomaceace family also capable producing secondary metabolite such as tannase and glucose oxidase. Therefore, further investigation could lead towards new producer of GA_3.

1.2 Problem Statement

GA_3 as one of the family members of gibberellins has obtained the utmost interest due to its wide range of usage and capable of regulating the growth of plant

and proficient of producing succession of benefit, valuable in agricultural sectors (Mander, 2002; Machado et al., 2008). Furthermore, implication of GA_3 employment has an effect on the whole plant growth development beginning from the seeds to fruits thus raising the crops yield. Therefore, GA_3 offered a great potential in enhancing agricultural sector due its global usage as growth promoter that increases the yield of various varieties of crops. However, a small quantity of GA_3 available in a normal plant provide no accelerate to the plant growth, and thus decreasing the crops yield (Cerdá-Olmedo et al., 1994). The addition of GA_3 is urgently required for speeding up plant growth as well as crops yields in agricultural sector. Although the government offers incentives, such as plant hormones to the farmers, this effort involved a lot of capital usage in agricultural sector due to higher cost required for transportation, and it needed to be imported from other countries with higher prices (Sponsel, 2010). Therefore its usage is limited to higher-premium crops only, yet the hormone demand was still elevated.

It was reported that GA_3 is a natural plant hormone, and was firstly isolated from culture filtrate of rice pathogen (*Gibberella fujikuroi*) (Tudzynski and Hölter, 1998). Production GA_3 via microbial fermentation were preferred due to its lower cost and higher yield compared to the plant origin. In a commercial scale, GA_3 can be produced by submerged fermentation (SmF) using *Gibberella fujikuroi* fungus. However, this process involved higher production cost with low product yield. In view of such a scenario, Bandelier et al. (1997) has carried out a study and showed that solid state fermentation (SSF) was capable of producing higher yield GA_3, a process, which was simpler and required less energy than the conventional processes.

In fact, SSF has been traditionally employed to produce a broad range of microbial products, including GA_3 (Kumar and Lonsane, 1990; Tomasini et al.,

1997; Wang, 1999; Machado et al., 2002; Rodrigues et al., 2009). In SSF processes, the microbes are grown on nutrients impregnated in solid substrate either with or without water. Utilization of lower cost biomaterial from agricultural residues such as coffee husk, cassava husk, cassava bagasse, sugarcane bagasse and apple pomace as the solid substrates or supports for nutrient in SSF is an alternative method that reduced the cost of production. Since SSF is relatively a simple process, used less energy, and could provide unique microenvironments which is conducive to microbial growth and metabolic activities, it could also help to solve environmental pollution problems (Soccol and Vandenberghe, 2003; Wang and Yang, 2007). With such potentials, a lot of residues from the agricultural sector that are yet to be explored, can be utilized as solid substrates for the production of value-added products. In this study, GA_3 produced via SSF using banana peel as the solid substrate is yet to be discussed and will be the main investigator of this project. In fact, optimization of significant parameters that affects GA_3 production using a conventional and statistical method let alone exploited.

Most of research study used microbial culture of *Gibberella fujikuroi* for GA_3 production in SSF and only some studies attempt to assess others species such as *Aspergillus niger* from Trichocomaceae family (Ates et al., 2006). Besides that, so far new producer for GA_3 production from Trichocomaceae family like *Penicillium variable* was less reported. In fact, production of GA_3 by *P. variable* grown on banana peel as solid substrate has not yet discuss elsewhere. Thus, kinetic study of GA_3 production by *P. variable* via SSF at different parameters can provide good starting point in research field.

Moreover, unstructured models used to describe the pattern of microorganism growth, GA_3 production and substrate utilization by *P. variable* were less reported.

Hence, this study would lead to a better understanding of the kinetic models for predicting higher yield of GA_3 by *P. variable* at different fermentation parameters using SSF in a static flask culture.

1.3 Research Objectives

This study addresses an alternative method for GA_3 production by *Penicillium variable* in a static flask culture using the selected food processing residues as substrates.

The measurable objectives are:

i. To screen the selected food processing residues as solid substrates and gibberellic acid producing fungus for GA_3 production in static flask cultures.
ii. To select critical media components that affect GA_3 production in static flask cultures using Plackett Burman Design (PBD).
iii. To optimize the GA_3 production using one-factor-at-a-one-time (OFAT) method and Response Surface Methodology (RSM) via Box Benhken Design (BBD).
iv. To select and validate the kinetic models for microbial growth, substrate utilization and GA_3 production in static flask cultures.

1.4 Scope of Study

In view of the demand for GA_3 as one of most popular plant hormones that can be put into practice for enhancing agricultural product in Malaysia, the production of GA_3 via solid state fermentation (SSF) is presented in this study. The production of GA_3 was carried out in SSF using *Penicillium variable* (*P. variable*) in

selected food processing residues as a cheap source of solid substrates in static flask culture.

In this study, five different selected food processing residues such as, corn bran, banana peel, mango peel, papaya peel and mango waste were analyzed for their capabilities to be used as solid substrates for the GA_3 production. The concentration of sugar, nitrogen and other elements present in each substrate were analyzed.

In order to choose an appropriate GA_3 producing fungus, a total of 16 selected micro- and macro-fungi including nine different strains from the Trichocomaceae family have been screened for the production of GA_3. Besides that, the critical media components that affected microbial growth and product formation were also determined. The critical media components tested include; glucose and sucrose from the raw table sugar, which act as an extra carbon source, soy waste and yeast extract for the nitrogen source, olive oil as precursor and various trace elements.

Process optimizations of different culture conditions were also carried out using "one-factor-at-a-time" method and response surface methodology (RSM). The culture conditions tested include; incubation times, inoculum sizes and amount of precursor (olive oil).

Different kinetic models for growth, GA_3 production and substrates utilization were fitted to the experimental data of static flask culture. For cell growth, the Logistic, Kono and Asai, Monod and Combined Continuous Logistic with Fermi were proposed. The Luedeking-Piret model incorporated with Logistic, Monod and Kono and Asai were fitted to the GA_3 production data, while Luedeking-piret like models incorporated with Logistic model, modified Ludeking-Piret incorporated with

Monod and Luedeking-piret incorporated with Combined Continuous Logistic and Fermi were proposed for substrate utilization. Each models were validated by determining the coefficient of determination (R^2) value and root mean square error (RMSE).

1.5 Organization of the Thesis

There are five chapters in this thesis and each chapter described the sequence of this study.

Chapter 1 emphasized the demand and the importance of plant hormone, gibberellic acid (GA_3) in the agricultural sector. Problem statements, research objectives, scope of research and thesis organization were also highlighted.

Chapter 2 covered an overview of the structure, properties, biosynthesis and application of GA_3. The production of GA_3 via solid state fermentation in batch, fed-batch and continuous system were explained in details. *Penicillium variable* (*P. variable*) as the selected microorganism for GA_3 production was also highlighted in the fermentation process. Besides, method of optimization of process parameters, kinetics and modeling that applied in this study was also discussed.

Chapter 3 illustrated the materials and methods that applied during this study, which covered the screening process, optimization studies, kinetic modeling studies and analytical procedures.

Chapter 4 presented and discussed the experimental results and data that have been analyzed during fermentation of GA_3 production. Preliminary study of GA_3 production in solid state fermentation were done by determining elements present the solid substrates and screening the suitability of GA_3 producing fungus. Influenced of

critical media components for GA_3 productions were screened via statistical analysis, Plackett Burman Design. The optimization of process parameters using RSM was also analyzed for GA_3 production in static flask culture. Kinetic models of GA_3 production in static flask culture were selected and validated.

Chapter 5 summarized the overall conclusion based on the results obtained and discussion in chapter four. Recommendations for future research were also highlighted in this section.

CHAPTER 2

LITERATURE REVIEW

2.1 Gibberellic Acid (GA_3)

2.1.1 Structure and Properties of GA_3

Gibberellins is an important class of natural growth regulators in plants since it naturally occurrs in wide varieties of plants and regulated many aspect of plants growth and development (Graebe et al., 1965). Gibberellins are diterpenoids based on the tetracyclic *ent*-gibberellane skeletal structure. It comprises of 19 or 20 carbon units grouped into either four or five ring systems and the fifth ring was a lactone ring that attached to ring A (Figure 2.1) (Tudzynski, 1999; Bömke and Tudzynski, 2009). Gibberellins consists of a large family that also occurred in fungi and bacteria (Tudzynski, 1999). Sponsel et al. (2010) reviewed about 136 different types of gibberellins which were fully characterized and 128 were identified from different species of vascular plants and also from seven bacteria and seven fungi.

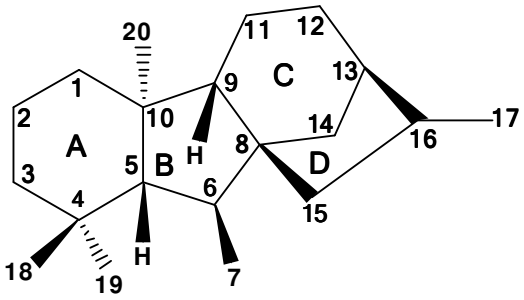

Figure 2.1: The structure of gibberellins (Tudzynski, 1999; Bömke and Tudzynski, 2009)

Among all the gibberellins, gibberellin A_3 also known as gibberellic acid or GA_3 played an important role towards plant growth, due to its broad application in agricultural sector, and has been commercially produced by fermentation using a fungus *Gibberella fujikuroi* (Brückner and Blechschmidt, 1991; Tudzynski, 1999; Mander, 2002; Shukla et al., 2003; Sponsel et al., 2010). The gibberellins biosynthetic pathway were first investigated in this fungus (rice pathogen) as it was able to produce larger amount of gibberellins, especially the bioactive compounds gibberellic acid (GA_3) and its precursors, GA_4 and GA_7 (Figure 2.2) (MacMillan, 1997; Tudzynski, 1999).

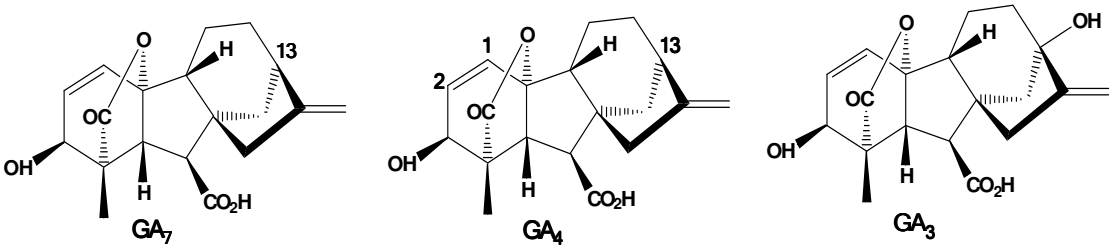

Figure 2.2: The structures of biologically the most active gibberellins (MacMillan, 1997)

Gibberellic acid (GA_3) with molecular formula $C_{19}H_{22}O_6$ is a colorless crystalline, optically active acid which decomposes at 233 °C to 235 °C. It is readily soluble in methanol, ethanol, acetone, sodium hydrogen carbonate and sodium acetate solution and moderately soluble in ethyl acetate but only slightly soluble in water and ether (Cross, 1954; O'Neil, 2006; Machado et al., 2008).

2.1.2 Biosynthesis of GA_3

Initially, gibberellins (GAs) were isolated and identified as secondary metabolites from the rice pathogenic fungus, *Fusarium fujikuroi*. The consequences of investigation concerning the abnormal growth of plants caused by *F. fujikuroi*, led

towards the discoveries that gibberellins were naturally existed in plant as natural growth regulator and developmental but with small quantity depending on the types of plants. Therefore, investigations on the basic biosynthetic pathways of gibberellins in *F. fujikuroi* have been intensively investigated and gibberellic acid (GA_3) was discovered as the end product of gibberellins biosynthesis (Bömke and Tudzynski, 2009).

In a report published by Tudzynski (2005), gibberellins (GAs) are synthesized from hydroxymethylglutaryl (HMG) coenzyme A via mevalonic acid, isopentenyl diphosphate, geranyldiphosphate (GDP), farnesyl diphosphate (FDP) and gerabylgeranyl diphosphate (GGDP), which is a precursor for GAs. The tectracyclic diterpene, *ent*-kaurene is produced in two cyclisation steps from GGDP via ent-copalyl diphosphate (CPP) by sequential oxidation at C-19 via ent-kaurenol and ent-kaurenal to give ent-kaurenoic acid which further oxidised to ent-7α-hydroxykaurenoic acid. Finally, an oxidation occurred at C-6β, resulting in a contraction of ring B, which then led to the formation of GA_{12}-aldehyde (Tudzynski, 1999; Hedden et al., 2001; Tudzynski, 2005).

In the gibberellins pathways step, the earlier step is from geranylgeranyl diphosphate (GGDP) up to the formation of GA_{12}-aldehyde and it is identical for both in the fungus *F. fujikuroi* and higher plants. Hence, the major pathways in fungi are differed from the higher plants starting from 3β-hydroxylation GA_{12}-aldehyde to GA_{14}-aldehyde (Bömke and Tudzynski, 2009).

GA_{12}-aldehyde is 3β-hydroxylated to GA_{14}-aldehyde, which then further oxidized at C-7 to form GA_{14} (Hedden et al., 2001; Tudzynski, 2005; Bömke and Tudzynski, 2009). GA_{14} is then converted to the 19-carbon gibberellin, GA_4 by 20-

oxidation. As the first biologically active GA, GA_4 is desaturated to GA_7 which then converted to GA_3 by late 13-hyroxylation, and is the major final product of *G. fujikuroi*. Conversely, GA_1 was formed as a minor product from side reaction by 13-hyrdoxylation of GA_4 (Tudzynski, 2005). Figure 2.3 illustrates the biosynthesis pathways formation of GA_3 in *F. funjikuroi* fungus.

Different strains produced different major final product of GA's (Figure 2.4). Beside *F. fujikuroi*, there are some other fungi that produced GA's. Rademacher (1994) reported that several species of *Sphaceloma* produced GA_4, but not 13-hydroxylated or 1,2-dehydro GAs like GA_3, GA_7 and GA_1. *Sphaceloma manihoticola* is an ascomycete similar to *F. fujikuroi* but differed as it belongs to the order of Myriangiales (Bömke and Tudzynski, 2009).

A species of *Phaeosphaeria*, which was another GA producing fungus with GA_1 as major of final product by a pathway in which 3β-hydroxylation occured after C_{19}-GA formation (Hedden et al., 2001; Bömke and Tudzynski, 2009). Other ascomycetous species also had been reported able to produce GAs namely *Aspergillus niger* and *Neurospora crassa* (Bömke and Tudzynski, 2009).

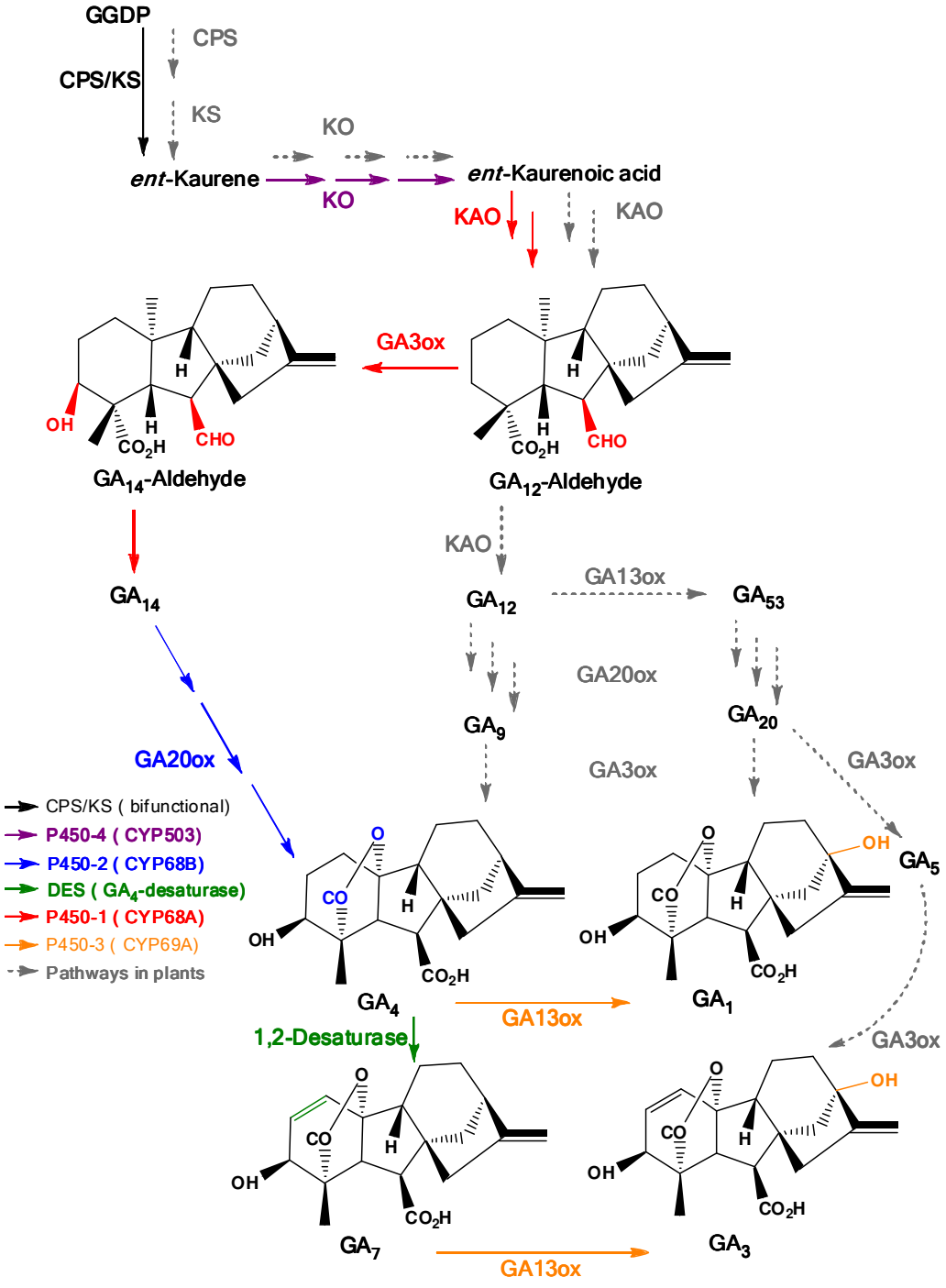

Figure 2.3: Biosynthesis pathways of GA_3 in fungus *F. fujikuroi* (Yamaguchi, 2008)

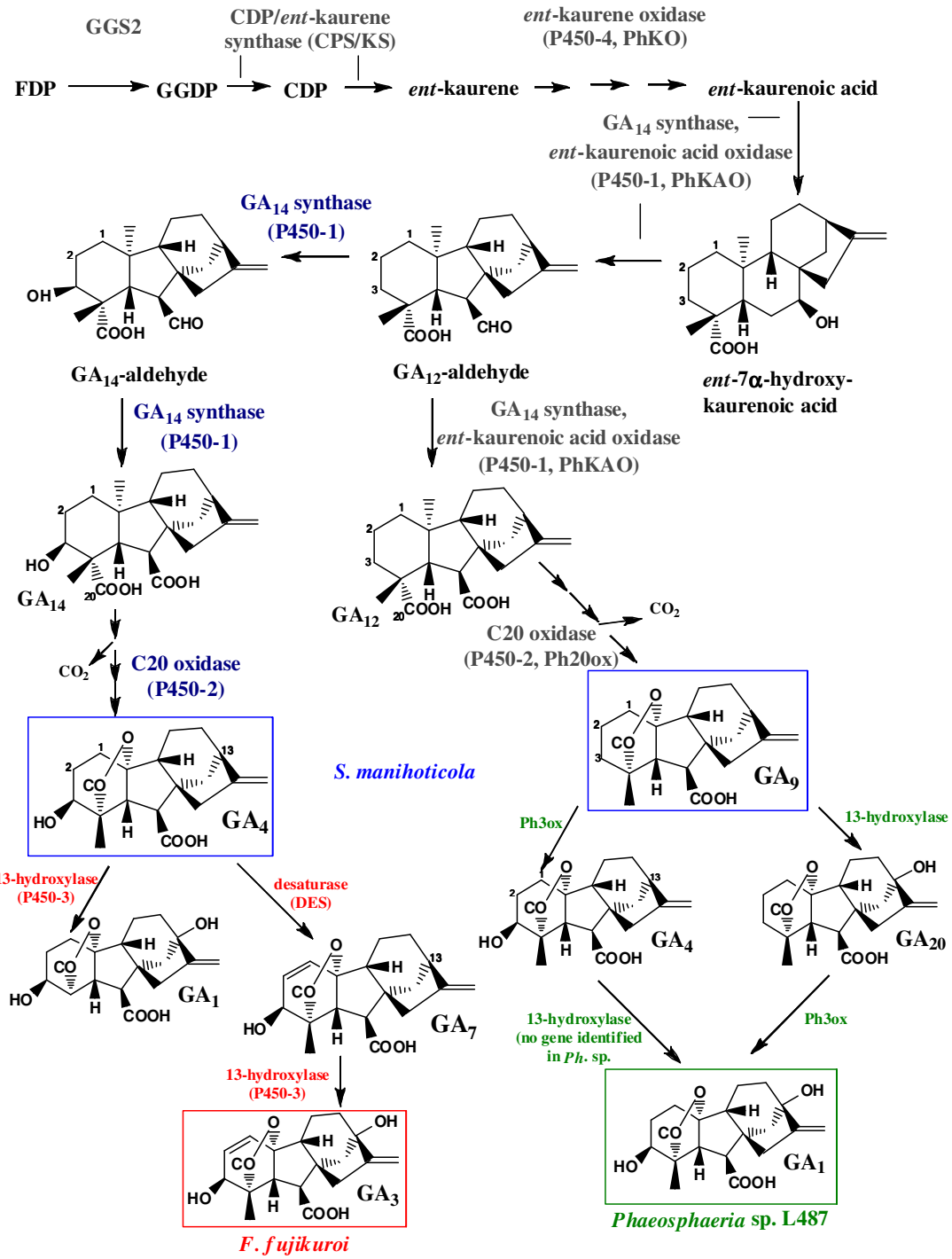

Figure 2.4: The gibberellins biosynthesis pathways with difference final product in *F. fujikuroi*, *S.manihoticola* and *Phaeosphaeria* sp. (Bömke and Tudzynski, 2009)

2.1.3 Application of GA_3

As a useful plant growth regulator, GA_3 can be used to promote seed germination, stem elongation, and fruit growth in varieties of agronomically and horticulturally important plants (Sponsel et al., 2010). As reviewed by Stowe and Yamaki (1957), it is evidently understood that the general applications of GA_3 as growth regulator, but it gives rise to different effects, which depend on the type of plants species and the time of it being used.

Previously, a few researchers have carried out studies to observe the effect of GA_3 towards plants development. In their studies, weed seedlings were grown in nutrient solution that contained GA_3 and acted in response to the increase in height that raise in length of both stem and leaves. Supplement of 10 µg/ml of GA_3 influenced the height of the plants to 50 cm compared to the untreated plant with only 20 cm. This showed that the treatment with GA_3 apparently increased the growth of shoot, which affected actively on growing tissues and organs with substantial enlargement in dry matter accumulation in the plant as a whole (Brian et al., 1954).

Mander (2002) stated that GA_3 have several useful commercial applications like most seedless table grapes, which are now grown with the utilization of GA_3. Besides, in the brewing of beer industry, times consume for product processing and cost of production were reduced by the addition of 25 – 500 µg of GA_3 for each kg of barley. GA_3 can also induce flowering of a variety of ornamental plants earlier than usual or during off-season (Mander, 2002). According to Garner et al. (2011), the Hass Avocado farm which dominated one of the worldwide avocado production

faced great problems with lower fruit set and smaller fruit size. However, by applying GA_3, the fruits yield accelerated from 178 g/fruit to 325 g/fruit.

Despite enhancing the development of plant growth, GA_3 is also able to improve the physical appearance of cactus pear with full of glochids (small spines), and negatively affected the harvesting operation and quality of this fruit. The treatment of cactus pear fruits with GA_3 (100 ppm) combined with ethephon, was significantly increased the length of glochids as well as pre-harvest number of glochids per areole compared with the control, and finally up to 93% of glochids were abscided after the mechanical effect of harvesting (Corrales-Garcia a and Gonzalez-MartÌnez, 2001). Usenik et al. (2005) also reported the influence of GA_3 on the physical properties together with chemical properties of sweet cherry fruits during ripening and fruit maturation. They have successfully proven the influence of GA_3 towards fruit dimensions, height, width and thickness of the sweet cherry, but the response depended on the properties of the cultivar. By spraying GA_3 to the slower fruit ripening, higher yield and better fruit quality was achieved.

Besides stimulated flowering, the application of GA_3 during flowering bud induction has also increased the capability of reducing flowering of 'Black Diamond' and 'Black Gold' Japanese plums has also successfully applied in many others trees for instance, the temperate fruit crops, citrus and mangos. A large number of blossoms produced a large number of fruits which affected the fruit size. In order to achieve a commercially appropriate size of fruit, it is necessitated to thin the fruits manually, which in turn involving higher cost, thus affecting carbohydrates partitioning and promoted vegetative growth and affected induction and differentiation of floral buds (Gonzalez-Rossia et al., 2006). Therefore the application of GA_3 during flowering bud induction interrupted the floral process and

partially reduced flowering and consequently, the number of developing fruitlets. However, the optimum effect depends on the GA_3 dosage, specific climatic conditions and date of treatment in order to avoid excessive depletion of the number of developing fruits, and consequently its final yield (Gonzalez-Rossia et al., 2006).

Jaleel et al. (2007) had explored the potential application of GA_3 for increasing the antioxidant potential and alkaloid production in medical plants like *C. roseus* by soil drenching method with only at lower concentration of GA_3.

Therefore, based on the varieties of GA_3 applications towards plant growth development, it is found that the crops yield improvement and plants resistance to environmental influence had increased very fast. Thus, shows that GA_3 could play an important role in enhancing the agricultural industry in the near future.

2.1.4 Production of GA_3 in Solid State Fermentation

Solid state fermentation (SSF) is characterized by the development of microorganism growth in an environment of low water activity, upon a damp insoluble material that acts as a physical support and a source of nutrient (Pastrana et al., 1995). In consideration of such characteristics, SSF process is capable of utilizing solid materials as substrates, especially from the by-products of agricultural or food processing industries that are abundantly generated with low nutritive values and contributed to pollution potential (Manpreet et al., 2005; Mitchell et al., 2006).

Substrates that have been traditionally employed in SSF include varieties of agricultural products such as rice, wheat, millet barley, grains, corn, beans and soy beans. Furthermore, non-traditional substrates which might also be of interest in the industrial process development include an abundant supply of agricultural, forest and

food-processing waste such as wheat bran, corn bran, rice husk and soy grits (Perez-Guerra et al., 2003). One important characteristic of SSF process is that, it made use of solid substrates with lower moisture content which is similar to the microbe's natural habitat which resulting into a rapid growth and higher metabolic activities (Manpreet et al., 2005).

There are two types of common materials which could be used as substrates for growth of microorganisms which are natural and inert solid material. The natural origin of inert solid material could only serve as an attachment point for the microorganism's growth. For instance, sugar cane bagasse, hemp, inert fibers, resins, polyurethane foam and vermiculite could be impregnated with liquid medium, which contained all the nutrients (sugar, lipids, organic acids, etc). Usually, cultivation on natural material is the most preferred choice and commonly used due to its nature which could serve both as a physical support and source of nutrient. Natural material that can be used as solid substrates could be grouped into starchy substrates (e.g. rice, cassava, wheat bran, banana meal, rice bran, corn meal and sweet potato residues), cellulose or lignocelluloses substrates (e.g. wheat straw, corn, rice stover, wheat bran, sugar beet pulp and wood) and soluble sugar substrates (e.g. grape pomance, sweet sorghum, pineapple waste and coffee pulp) (Pandey, 1992; Ooijkaas et al., 2000; Manpreet et al., 2005; Pandey et al., 2008a).

In view of the fact that most of the natural material substrates are taken from cheap source of agro based residues, pretreatment is needed to convert the raw substrates into a suitable form that could be fully utilized by the microorganisms. Some of the treatments are size reduction by sieving, grinding, rasping or chopping, damage to outer substrate layer by grinding, pearling or cracking, chemical and enzymatic hydrolysis polymers. In fact, supplementation with nutrients (phosphorus,

nitrogen, salts) and setting the pH and moisture content, using a mineral solution, and cooking or vapor treatment for macromolecular structure pre-degradation and elimination of major contaminants are also used to increase the products yield (Mitchell et al., 2000; Perez-Guerra et al., 2003; Manpreet et al., 2005).

Besides, there are some other advantages that made SSF technique as unique and favorable process for the production of microorganism metabolite which could be summarized as in Table 2.1.

Table 2.1: Some unique characteristics and advantages of SSF (Bandelier et al., 1997; Wang and Yang, 2007)

Characteristics	Advantages / Comments
Low moisture content	• Lower reactor volume required for a given productivity • Lower purification costs because of higher product concentrations • Lower costs for treatment of liquid effluent • Inhibition of contaminants
High interfacial surface to liquid volume ratio	• Aeration is easily achieved • Generally has lower energy requirements
Simulates the natural environment for microbial growth	• Allows more complete genetic expression in the microbe • Some products are produced at higher rates in SSF • Some products are produced only in SSF • Yields are reliable and reproducible
Simple media	• Often no more than unprocessed grains with minimal mineral supplementation or no supplementation at all • May consist of agro-industry wastes such as corn fiber and bagasse
Substrate availability	• May increase during fermentation (or decrease or remain constant, as well) rather than always decrease as it does in SmF

Conventionally, the production of GA_3 has been carried out via a submerged fermentation (SmF), which resulted into a number of difficulties in particular, an increase in production cost. Example was the define media that required synthetic glucose as carbon source, ammonium sulfate as nitrogen source and other minerals

sources, of which sometimes produced lower yield and a number of downstream processing (Pastrana et al., 1995). Conversely, Bandelier et al. (1997) reported that by using SSF technique, higher yield of GA_3 could be produced. In another study by Tomasini et al. (1997) also showed that higher production of GA_3 from the SSF process with 250 mgGA_3/kg of cassava flour only took shorter time (36 h) compared to the SmF with only 23 mg GA_3/ml in 120 h. In fact, the production of GA_3 using wheat bran as a substrate has been improved by enhancing its nutritional factor with urea and $MgSO_4$, and the physical factor, which were moisture content, autoclaving time, inoculum ratio and moist medium. Since the wheat bran has enough carbon source for microorganism growth, extra carbon source from the synthetic glucose is not required and production cost also can be reduced (Kumar and Lonsane, 1990). Rodrigues et al. (2009) had screened some agro industrial residues namely citric pulp, soy bran, sugarcane bagasse, soy husk, cassava bagasse and coffee husk to be used as solid substrates for the production of GA_3. Among these substrates, citric pulp (CP) was found to be the best substrate for GA_3 production by SSF which reached 5.9 g GA_3/kg of dry CP after only 3 days of fermentation. Table 2.2 provides a summary of the usage of various varieties of natural substrates that can be used in SSF process for GA_3 production.

However, there are some difficulties that made SSF processes incapable to be applied in industrial scale such as difficult in maintaining an aseptic culture conditions along the processing period and complexity in controlling the important parameters such as temperature, moisture content and mass transfer (Shukla et al., 2003). In view of such difficulties, current research is concentrated on overcoming these problems using different modes of cultivations such as batch, fed batch, and continuous process. Appropriate kinetic models for each process are also part of this

presence work. However, most of researchers conducted their study in batch mode since it is the most suitable method especially for product optimization in SSF process.

Table 2.2: Solid substrates employed for GA_3 production in SSF processes

Fungi	**Substrates**	**GA_3 concentration**	**References**
Gibberella fujikuroi P-3	Wheat bran	1116 mg GA_3/kg dry mouldy bran	(Kumar and Lonsane, 1990)
Fusarium moniliforme M-7121	Maize flour (80% w/v) mixed with wheat bran	18 – 21 g GA_3/kg dry culture	(Qian et al., 1994)
Gibberella fujikuroi (NRRL 2284)	Cassava flour	250 mg GA_3/kg dry solid medium	(Tomasini et al., 1997)
Gibberella fujikuroi ATCC 12616	Wheat bran mixed with soluble starch	5- 5.5 g GA_3/kg final dry substrate	(Gonzalez-Sepulveda and Agosin, 2000)
Gibberella fujikuroi ATCC 12616	Wheat bran mixed with soluble starch	4.5 – 5 g GA_3/kg dry basis	(Corona et al., 2005)
Gibberella fujikuroi LPB-06	Coffee husk mixed with cassava bagasse (7:3, dry wt)	492.4 mg GA_3/kg of substrate	(Machado et al., 2002)
Gibberella fujikuroi LPB-06	Coffee husk mixed with cassava bagasse (7:3, dry wt)	0.925 g GA_3/kg of substrate	(Machado et al., 2004b)
Fusarium moniliforme	Jatropha seed cake	225 g GA_3/kg of substrate	(Rangaswamy and Balu, 2008)
Gibberella fujikuroi	Wheat bran	3 g GA_3/kg of dry matter	(Bandelier et al., 1997)
Fusarium moniliforme LPB03	Citric pulp (CP)	5.9 g GA_3/kg of dry CP	(Rodrigues et al., 2009)

2.1.4 (a) Batch Process

In a batch cultivation technique, all nutrients are required during one run of cultivation. However, for molecular oxygen in aerobic process or other chemicals for pH adjustment, nutrients are added to the medium before the operation started, and

the final products are removed at the end of each batch run (Yamanè and Shimizu, 1984). A number of the studies on GA_3 production have been conducted in Erlenmeyer flasks in batch mode. As reported by Kumar and Lonsane (1990) who studied the influenced of GA_3 production by various nutritional and physical factors, the formation of the product increased tremendously in flask culture under a batch process. Nutritional factors such as urea nitrogen and $MgSO_4$ concentration and physical factors namely moisture content, autoclaving time, inoculum ratio and moist medium to culture flask volume were selected and the results showed that the yield of GA_3 improved by 2.9 times. In batch mode process, the GA_3 production is exploited via solid state culture in Erlenmeyer flask and horizontal rotary reactor with the effects of pH, moisture contents and oxygen concentration. The highest GA_3 was detected after 10 days of incubation with concentration up to 19.3 mg/g dry culture and the lag phase was short with rapid growth continued for up to 2 days in the rotary reactor (Qian et al., 1994).

Tomasini et al. (1997) also carried out a study on GA_3 production in batch mode using different technique of fermentation like SmF and SSF. A submerged culture was performed in 250 ml Erlenmeyer flask, whereas solid state cultures were performed in a column fermenter unit in batch process. It was found that SSF on cassava flour presented higher production of GA_3 with shorter time. Therefore it was proven that although the fermentation conducted in batch mode, the solid state cultivation with natural material was capable in generated higher yield. Gelmi et al. (2000) also cultivated *G. fujikuroi* on an inert material via SSF. Nevertheless, the formation of GA_3 was only 0.73 mg/g inert support which was lower than the natural material as solid substrates. These studies were carried out in SSF in glass column under different conditions of temperature and total moisture content in batch mode

process. Besides that, Tomasini et al. (1997) and Machado et al. (2002) had also conducted a comparative studies on different types of fermentation processes and optimization of some physical and nutritional factors in Erlenmeyer flask under the batch mode. SmF and SSF were performed using extract coffee husk and pretreatment of coffee husk mixed with cassava bagasse respectively at various pH and supplementation of saline solution. As expected, SSF process with a pH 5.3 and optimized saline solution containing 0.03% $FeSO_4$ and 0.01% $(NH_4)_2SO_4$ gave a maximum of 492.5 mg of GA_3/kg of dry substrate which was incubated at 29^oC and 75% of moisture.

Hence, the exploration on the capability of different strains and substrates for GA_3 production was carried out in batch mode process due to its practicalities in research study as reported by Rodrigues et al. (2009). The fermentation assay was performed in Erlenmeyer flask using citric pulp extract supplemented with sucrose and initial moisture of 70-80% at 29^oC, the GA_3 production had accelerated up to 5.9 g/kg of dry substrate after 3 days of the fermentation period. Thus showing that, the batch process is more favorable and practical method for preliminary and optimization study.

2.1.4 (b) Fed-Batch Process

The fed batch or semi batch operation were classified according to the mode of nutrient feeding whether constant (fixed volume) or intermittent (variable volume) of nutrients feeding. In this process, one or more nutrients were provided throughout the cultivation and the product remained in the containment until the end of the experiments (Yamanè and Shimizu, 1984). The fed batch culture was effective to