SUBMERGED FERMENTATION PROCESSES FOR THE PRODUCTION OF BENZALDEHYDE BY A LOCALLY ISOLATED *Rhizopus* sp. D133

BY

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UNIVERSITI SAINS MALAYSIA

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DEDICATION

This work is dedicated to the memories of my late father Danjuma, my late brother Idris and my late sister Amina (Azumi). May all the mercies of ar-Rahim be with them.

….and to the most important person in my life….my dear mother, Hajara.

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ABSTRACT

The study focuses on the selection and production of an almond cherry flavour, benzaldehyde via direct submerged fermentation processes using a locally isolated fungus. Based on the screening program, a total of 56 isolates were obtained of which 44 were found to produce benzaldehyde. Among them, isolate D133, which was identified to be *Rhizopus oryzae* produced the highest benzaldehyde concentration of 13.12 mg L^{-1}. *Rhizopus oryzae* D133 was selected for further work for benzaldehyde production. The optimization of modified production medium revealed that the production of benzaldehyde by *Rhizopus oryzae* D133 was maximum using the medium composition and cultivation conditions consisting of (g L^{-1}): glucose 13, yeast extract 0.5, L-phenylalanine 2, KH_2PO_4 0.3 and $CuSO_4 \cdot 5H_2O$ 0.02, while the optimum temperature, pH, inoculum size, and agitation rate were 25^{o}C, pH 5.5, 2 % (v/v), and 150 rpm, respectively. The maximum concentration of benzaldehyde obtained was 22.82 mg L^{-1} with the growth of 2.41 g L^{-1}, which demonstrated an increase of 73.9 % benzaldehyde production by *Rhizopus* sp. D133 compared to before optimization. Under high agitation rate of 200 rpm or more, the alteration of the morphological structure of the fungal hyphae resulted in a decrease in benzaldehyde production. Fermentation processes revealed that benzaldehyde production by free cells was higher using the loop airlift fermenter system with benzaldehyde production of 33.11 mg L^{-1} compared to the shake flask, stirred tank and tubular airlift fermenter systems with benzaldehyde concentration in the range of 21.65 – 26.42 mg L^{-1}.The result obtained so far indicated that benzaldehyde production by *Rhizopus* sp. D133 was growth dependent. The batch fermentation kinetics of free cells in the loop airlift fermenter system gave the highest specific growth rate (μ) of 0.15 hr^{-1}, doubling time (t_d) of

4.62 hr, (*dp/ds*) of 20.31 mg g^{-1} and product formation rate (*dp/dt*) of 0.17 mg L^{-1} hr^{-1}, with substrate consumption rate (*ds/dt*) of 0.008 g L^{-1} hr^{-1}, product yield (*dp/dx*) of 7.41 mg g^{-1}, growth yield (*Y*) of 2.74 and metabolic quotient (*q*) of 0.05 g hr^{-1}. Other fermenter systems exhibited lower performance as indicated by μ in the range of 0.03 – 0.11 hr^{-1}, t_d were in the range of 6.3 – 23.10 hr, *ds/dt* and *dp/dt* in the range of 0.008 - 0.03 g L^{-1} hr^{-1} and 0.15 - 0.16 mg L^{-1} hr^{-1}, respectively. The single feeding fermentation of 25 % by free cells in loop airlift fermenter gave higher μ of 0.09 hr^{-1}, *ds/dt* of 0.02 g L^{-1} hr^{-1} with higher *dp/dt* of 0.23 mg L^{-1} hr^{-1}, *dp/ds* of 12.93 mg g^{-1}, growth yield (*Y*) of 1.23 and biomass doubling time (t_d) of 7.53 hr, with the metabolic quotient (*q*) of 0.07 g hr^{-1}. Benzaldehyde production by immobilized cells was also found to be higher using the loop airlift fermenter system than in the shake flask, stirred tank and tubular fermenter systems with the benzaldehyde production of 26.64 mg L^{-1} while others showed benzaldehyde production in the range of 21.75 – 24.3 mg L^{-1}. The results obtained indicated that free cells showed higher benzaldehyde production than immobilized cells and loop airlift fermenter exhibited significance performant than other fermenter systems. The benzaldehyde was extracted using chloroform and purified using either florisil or activated carbon. The recovery of benzaldehyde using florisil column was 99.8 % with 98 % purity. Studies on the toxicity effect of the purified benzaldehyde at concentrations 0.75 x 10^{-9}, 0.50 x 10^{-9}, and 0.25 x 10^{-9} mg kg^{-1} on test mice suggested that the purified benzaldehyde is safe to be used as an ingredient in the flavour formulation. However, the purified benzaldehyde revealed that the lethal dose LD_{50} on test mice was found to be 0.028 mg kg^{-1}, and 0.07 mg kg^{-1} for intravenous and intraperitoneal administration, respectively.

PROSES PEMFERMENTASIAN KULTUR TENGGELAM UNTUK PENGHASILAN BENZALDEHIDA OLEH PENCILAN TEMPATAN *Rhizopus* sp. D133

ABSTRAK

Kajian ini memberi tumpuan kepada pemilihan dan penghasilan benzaldehida, sejenis perisa ceri badam, melalui proses pemfermentasian secara langsung dalam kultur tenggelam menggunakan kulat tempatan. Berdasarkan program penyaringan, sejumlah 56 pencilan diperolehi dan 44 pencilan berupaya menghasilkan benzaldehida. Antaranya adalah pencilan D133 yang dicamkan sebagai *Rhizopus oryzae* berupaya menghasilkan benzaldehida tertinggi sebanyak 13.12 mg L^{-1}. *Rhizopus oryzae* D133 dipilih untuk kajian lanjutan untuk penghasilan benzaldehida. Pengoptimuman ke atas medium penghasilan terubah suai menunjukkan penghasilan benzaldehida oleh *Rhizopus oryzae* D133 adalah maksimum menggunakan komposisi medium dan keadaan pengkulturan yang terdiri daripada (g L^{-1}): glukosa 13, ekstrak yis 0.5, L-fenilalanina 2, KH_2PO_4 0.3 dan $CuSO_4 \cdot 5H_2O$ 0.02, sementara suhu, pH, saiz inokulum dan kadar goncangan yang optimum adalah 25^0C, pH 5.5, 2% (i/i) dan 150 rpm, masing-masing. Penghasilan benzaldehida yang maksimum adalah 22.82 mg L^{-1} dengan pertumbuhan 2.41 g L^{-1}. Ini merupakan peningkatan sebanyak 73.9 % penghasilan benzaldehida oleh *Rhizopus oryzae* D133 berbanding sebelum pengoptimuman. Di bawah kadar pengadukan yang tinggi melebihi 200 rpm, perubahan dalam struktur morfologi hifa kulat menyebabkan pengurangan dalam penghasilan benzaldehida. Proses pemfermentasian menunjukkan bahawa penghasilan benzaldehida maksimum sebanyak 33.11 mg L^{-1} diperoleh menggunakan sel bebas di

dalam sistem fermenter angkut udara jenis gelung berbanding dengan sistem kelalang goncangan, tangki teraduk dan angkut udara jenis tubular, yang memberikan kepekatan benzaldehida dalam julat 21.65 – 26.42 mg L^{-1}. Keputusan yang diperolehi sehingga kini menunjukkan perhasilan benzaldehida adalah bergantung kepada pertumbuhan. Kinetik pemfermentasian sel bebas secara sekelompok di dalam sistem fermenter angkut udara jenis gelung memberikan kadar perturmbuhan spesifik (μ) 0.15 jam^{-1}, masa penggandaan (t_d) 4.62 jam, (*dp/ds*) 20.31 mg g^{-1}, kadar pembentukan hasil (*dp/dt)* 0.17 mg L^{-1} jam^{-1} dengan kadar penggunaan subtrat (*ds/dt*) 0.008 g L^{-1} jam^{-1} hasil pembentukan benzaldehida (*dp/dx*) 7.41 mg g^{-1}, hasil pertumbuhan (*Y*) 2.74, dan kuosien metabolik (*q*) 0.05 g jam^{-1}. Sistem fermenter yang lain menunjukkan prestasi yang rendah seperti ditunjukan dengan μ dalam julat 0.03 - 0.11 jam^{-1}, t_d dalam julat 6.3 - 23.10 jam, *ds/dt* dan *dp/dt* dalam julat 0.008 – 0.03 g L^{-1} jam^{-1} dan 0.15 - 0.16 mg L^{-1} jam^{-1}, masing-masing. Pemfermentasian suapan tunggal sel bebas pada 25 % di dalam sistem fermenter angkut udara jenis gelung memberikan μ tertinggi 0.09 jam^{-1}, *ds/dt* 0.02 g L^{-1} jam^{-1}, *dp/dt* 0.23 mg L^{-1} jam^{-1}, *dp/ds* 12.93 mg g^{-1}, hasil pertumbuhan, *Y* 1.23 dan masa penggandaan biojisim (t_d) 7.53 jam dengan *q* 0.07 g jam^{-1}. Penghasilan benzaldehida oleh sel tersekat gerak juga didapati lebih tinggi menggunakan sistem fermenter angkut udara jenis gelung berbanding dengan sistem kelalang goncangan, tangki teraduk dan angkut udara jenis tubular dengan penghasilan benzaldehida sebanyak 26.64 mg L^{-1}. Manakala, sistem lain menunjukkan penghasilan benzaldehida dalam julat 21.75 – 24.3 mg L^{-1}. Benzaldehida diekstrakkan menggunakan kloroform dan ditulenkan menggunakan sama ada florisil atau karbon teraktif. Perolehan benzaldehida adalah sebanyak 99.8 % menggunakan turus florisil, dengan ketulenan 98 %. Kajian kesan ketoksikan benzaldehida tulen pada kepekatan 0.75 x 10^{-9}, 0.5 x 10^{-9} dan 0.25

x 10^{-9} mg kg^{-1} mencadangkan benzaldehida tulen adalah selamat untuk digunakan sebagai ramuan dalam performulaan perisa. Walau bagaimanapun, benzaldehida tulen mempunyai takaran maut, LD_{50} pada mencit ujian setinggi 0.028 mg kg^{-1} dan 0.07 mg kg^{-1} apabila diberi secara intravenous dan intraperitonium, masing-masing.

CHAPTER 1 INTRODUCTION

1.1 Flavours and Foods

What would foods and drinks taste, smell or look like without flavour, aroma or colour? Men throughout history, have always sought to make their food more appetizing, firstly by using spices, herbs and then by the "spirits" of fruits and aromatic plants or essential oils. Flavours are extremely important for the food, feed, and pharmaceutical industries. The industrial exploitation of microorganisms for the production of flavours is another extension of traditional processes mainly in the production of primary metabolites such as amino acids (Aida *et al.* 1986) or secondary metabolites, such as antibiotics (Van Dame 1984). Secondary metabolites are compounds produced by the cells, however, they are not required for growth. Most of the volatile flavour compounds such as terpenes, ketones, lactones, alcohols and aldehydes are classified as secondary metabolites. In many cases, these volatile compounds were produced as a result of the detoxification process developed within the cell to compensate with the effect caused by unfavourable environmental conditions such as when there are excessive concentration of nutrients or metabolites. Examples of such flavour compounds include; benzaldehyde and vanillin. Secondary metabolites are present in low concentrations during the logarithmic growth phase, but appear in large quantities during the stationary phase. Most flavour compounds available at present are produced via chemical synthesis or extraction, for example benzaldehyde extracted from fruit kernels such as apricots, leading to the formation of undesirable toxic by-products such as hydrocyanic acid. The formation of undesirable racemic mixtures or toxic by products such as the hydrocyanic acid, are considered drawbacks in chemical processes. The growing aversion of the consumer towards chemicals added to his food must also be taken into consideration. Of all available flavour compounds, 84 % are

produced by chemical synthesis (Unger 1989). This has caused flavour companies to direct their attention towards the production of flavour compounds of biological origin, so called natural- or bio-flavours. Up to now, plants are important source of essential oils and flavours for example, 4-decalactone, from peach and eugenol from cloves. However, these flavour compounds are often present in minor quantities or are only found in exotic plants, making isolation difficult and the flavour products expensive. Apart from plant cells and tissue culture techniques, a direct viable alternative route for flavour synthesis is the microbial process, that is the fermentation 'process' *de novo* or biosynthesis, or bioconversion and precursor biotransformation (Scharpf *et al.* 1986). It is important to establish whether or not the biotechnologically produced aromas can be considered to be natural. With the exception of traditional applications such as cheese and beer, the use of biotechnological methods for the production of food ingredients is fairly recent. It is regrettable in many countries that the legislative authorities lag behind in regulating new developments for biotechnological approaches. In Europe, attempts are now being made by the European Commisssion to work out a common legislation (Janssen *et al*. 1992). In the USA, flavour compounds can be classified as natural and artificial. The code of Federal regulations include a natural flavour may either be as essential oils, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavour constituents derived from spices, fruit juices, vegetable or vegetable juices, edible yeasts, herbs, buds, barks, roots, leaves or similar plant material, meat, sea food, poultry, eggs, dairy products and fermentation products thereof, whose significant function in food is flavouring rather than nutrition (Dubois 1988). Therefore, based on this classification, natural flavours comprise of conversion products, by living cells or part thereof, including enzymes. A third classification, namely the nature-identical flavours, exists in

most of the European countries. These compounds are synthesized via chemical processes, but are in all chemical aspects identical to aromas identified in nature. The distinction between natural and synthetic flavours is analytically possible via GC/MS, in particular by determining isotope ratio (Stofberge 1986). In this respect, flavours produced by microorganisms are natural if the precursor material is also of natural origin. There is however a great distrust of biotechnological products with applications in food, especially when genetic manipulation is also involved, which requires supplementary regulation. In USA, admissions for such new products are given by the Food and Drug Administration (FDA). Compounds with a GRAS-status (Generally Recognized As Safe) not only include those from natural resources, but also products, which are produced by microbial, or enzymatic processes (McNamara 1989). The GRAS-label is important because these compounds are not considered as additives. GRAS refers to strains of microorganisms from traditional fermentations such as tempe, soy sauce, and other fermented foods.

The generation of volatile flavour compounds from microorganisms on an industrial scale is in its infancy, and is still carried out using complicated and very tedious empirical technologies. An increasing number of publications and patents indicate a surge of scientific and economic interest in the use of biotechnology to produce volatile flavour compounds (Janssens *et al.* 1992). Numerous studies have shown that enzyme technologies and microorganisms cultivated in submerged culture may yield complex plant-typical volatiles such as terpenes, aliphatic esters and other carbonyls, phenylpropanoids and lactones. Some fungi, in particular, possess an impressive metabolic diversity which, by means of *de novo* synthesis or bioconversion, opens direct access to the production of industrially important volatile compounds. The exploitation of microbial biosynthetic pathways for the production of natural flavours

has proved to be both feasible and of economic interest. The production of γ-decalactone is a good example. Its price decreased from US$20000/kg in the early 1980s to only US$1200 in 1995 because of the move to microbial production system. It is currently estimated that about 100 compounds could be produced using microbial processes (Delest 1995). Of these, only a few are produced on industrial scales. This is largely due to the high cost of the process currently used, which means that applications focus mainly on the production of added-value products such as γ- and δ-lactones (from US$6000/kg to US$1200/kg), vanilla extracts (about US$4000/kg) and various esters. Even for these molecules, our ignorance of their biosynthetic pathways is one of the main bottlenecks for industrial production. In the case of cheaper molecules, such as benzaldehyde (about US$240/kg), it will also be necessary to develop low-cost processes with improved production yields. In quantity, benzaldehyde is the second most important molecule after vanillin for its use in the flavour and fragrance industries (Welsh *et al.* 1989). For all these reasons, basic research on the microorganisms is required in order to better control and direct the metabolic pathways; the exploration and development of alternative production technologies, such as the use of immobilized cells.

1.2 Rationale for selection of benzaldehyde

Benzaldehyde is the second most important compound after vanillin for its use in the flavour and fragrance industries (Welsh *et al.* 1989). The demand for benzaldehyde is high of approximately 5000 kg/year and a price of approximately US$240/kg encourages the search for an alternative way of producing it in order to meet the market demand, and consumer's preferences of natural than chemical synthetic flavours. One of the uses of natural benzaldehyde is as ingredient in cherry, almond, and other fruit

flavours. The natural benzaldehyde extracted from fruit kernels such as apricots leads to the formation of undesirable toxic by products such as hydrocyanic acid, and its classification as 'natural' is questionable. Therefore, the fermentation processes via natural precursors such as phenylalanine gives an alternative route for the natural benzaldehyde biosynthesis without the production of such toxic by-products and with the benefits of a 'natural' label.

There is growing fear from the public when it comes to the use of chemically synthesized flavour compounds as compared to that of natural origin. There is also a drawback of chemical synthesis, which includes the formation of isomers, mainly hydrocyanic acid (Feron *et al.* 1996). Indeed, some kind of 'chemophobia' can be noticed for any compound that is chemical or synthetic, an example of a synthetic flavour is ethyl maltol (Kuentzel and Bahri 1991). Foodstuffs containing synthetic aromas are often avoided, because the consumer suspects these compounds to be toxic or harmful to his health. Because of the fact that benzaldehyde is the second most important compound after vanillin for its use in the flavour and fragrance industries (Welsh *et al.* 1989), and highly demanded, the search for an alternative way of producing the compound is inevitable. Furthermore, benzaldehyde produced through the use of natural phenylalanine as a precursor can be labeled as 'natural' there by giving consumer a satisfaction of taking natural product rather than chemical. Based on that, the search and screening of indigenous sources of potential microorganisms capable of producing natural benzaldehyde is to be carried out. However, not only its production but also the process has to be optimized to ensure maximum production. It is important to further explore its production in various types of fermenter systems including kinetic studies so as to identify the optimal fermention systems that give the highest production rate.

1.3 Research scope

This research emphasizes on the exploration of an alternative route of producing a natural benzaldehyde, as a flavour ingredient in food. The approach undertaken was via submerged fermentation process, using locally isolated microorganisms. Potential isolate for the production of benzaldehyde was selected and identified. Fermentation processes for the production of benzaldehyde were examined through production medium formulation and physical conditions optimization. Lapadatescu and his colleagues (1997) reported microbial biotransformation of L-phenylalanine to benzaldehyde using white-rot basidiomycetes, while production of benzaldehyde could also be carried out enzymatically as reported by Groot *et al.* (1998). In this work, direct fermentation for benzaldehyde production was carried out using medium composition containing L-phenylalanine, which was transformed to benzaldehyde throughout the fermentation process. The supplementation of L-phenylalanine at the beginning of the fermentation was performed and growth, pH, benzaldehyde concentration and residual glucose were monitored until the end of the process. The optimized medium composition and physical conditions were applied in various fermenter designs for benzaldehyde production, which include fermentation processes in shake flasks, stirred tank, and airlift (loop and tubular) fermenter systems. Batch and single step feeding modes were carried out. Immobilization of the fungus on scouring mesh allowed a comparison of the growth and benzaldehyde production as compared to the free cells. The kinetic studies of the fermentation were carried out to compare the performances of the fermenters. Extraction, purification and characterization of the benzaldehyde produced were studied and comparison with commercialy available benzaldehyde was also carried out. The production of benzaldehyde was examined for possible toxicity

effect using animal model to ensure the safety of benzaldehyde as a flavour ingredient in food.

1.4 Research objectives

Based on the scope of this work, the objectives of this research include:

1. To screen potential microorganisms from indigenous sources for the production of a natural benzaldehyde.
2. To optimize the fermentation medium and physical conditions for benzaldehyde production by the selected benzaldehyde producer
3. To study fermentation processes for benzaldehyde production using shake flask, stirred tank, loop and tubular airlift fermenter systems.
4. To compare the fermentation kinetics parameters under different fermenter designs and fermentation conditions
5. To purify, characterize and evaluate the toxicity of the benzaldehyde produced by the selected potential producer.

CHAPTER 2 LITERATURE REVIEW

2.1 Definition of flavour compounds and their sources

Food flavourings and flavour in food are terms, which vary in definition though they are related in terms of their purposes. Flavour is created by aromatic substances, which have been developed in living matter grown in nature. Flavour in food represents the quality of aromatic substances, which impart odour and taste, hence make the food more palatable for human consumption. Food flavourings are man-made. They aim to impart a flavour of particular choosing and also to affect the palate with similar enjoyment. In the manufacture of food flavourings, therefore, it is imperative to know food flavour and its chemistry as well as the properties of the biological materials in food products, for they influence the development of flavour (Merory 1968). In another definition, flavour could be defined, as thus not the volatiles in food; it is an interaction of the components in the aroma headspace above the food and the consumer (Piggott and Paterson 1994). Food has been the ever-dominant concern of the human race. The whole human structure is influenced by food. Consequently, man himself and his history have to be known. Also, man's chemistry, biology and physiology should be studied in order to comprehend his interest in flavour and to understand his desires in food. The science and technology of food flavourings require that the chemistry and properties of aromatic substances of natural and man-made origin be studied, as well as their analysis and interpretation. Sources of flavour compounds include; microbial fermentation products, plant extract, and synthetic flavours (Fig. 2.1).

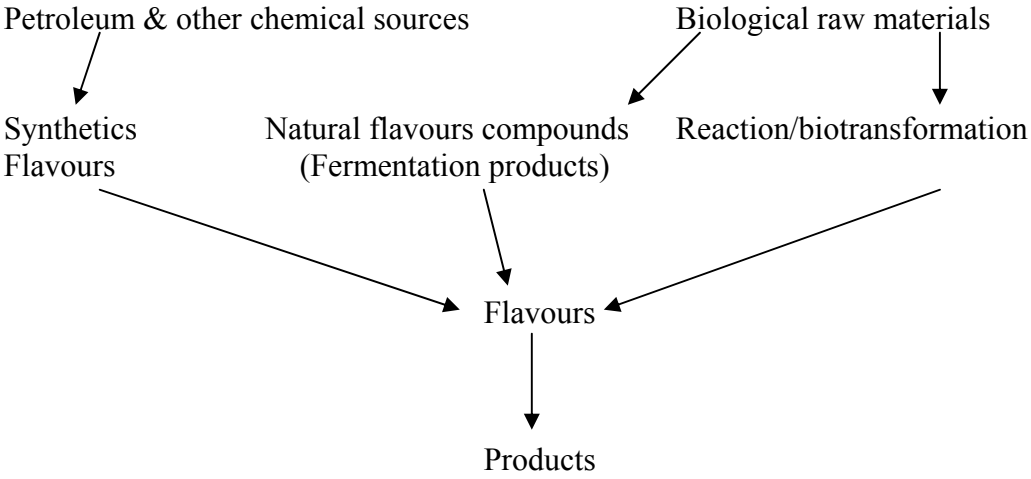


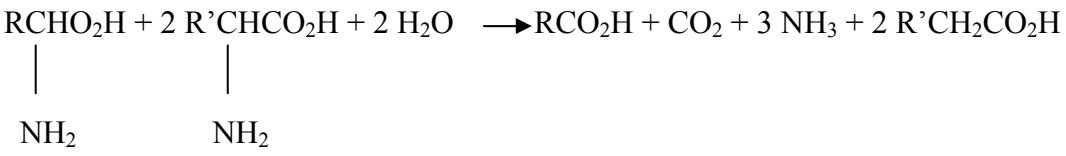
Fig. 2.1: Sources of raw materials for the flavour industry

2.2 Production and applications of flavouring compounds

Nearly all important flavour companies declare indeed that they regularly use fermentation techniques for the production of aroma compounds, yet only few of them mention the specific products (Van Brunt 1985). Recently, a German company with a trademark name BASF, started the microbial production of 4-decalactone, a peach aroma that is distributed by its subsidiary company Fitzsch, Dodge and Olcott. The process involves the bioconversion by *Yarrowia lipolytica* of castor oil, an oil that is pressed from the seeds of *Ricinus communis* and is composed of 80 % of triglyceride of 12-hydroxy-9-octadecene acid, also known as ricinoleic acid (Tyrrell 1987). The yeast lipolyses the castor oil, after which the liberated hydroxyacid is metabolized via the β-oxidation pathway, resulting in the formation of 4-hydroxydecanoic acid. This compound lactonizes easily. In the United Kingdom, (R)-δ-dodecanolide is prepared by Unilever on a commercial scale using baker's yeast using 5-ketododecanoic acid as substrate (Janssens *et al.* 1992). This process takes place in a 30,000-litre fermenter and the lactone produced can be applied as a butter flavour in margarines. Butyric acid and ethyl butyrate are produced microbiologically by the American company Hercules Inc. (Tyrrell 1987). *Clostridium butyricum* converts glucose under anaerobic conditions into butyric acid, the concentration of which can reach 1.2 % in the fermentation broth. Butyric acid, a component naturally present in butter and some cheeses, can be applied for instance as a natural cheese aroma (Sharpell Jr. 1985).

Beside the biological methods, the chemical synthesis of a flavour compounds was also reported. Isovaleric acid can be synthesized by the oxidation of isopentyl alcohol. Subsequent direct esterification leads to the formation of various esters. Isovaleric acid can also be obtained via methods namely; the microbial oxidation of

isopentyl alcohol, and the conversion of leucine to isovaleric acid. Numerous investigators have demonstrated that this is possible via the Stickland reaction shown below (Sharpell Jr, 1985).



The reaction employs amino acid utilization by anaerobes to facilitate coupled oxidation-reduction between pairs of amino acids. One amino acid is oxidatively deaminated and decarboxylated; the other is reductively deaminated.

2.3 Classification and nomenclature of flavouring compounds

The nomenclature and classification of flavour ingredients are based on characteristics that have been widely used not only in technical literature, but also commercially and by various regulatory agencies. The physical appearance of a flavour ingredient (solid, liquid, and paste) may be considered as the first criterion of classification (Table. 2.1). Flavour ingredients also can be classified as either *simple* or *compounded*. Simple flavours are those consisting of a single ingredient *per se* or diluted in an appropriate neutral carrier. Compounded flavours are blends of several ingredients; as in the case of simple compounds, these may also be diluted in carriers (solvents). The presence of alcohol as a solvent can be of paramount importance for both technical and commercial reasons. Therefore, alcohol could be substituted whenever feasible with carbitols, glycerin, or other functional carriers approved by regulatory agencies (Weast 1971).

Table. 2.1: Classification of flavour ingredients

Solid	**Liquids**	**Pastes**
Crystals Powder Freeze-dried Spray-dried Dried extracts Plated Encapsulated flavours	Essential oils Folded Rectified Oleoresins Absolutes Fluid extracts Compounded oils Alcoholates Alcolates Tictures Infusions Distillates Spirits Soluble essences Emulsions Fractions and isolates Concentrated juices Single-strength juices	Soft extracts Resins (natural or prepared) Resinoids Concretes

Source: (Wright 1995; Moyler 1995; Ashurst and Taylor1995; Mathews 1995; Weast 1971)

The solid flavour ingredients include the crystalline aspect of flavours such as vanillin, coumarin, propenylguaethol, and ethyl vanillin. Flavour ingredients in powdered form (more or less hygroscopic) are more common. Dried extracts are obtained by total removal of solvent from an extract. Freeze-dried (lyophilized) flavour ingredients are characteristically very hygroscopic. Powdered flavour ingredients are obtained by plating concentrated essences onto lactose, magnesium carbonate, or other solid carriers (Amoore and Venstrom 1967; Ashurst and Taylor 1995; Beets 1961; Mathews 1995; Moyler 1995; Naves 1957; Weast 1971; Wright 1995). Liquid flavours can be oily and oleoresins, or non-oily when obtained by dissolving the active flavour principles in an appropriate solvent, example alcohol of various strengths. However, Weast (1971) indicated that flavour compounds can also be classified according to their taste and flavour type, such as allyl benzoate which has a bitter sweet taste with characteristic cherry flavour type (Table 2.2). Others include diacetyl, which has a sweet taste with a butter flavour type, and vanillin, with a bitter taste and vanilla flavour type.

Table 2.2: Classification of flavour ingredients by primary taste and flavour type

Flavour ingredient	Taste			Flavour type
	Sweet	Bittersweet	Bitter	
Allyl benzoate		x		Cherry
Allyl butyrate		x		Apple, apricot
Allyl caprote		x		Pineapple
Allyl cyclohexylvalerate		x		Peach, apricot, apple
Anisyl formate	x			Strawberry
Benzyl cinnamate	x			Honey
Benzyl salicylate	x			Raspberry
Cyclohexylbutyrate		x		Banana, apple, current
Decanal dimethyl acetal			x	Citrus
Diacetyl	x			Butter
Dimethyl hydroquinone			x	-
γ-Dodecalactone		x		Apricot, peach
Ethyl acetate		x		Wine
2-ethyl-3-furylacrolein		x		Cola
Ethyl vanillin		x		Vanilla
Linalyl anthranilate	x			Orange
γ-Nonalactone		x		Coconut
Phenethyl alcohol			x	Peach, rose
Phenylpropyl cinnamate	x			Cocoa
Phenylpropyl ether		x		Grape
Rhodinyl isovalerate		x		Cherry
Santalyl acetate		x		Apricot
Styralyl acetate			x	Grapefruit
Tolualdehyde		x		Cherry, almond
γ-Undecalactone	x			Apricot, peach
Vanillin			x	Vanilla
Vanillylidene acetone		x		Vanilla

x = taste type, - = not determined

Source: Weast (1971)

2.4 Biological processes for the production of flavouring compounds

Biological sources of flavour compounds derived mainly from plant or part of plant extracts. However, microbial sources originated from specific substrates, through different types of reactions such as oxidations, reductions, hydrolytic reactions, dehydrations, formation of new C-C bonds and several degradation reactions, which can be performed by microorganisms for the production of flavours (Scharpf *et al.* 1986).

The ability of some microorganisms in generating pleasant odours has long been known. Odour description has also been used for many years in the taxonomic classification of some microorganisms (Badcock 1939). More volatiles flavouring compounds from microbial sources were identified following the introduction and the improvement of several analytical techniques in organic chemistry, such as gas chromatography and mass spectrophotometry.

2.4.1 Lactones

Lactones are associated with odour impression such as fruity, coconut-like, buttery, sweet or nutty. Lactones are internal (cyclic) esters of primarily γ- and δ-hydroxy acids (Fig. 2.2). They are mostly produced chemically but the use of microorganisms can have several advantages in comparison with chemical synthesis especially for the production of optically active lactones. *Trichoderma viride*, a soil fungus generates a strong coconut flavour on a simple growth medium. The compound produced by fungus is 6-pentyl-2-pyrone, which is produced to a maximum concentration of 170 mg/L (Collins and Halim 1972; Welsh *et al.* 1989). *Sporobolomyces odorus* is a yeast which produces *de novo* up to 1.6 mg/L 4-decalactone, resulting in an intense peach odour.

An enantioselective lipase-catalyzed lactonization of racemic methyl 4-hydroxybutyrate is done to give approximately 94% (−)-(S)-4-pentanolide, an enantioselective reduction of prochiral 3- and 4-oxocarboxylic acids to corresponding hydroxyl acids of (R)-configuration which are then chemically transformed into the corresponding optically active γ- and δ-lactones of (R)-configuration shown in Figure 2.3 (Naf and Uhde 1990; Utaka *et al.* 1987). A coconut aroma is highly desired by flavourists, γ-octalactone and γ-nonalactone possess this aroma.

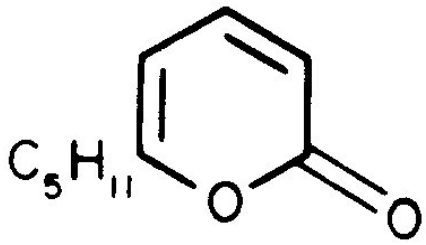


Fig. 2.2: 6-pentyl-2-pyrone. 6-pentyl-2-pyrone (Coconut odour) (Sharpell Jr. 1985)

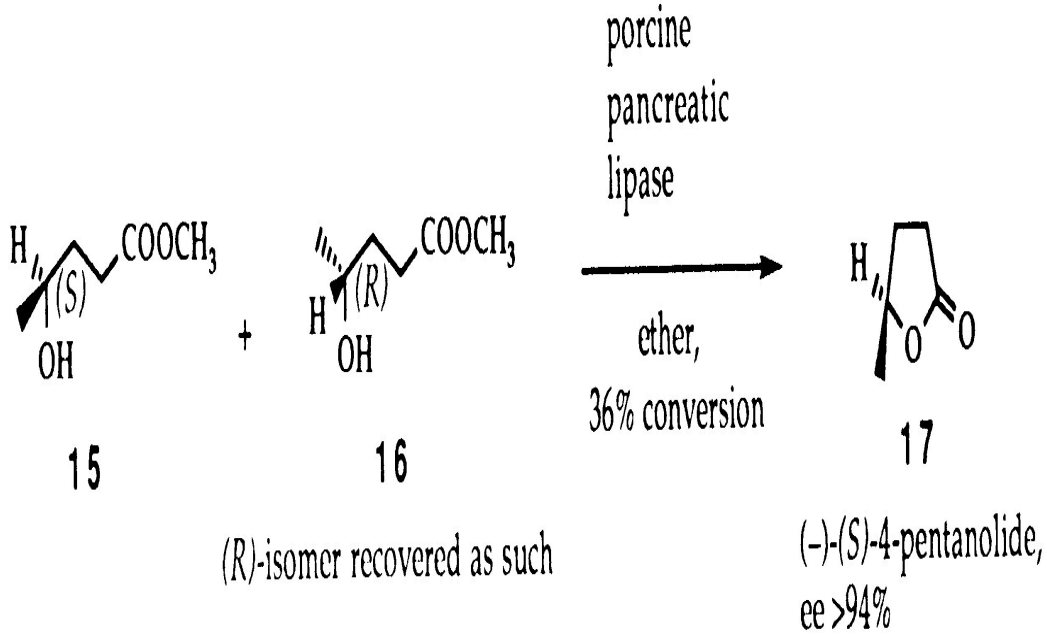


Fig. 2.3: Enantioselective, lipase catalyzed lactonization of methyl 4-hydroxybutyrate (Naf and Uhde 1990; Utaka *et al.* 1987)

15, 16 = racemic methyl 4-hydroxybutyrate, 17 = (-)-(S)-4-pentanolide

2.4.2 Esters

Esters are another important group of flavours. They are important aroma compounds of fruits, in which they are present in fairly low concentrations, mostly between 1 and 100 ppm. They were produced as the first synthetic flavours, but it is also known that these compounds can be synthesized by microorganisms. Diaz *et al.* (2003) and Janssens *et al.* (1992) reported that some microorganisms that generate an apple aroma, was probably due to the formation of 3-methylbutyl 3-methylbutyrate (Table 2.3). Also included was the formation of ethyl acetate, 3-methylbutanol, 3-methylbutyl acetate, 2-phenylethanol, and 2-phenylethyl acetate by *Geotrichum candidum*, which give melon aroma. The formation of off-flavours in pasteurized milk and cheese, due to the production of ethylbutyrate and ethyl hexanoate by certain lactic acid bacteria and *Pseudomonas* species is also well known (Pereira and Morgan 1958).

Table. 2.3: Esters and flavours produced by microorganisms

Microorganism	Aroma group	Products	Flavour characteristics
Geotrichum spp.	Esters	Ethyl isobutyrate, ethyl propionate, butyl acetate, 2-phenylethyl acetate	Apple
Geotrichum candidum	Esters, alcohols	Ethyl acetate, 3-methylbutanol, 3-methylbutyl acetate, 2-phenylethanol,2-phenylethyl acetate	Melon
Dipodascus spp.	Esters and alcohols	Higher alcohols and esters	Apple, pine apple
Hansenula mrakii CBS 500	Esters and alcohols	2, and 3-methylbutyl acetate, isobutyl acetate	Fruity, banana
Pseudomonas fragi CRDA 07	Esters	Ethylbutyrate, ethyl 3-methylbutyrate, ethyl hexanoate, ethyl 2-hexanoate, ethyl crotonate, ethyl 2-methylhexanoate	Fruity, strawberry-like

Source: (Diaz *et al.* 2003, Janssens *et al.* 1992)

2.4.3 Acids and alcohols

Butyric acid and ethyl butyrate are produced by microorganisms commercially, by the American company Hercules (Dzieczak 1986; Yang *et al.* 2002). In this process, *Clostridium butyricum* converts glucose under anaerobic conditions into butyric acid. Butyric acid, a component naturally present in butter and some cheeses, can be used as a natural cheese aroma (Sharpell Jr. 1985). Esterification with ethanol gives rise to ethyl butyrate, an important fruity flavour with a low odour threshold. Pentyl butyrate provides a strong, ethereal, fruity odour reminiscent of apricot, banana and pineapple. Isobutyl butyrate gives an ethereal, fruity, somewhat pungent odour suggestive of pear, pineapple and banana (Arctander 1969). The mechanism for butyric acid production has been summarized in Figure 2.4 (Sharpell Jr. 1985). After vanillin and benzaldehyde, β-phenethyl alcohol is another important group of flavouring compound, in terms of market value. It is mainly obtained from roses, but the resulting extracted molecules show artifact flavours that are undesirable for finished food flavour (Feron *et al.* 1996). β-Phenethyl alcohol could also be obtained from the bioconversion of phenylalanine via phenylacetaldehyde shown in Figure 2.5 (Akita *et al.* 1990; Feron *et al.* 1996).

Another important acid used as flavouring compound is citric acid. About hundreds of thousands of tonnes of citric acid are produced every year using *Aspergillus niger* fermentation of glucose. The first commercial process dated back to 1923 when Pfizer began producing citric acid using a surface fermentation method. Citric acid is the most widely used as food acidulant with a total world volume of more than 500,000 tons (Blair and Staal 1993). The process involves the fermentation of glucose to citric acid using *Aspergillus niger* through either submerged or solid-state fermentation (Blair and Staal 1993; Goldberge *et al.* 1991). The process depends on using limiting amount of iron, magnesium, and zinc in the fermentation medium (Kapoor *et al.* 1992). Citric

acid is then recovered from the fermentation broth by precipitation as the calcium salt, acidified and recrystallized as the free acid. Glutamic acid is another example of a flavour ingredient produced by fermentation in large volume (Fig. 2.6a). It was first discovered as a major flavour constituent of Japanese seaweed. Glutamic acid (monosodium glutamate-MSG) is produced today largely through fermentation of about 300,000 tons annually (Kumon and Kawakita 1991).

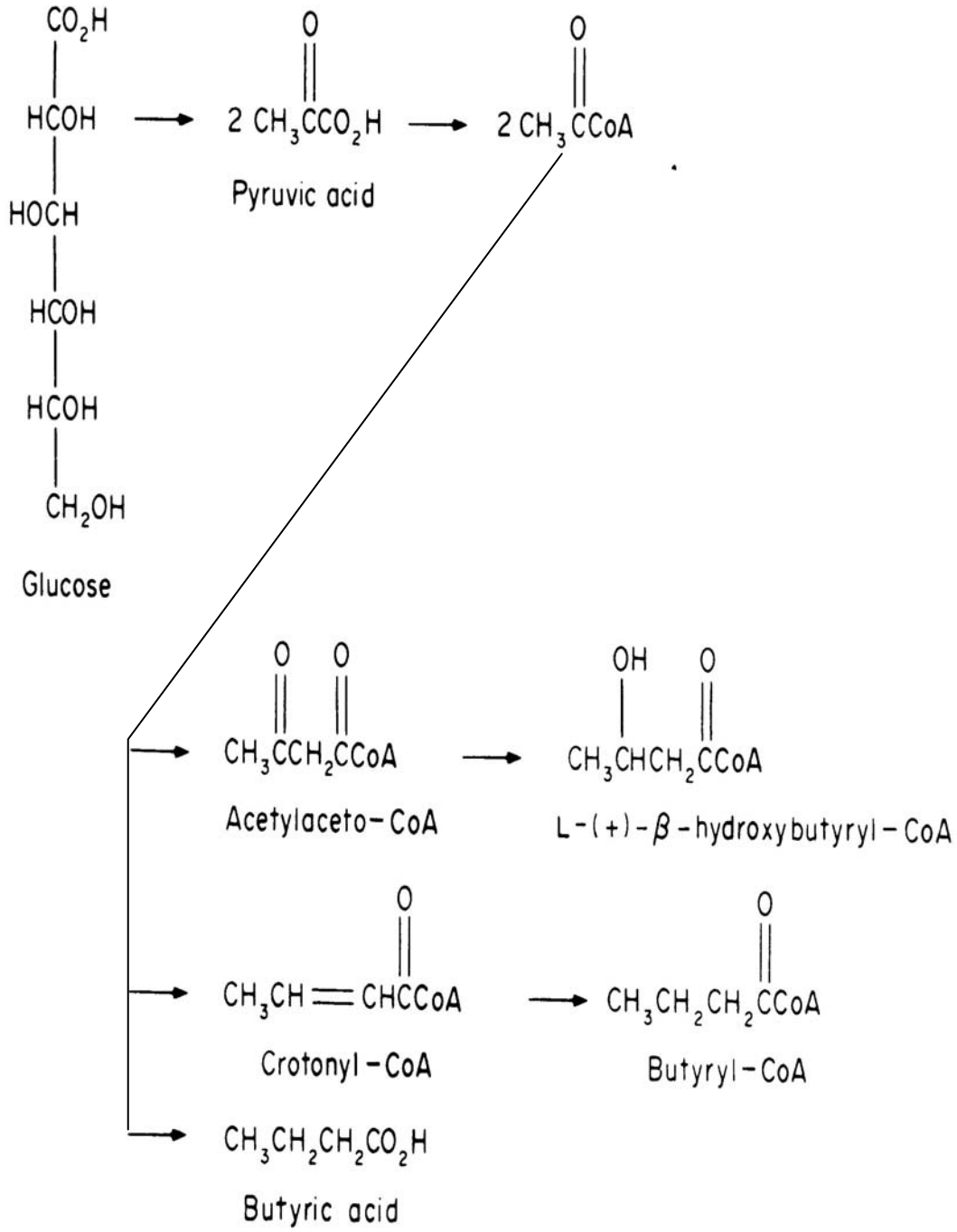


Fig. 2.4: Mechanism of butyric acid production from glucose, using *Clostridium butyricum* (Sharpell Jr. 1985)

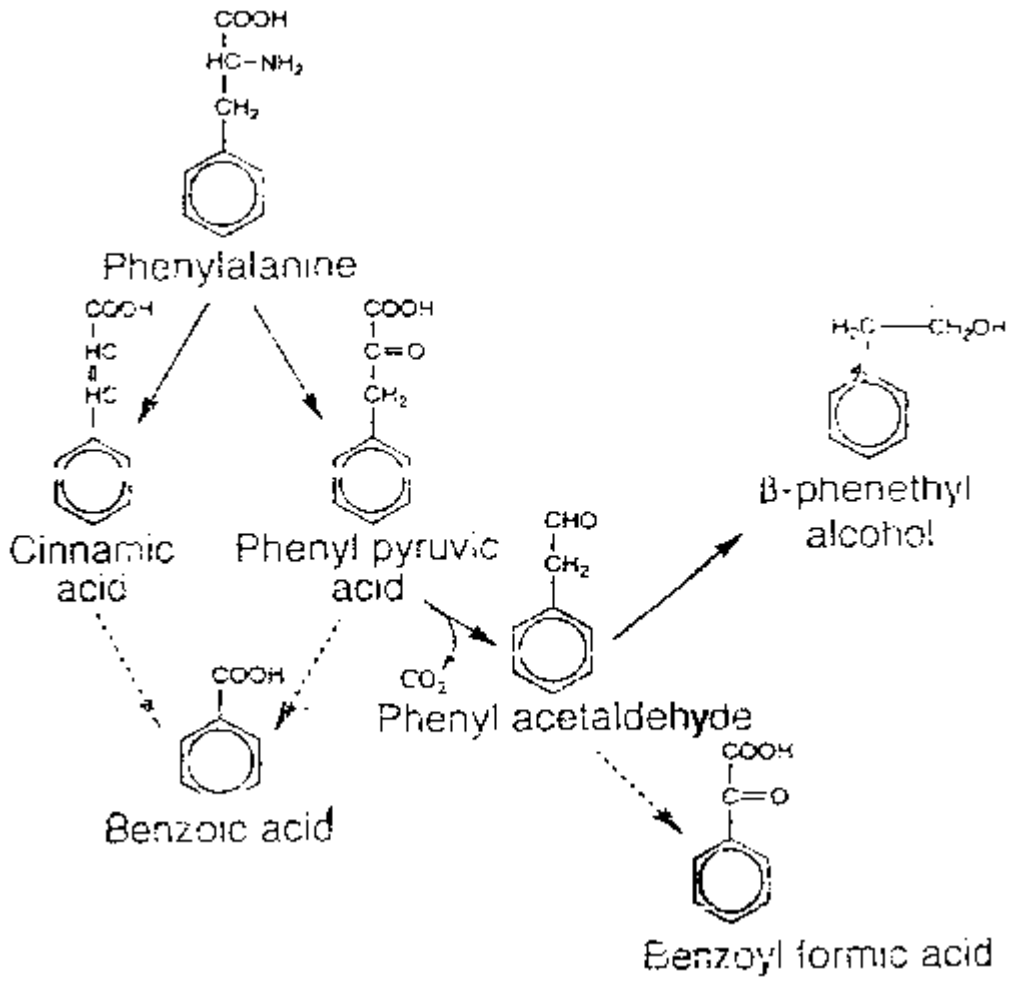


Fig. 2.5 Pathway for the synthesis of β-phenethyl alcohol

Source: (Akita *et al.* 1990; Feron *et al.* 1996)

Glutamate gives foods a savory character often referred to as the *Umami* flavour attribute. In 1957, Ajinomoto commercialized fermentation for the production of glutamic acid using mutant of *Brevibacterium* sp. and *Corynebacterium* sp. Monosodium glutamate is the monosodium salt of L-(+)- glutamic acid (Fig. 2.6b)

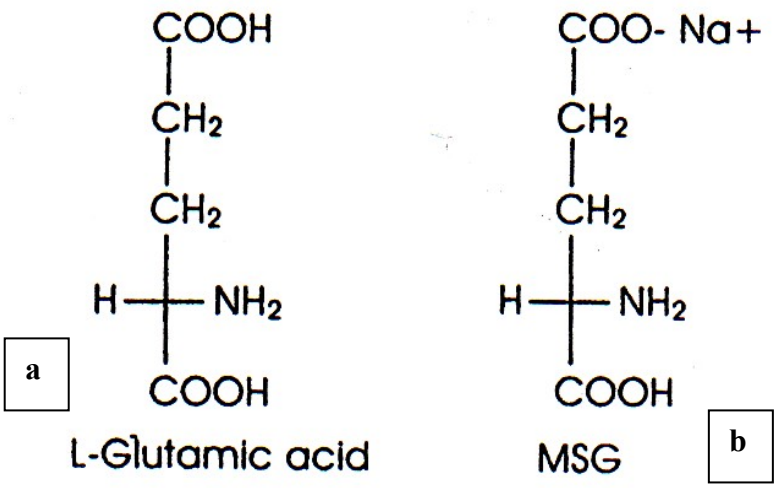


Fig. 2.6: L- glutamic acid (a), Monosodium glutamate (b),

Source: (Matheis 1999)