STRATEGIES FOR 2-HEPTANONE BIOSYNTHESIS FROM OCTANOIC ACID BY *Penicillium roqueforti* SPORES

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ABSTRACT

This paper reviews two strategies of 2-heptanone (blue cheese aroma compound) biosynthesis from octanoic acid by Penicillium roqueforti spores. First, the production and preparation of fungal spores are discussed a long with effect of spores treatment on their biocatalytic activity. Following this the first strategy of 2-heptanone production i.e batch production of 2-heptanone by submerged bioconversion process is discussed. Furthermore 2-heptanone loss by air stream stripping due to its hight volatility is evaluated and then the second strategy, that is continuous biosynthesis in aerated stirred reactor, is addressed. An option for controlling pH of biosynthesis medium is also discussed. Finally, in the concluding paragraphs, utility of the strategies are presented.

Key words: Spore, penicillium roqueforti, 2-heptanone

INTRODUCTION

The consumption of compounds possessing a blue-cheese-like flavor is continuously increasing (Tallu, 1986; Creuly et al., 1992; Molimard et al., 1997). This kind of flavoring allows decrease in the traditional blue cheese ripening time and the manufacture of new cheeses presenting a blue taste without the presence of any fungus (Larroche et al., 1988; Gallois and Langlois, 1990).

Typical flavor of the traditional Blue and Roquefort-type cheeses is derived from the milkfat in the natural cheese during ripening for 90 days or more (Moinas et al., 1973; Berger et al., 1999). The milk fat is hydrolyzed, oxidized and decarboxylated by enzymes produced by spores and mycelium of the fungus *P. roqueforti* to produce methyl ketones (Kinsella and Hwang, 1976; Molimard et al., 1997; Berger et al., 1999), which have aromas characteristic of Blue cheese (Gallois and Langlois, 1990). When the fungus is grown in an aerated liquid culture containing milkfat, the reactions can be completed in days rather than months. In this way, a natural flavor that is suitable for making salad dressing, soups, crackers and cakes or other prepared foods can be produced at a more convenient way (Arpah, 1987; Creuly et al., 1989; Creuly et al., 1990).

The blue cheese aroma is mainly due to the lipolytic activity of the fungus, *P. roqueforti*, during the ripening of the cheese (Dartey and Kinsella, 1971; Kinsella and Hwang, 1976; Berger et al., 1999). This flavoring agents essentially consist of methyl ketones, where 2-heptanone and 2-undecanone compounds are predominant (Molimard et al., 1997). Both mycelium and spores are able to synthesize methyl ketones but spores have a predominant role at the end of the cheese ripening period through the synthesis of flavor compounds from fatty acids (Tallu 1986; Arpah 1987), *P. roqueforti* spores alone has also been reported to carried out the conversion of non milk origine-fatty acids to methyl ketones (Tallu, 1986; Arpah 1987; Larroche and Gros, 1997). This promising reaction is of great practical importance due to the control of subsrate conversion and a lack of mycelium proliferation of the fungus (Larroche et al., 1988).

The production of fungal spores with specific biocatalytic activity has been widely explored recently, it was depelopped from SSF or solid substrate fermentation (Desfarges et al., 1987; Larroche, 1996a) which were mainly for fungal mycelium production (Maheva et al., 1984). The main utility lies in the fact that the biocatalytic activities of the spores are 3 to 10 times to that of mycelium (Maheva et al., 1984; Larroche, 1996a; Larroche et al., 1994), they are generally stable and can be stored or transported as a biochemical catalyst, they give minimal amounts of undesirable products and the lack of mycelium proliferation during biosynthesis may lead to an easier product recovery. (Larroche and Gros, 1989b),

consequently they give great interest as biochemical catalyst for the production of novel organic compounds.

The spores are generally recolted from incubation of fungal starter by SSF (Maheva et al., 1984; Desfarges et al., 1987; Larroche and Gros, 1989b). This method presents several advantages over others techniques such as submerged culture techniques because it gives rise to better yields of homogeneous and pure spores (Larroche and Gros, 1989b), furthermore, the spores may be both served as biocatalyst directly or may be recolted for strain conservation and dissemination which is readily available when required (Arpah, 1987; Larroche, 1996a).

This paper reviews strategies to synthesize 2-heptanone from sodium salt of octanoic acid using *P.roqueforti* spores as biocatalyst, a long with the production and preparation of biocatalyst for bioconversion process, effect of spores treatment on their biocatalytic activities and product loss by air stream stripping are also discussed.

BIOCATALYST PREPARATION AND ENUMERATION

Spores production

The main steps of spores production generally consists of medium (cereal dry seeds) sterilization, inoculation with a suspension of spores starter, incubation and spores extraction (Desfarges et al., 1987).

Almost all starchy cereal may be used as medium or substrate for SSF (Maheva et al., 1984). The substrate choice however has to encounter three main limitations which may occur during a cultivation. They are carbon source limitation, nitrogen source limitation and water content limitation (Desfarges, 1988). The use of several cereal and natural starchy substrates to favor spores production give fairly comparable yields as can be seen in the following table.

Table 1. Production in petri dishes of spores of *P.roqueforti* on different solid starchy substrate after 230 hours of fermentation at 25°C.

Substrate	(Spore/g .DM). 10^8	Substrate	(Spore/g. DM). 10^8
Wheat Bran	18	Triticale	13
Oats	17	Wheat	12
Buckwheat	16	Rice	12
Sorghum	15	Barley	11
Maize	14	Millet	11
Hulled wheat	14	Potato	8
Rye	13		

(Maheva et al., 1984).

Maheva et al., (1984) reported that starchy substrate highly deficient in nitrogen-containing compound, such as potato, naturally give lower yields (Table 1). In such cases mineral salts as ammonium sulfate or potassium phosphate, and urea may be added into the substrate (Raimbault and Alazard, 1981; Larroche, 1996a; Larroche, 1996c).

For ensuring reliable growth of the amylolytic microorganism, starch content of the cereal dry seeds (substrate) must be under gelatinized condition. Therefore, prior to inoculation, dry cereal seeds should be sorted and soaked in water for at least 24 hours (to give final water content of the seeds about 0.5 g H_2O per gram dry matter) followed by steam sterilization for 15 minutes at 120°C (Maheva et al., 1984; Raimbault and Alazard, 1981). Cereal dry seed can also be gelatinized solely by steam sterilization provide that there is enough water available surrounding the starch granules.

Maheva et al., (1984) reported utilization of a fixed bed reactor for pilot scale spores production as shown in Figure 1 that allowed the use of about 100 g dry matter substrate. It is known that the temperature inside a bulk substrate increases as the fermentation proceeds which is directly linked to the thickness of the substrate and the metabolic activity of the microorganism (Larroche and Gros, 1986). Therefore the reactor has to ensure a favorable condition for sporulation of microorganism (Larroche, 1996a). This can be achieved through control and regulation of three main parameter, they are the temperature optimum for sporulation, aeration rate and water content of the substrate (Desfarges et al., 1987). The aeration is need to ensure O_2 supply to the microorganism (for aerobic metabolism) and removal of CO_2, water vapor and any secondary volatile metabolites.

Uniform temperature within the substrate may be obtained by circulating water in the jacket (Maheva et al., 1984), whereas adequate aeration is usually achieved by a continuous supply of air saturated with water and free of carbon dioxide (by passing it through potassium hydroxide solution) to the reactor (Desfarges et al., 1987; Larroche and Gros, 1986; Maheva et al., 1984).

Several variation on SSF technique in others fermentor types and the use of synthetic medium rather than natural starchy substrate have also been reported for *P.roqueforti* spores production (Larroche and Gros, 1989b). Desfarges (1988) showed that the use of drum fermentor and a column fermentor for spores production of *P. roqueforti* in batch and semi continuous cultivation on buckwheat seeds substrate gave a maximum productivity close to 9.2 10^6 external spore/g dry matter substrate per hour (Desfarges, 1988). However, it was also reported that the rotation of the drum fermentor disturbed the growth and the sporulation during cultivation. While Larroche and

Gros (1989b) reported that cultivation on inert porous particle-impregnated and continuously fed with a concentrated liquid substrate and the use of alginate coated seeds as substrate gave lower result than the natural starchy materials. Thus, the natural starchy substrate rich in nitrogen-containing compound and the use of fixed bed column fermentor represent the best way to perform cultivation of the fungus.

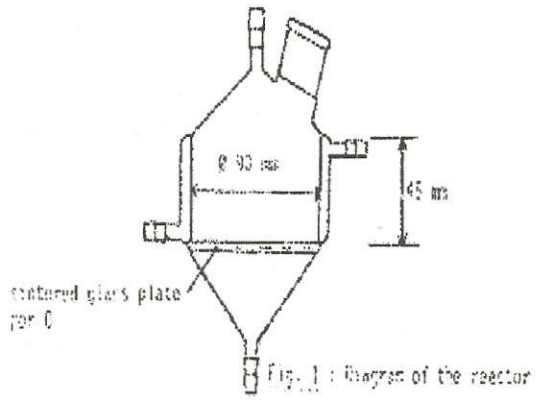

Figure 1. Diagram of the fixed-bed reactor. (Maheva et al., 1984)

The development of the *P. roqueforti* on natural starchy grains proceeds roughly into four steps, involving by both growth and sporulation in the external and the internal part of grains. First, it appears as a spore germination and external colonization of the grains by the mycelium. Then mainly external sporulation and internal colonization of the seeds and finally internal sporulation takes places (Pomeranz, 1983; Desfarges et al., 1987; Larroche and Gros, 1989b).

Biomass production (the growth of microorganism) during the course of fermentation may be estimated by the protein evolution of the medium (Raimbault and Alazard, 1981; Desfarges et al., 1987). Kinetic studies of the development of filamentous fungi on seeds or grains cultivation have been also widely reported (Pomeranz, 1983; Desfarges et al., 1987; Larroche and Gros, 1989b). Desfarges et al., (1987) and Larroche and Gros (1989b) reported that the biomass production of a cultivation on buckwheat seeds of *P. roqueforti*, expressed as protein content of the medium first occur with an active growth phase (approx. μ_{max} =0.03 h^{-1}) followed by a stationary period corresponding to a maximum protein synthesis of environ 35.61 mg g^{-1} DM and later, lysis of the mycelium generally take place after the stationary phase.

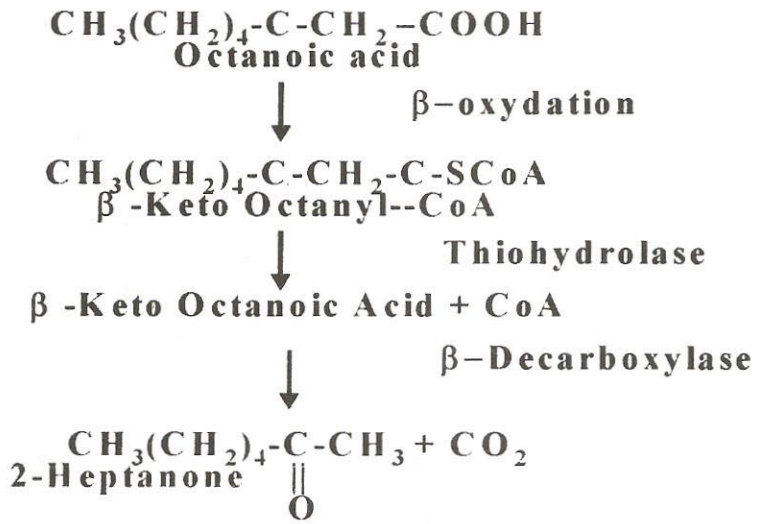

Figure 2. Biosynthesis pathway of 2-heptanone (Arpah, 1987)

Spores Extraction, Enumeration and Immobilization

Colony growth and differentiation of a filamentous fungi on the grains are well understood. First, a young sterile mycelium grows at the periphery of the colony, and then older mycelium which sporulate. These external (of the grains) biomass then lays their spores on the periphery of the grain, whereas internal colonization which proceeds later, produces spores at internal part of the grains (Descartes et al., 1987). It is interesting to mention that the placement of spores during sporulation of *P.roqueforti* in grains are mainly on the outer parts of the grains (Maheva et al., 1984).

Therefore, the external spores of *P.roqueforti* are best recolted by extraction using vigorous agitation of the colonized substrate, but the internal spores can only be obtained after the rupture of substrate matrix and allow suspension of the spores in solution, so homogenization of the sustrate with a blender (Polytron or an Ultra Turrax blender) is necessary to ensure the extraction of internal spores (Desfarges, 1988). A 1% Tween 80 solution is usually used as extraction liquid (Maheva et al., 1984; Desfarges et al., 1987).

Enumeration of the spores on the supernatant usually approached by hematimetric counting under the microscope using a Malassez cell (enlargement 100 to 400). Naturally, this gives the number of external spores in the substrate (Maheva et al., 1984). Total spore can be estimated from addtion of the external spores with the spores obtained after homogenization of the substrate i.e. the internal spores (Desfarges, 1988). A supernatant containing spores (which are generally adjusted to 7 10^6 spores/ml using sterilized water) may then be used as biocatalyst directly or otherwise, preserved. Alternatively, this suspention of spores may be subjected to treatment prior to biosynthesis (Tallu, 1986; Arpah, 1987; Larroche et al., 1988; Creuly et al., 1992) in order to enhance their biocatalytic activity.

Several treatments that have been reported including: preservation at –20°C, equilibration, rehydration and immobilization (Larroche et al., 1989a). Arpah (1987) reported that immobilization of the spores prior to used as biocatalyst prolong usage of the biocatalyst for several days.

BIOSYNTHESIS OF 2-HEPTANONE

During the ripening of blue cheese the milk fat is hydrolyzed by lipase activity of the fungus to free fatty acids (Molimard et al., 1997). These free fatty acids are then enter the β-oxidation pathway where they are oxidized and decarboxylated to produce methyl ketones (Dartey and Kinsella, 1971; Kinsella and Hwang, 1976). A fatty acids with short carbon chain give rise to a methyl ketone with one carbon chain less to their corresponding fatty acid (Smith and Alford., 1969). The use of a long chain fatty acid, however produce several methyl ketones. This has

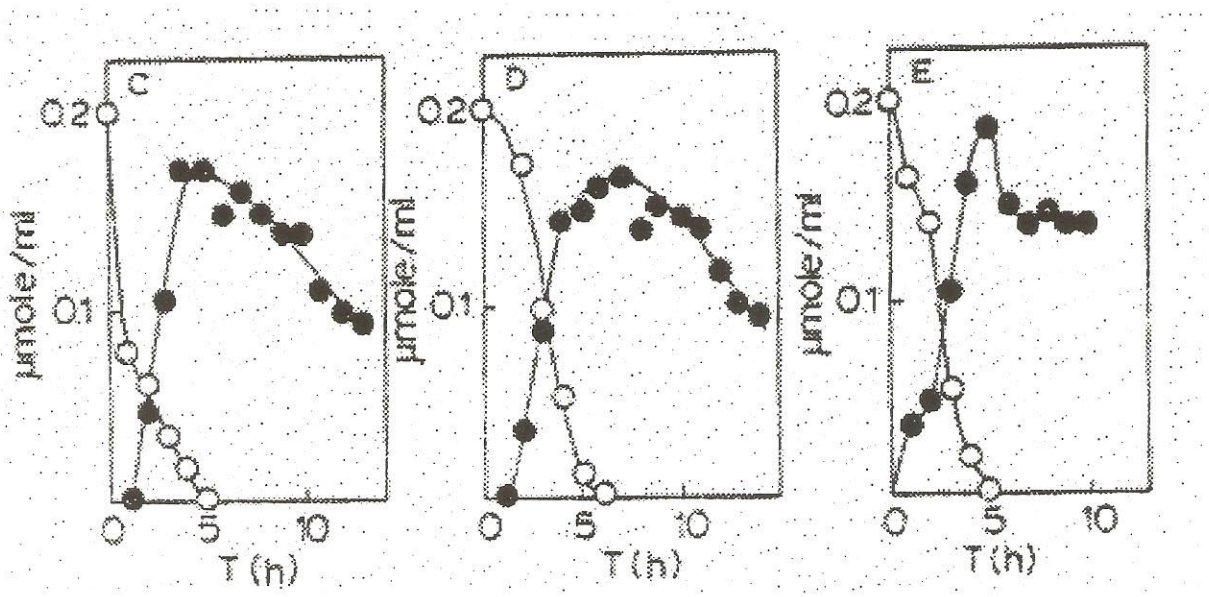

Figure 3. Utilization of sodium octanoate (O) and production of-heptanone (•) during reactions of three strains of *P. roqueforti* spores in erlemeyer flasks (Tallu., 1986).

been suggested as a result of a recycling in β-oxidation (Dartey and Kinsella, 1973). However, the use of octanoic acid as substrate have been reported to give rise exclusively 2-heptanone as the sole methyl ketone (Arpah, 1987; Larroche et al., 1989a).

The production of 2-heptanone can be performed either by batch or continuous production in a submerged bioconversion process (Tallu, 1986; Arpah, 1987; Larroche et al., 1988). The time needed for a complete batch production cycles is 10 h to 14 h, whereas for a continuous production, a duration of as long as 7 days have been reported by maintaining the substrate concentration of the medium at constant level (Arpah, 1987).

Batch production by submerged bioconversion process with phosphate buffer medium

The first strategy of 2-heptanone production comprised, in most places, of a flask cultures placed on rotating water bath filled with medium and biocatalyst.. It deals frequently with the utilization of a reaction volume (Erlemeyer flask), suitable for 150 ml medium, mounted on a rotating water bath for ensuring an additional aeration through agitation during incubation at a constant temperature (Tallu, 1986; Arpah, 1987; Larroche et al., 1988). This batch production of 2-heptanone by submerged bioconversion process may also composed of a Bioflo type reactor with 400 ml working volume associated with either an aerator or with an additional agitator (Arpah, 1987).

The bioconversion medium usually consist of appropriate concentration of substrate i.e octanoic acid-sodium salt solution (environ 5 μmol/ml medium) in a phosphate buffer medium and pH maintained at constant level near the optimum sporulation condition of the fungus (Arpah 1987; Creuly et al., 1992). This medium can be enriched with the addition of activators, such as L-alanine, casamino acid, ethanol or glucose at a concentration level approx. 0.5mg/ml medium respectively or 0.2 μl/ml medium for ethanol (Arpah 1987; Creuly et al., 1992). Larroche et al (1989a) showed that both free spores and immobilized spores performe well in those mediums.

Influence of spores treatments

The course of utilization of sodium octanoate and production of 2–heptanone during reactions by batch submerged bioconversion process is influenced mainly by strains used, storage duration of the spores prior to utilization, medium used for preservation, presence of an activator and in case of immobilized spores is used, the immobilization procedures.

Two effects of these treatments on the course of utilization of sodium octanoate that is commonly reported are prolonged lag phase on the beginning of the process and germination of the spores in the medium. These may both lead to diminuation of biosynthesis rate. However germination of the spores in the medium is more frequently reported on free spores than immobilized spores (Tallu, 1986). But it seems evident from several reports that in all case of treatments the spores usually give a fairly high catalytic activity. Consequently, optimum rate of the reaction (product formed in a given time range) can be achieved by proper spores treatment (Arpah 1987; Creuly et al., 1992). Larroche et al., (1989a) reported that washing the free spores with sterilized water prior to biosynthesis influence their catalytic activity. Non washed spores is the spores suspension which is directly obtained from extraction in solution used (usually a 1% Tween solution), whereas a washed spores is obtained by centrifugating this suspension and rinsing the peilet with steriie water.

Washed spores stored 67 days at –20°C have been also reported to exhibited a loss of activity with respect to the same biocatalyst stored for 40 days (Arpah, 1987). However, their residual activity may be enhanced by activator compounds such as L-alanine, casamino acid or ethanol (Larroche et al., 1989a). But these catalytic activities are remain less important than in the case of young non-washed spores, except for ethanol (Table 2). Arpah (1987) and Larroche et al (1989a) showed that utilization of an old suspension of biocatalyst, stored for 23 days at –20°C, exhibited the strong activator effect of ethanol on "old" free spores in comparison with L-alanine and casamino acid as activator.

The theory and the nature effect of activators on old spores are poorly understood, but it can be related to the enzyme activation hypothesis. Lawrence (1966) and Lawrence and Bailey (1970) postulated an enzyme addition model where permeases allow the substrate to enter the spore through the cell wall. However, details of these mechanisms on a molecular basis is also poorly known.

It may be concluded that good initial conditions of spores for carrying out a biotransformation in a batch submerged bioconversion process are: the use of spores stored for less than 40 days and activated with ethanol (Arpah 1987; Creuly et al., 1992).

The time of exposure of the spores to a liquid medium after thawing and prior to the beginning of the process may enhance biocatalytic activity of the spores (Arpah, 1987). This is due to the hydration of the biocatalyst that lead to permeability changes of the cell wall during the dry and frozen storage state of the spores (Lawrence, 1966; Larroche et al., 1989a). However, Larroche et al. (1989a) reported that only small biocatalytic activity decrease is observed with increasing spores hydration duration and that this phenomenon of permeability

changes was observed for immobilized spores as well as for free spores. The hydration effect and activation by ethanol therefore are additive on enhancing the biocatalytic activity of the spores.

Immobilization of the spores may prolong usage of the biocatalyst during a batch submerged bioconversion process. However unproper immobilization techniques applied can create a diffusion barrier of substrate into the beads (Larroche et al., 1989a). The most common carrier used to immobilized a biocatalyst is Ca-alginate gel, which are hardened, coated or polymerized to enhanced their mechanical properties (Larroche and Gros, 1997). Immobilization of spores for this purpose is best performed by entrapment of the spores in Ca-alginate beads having a diameter of environ 1.5 to 2.5 mm.

Arpah (1987) reported detail of the procedures of this technique using four hardening solutions they are solution of 0.5 M and 0.05 M pure calcium dichloride, a 0.5 % Eudragit RL (acrylate/methacrylate copolymer) emulsion in 0.05 M $CaCl_2$, and a glutaraldehyde solution. Performance comparison of these four entrappement methods during bioconversion process showed (Table 2) that the hardening solutions give different productivity and mechanical stability. According to Larroche et al., (1989a), Eudragit RL (acrylate/methacrylate copolymer) may enhance mechanical properties of alginate beads without altering biocatalytic activity of the spores entrapped inside the beads, and without diffusion barrier for substrate entering those beads. This is supported by Tanaka et al., (1984) by postulating that compounds of molecular weight less than 2×10^4 exhibit the same diffusivity in alginate beads as in pure water. By assuming the molecular weight of octanoic acid is close to 144 and average diameter of alginate beads is equal to 1.5 mm Larroche et al., (1989a) and Grivel et al., (1999a) calculated the effectiveness factor of this reaction diffusion and reported a value equal to 1. This means that there are no substrate barrier entering those beats.

Mechanical stability of gel beads in submerged bioconversion process are highly characterized by their hardening solutions. Arpah (1987) and Creuly et al., (1992) reported that the use of a high calcium chloride concentration (max. 0.5M) as hardening solution lead to a strong turbidity development in the supernatant after 14 h

Table 2. Influence of the treatment of 2% alginate solution on the biotransformation process in the presence of activator in phosphate buffer medium[1)]

Activator	Hardening procedure	Average substrate consumption rate (μmol/ml.h) [a)]	Maximal average ketone productivity (μmol/ml.h)[b)]	Maximal molar yields (%) [c)]	Time of turbidity appearance in supernatant (h)
L-alanine (0.5 ug/ml)	$CaCl_2$ 0.5 M [d)]	---	0.0708	---	14
	$CaCl_2$ 0.05 M	0.339	0.234	68.2	14
	Glutaral-dehyde 1%	0.272	0.194	72.4	24
	$CaCl_2$ 0.05 M + Eudragit 0.5%	0.321	0.240	69.7	22
Ethanol (2 μl/ml)	Free spores	0.339	0.192	74.7	---
	$CaCl_2$ 0.05 M + Eudragit 0.5%	0.349 [e)]	0.197 [e)]	51 [e)]	16 [e)]

Adapted from: Arpah (1987) and Larroche et al., (1989a)

1): spores stored for 23 days at –20°C; initial substrate concentration 5 μmol/ml; reaction volume 150 ml; 2.10^7 spores/ml; T = 27°C; agitation 232 rpm; experiment performed at 250 ml Erlenmeyer flasks; pH = 5.5.

a). Average substrate consumption rate is taken as the ratio of the octanoic acid consumed versus the reaction time. When the substrate is exhausted, it is the ratio of the initial fatty acid concentration versus the time at which it is exhausted.

b). Maximal average ketone productivity is defined according to Wang .et.al (1979).

d). Maximal methyl ketone productivity was not attained at t= 30 h, the value is given at t=30 h.

e). Experiment performed with old spores stored for 60 days at –20 °C.

due to the rupture of beads matrix and rendered a decrease of the catalytic activity of *P. roqueforti* spores. The use of a lower $CaCl_2$ concentration (environ 0.05 M) solution with Glutaraldehyde addition along with the acrylate/ methacrylate copolymer, allow an increase in the beads stability. In this latter case, the catalytic activity of the entrapped spores remained of the same order of magnitude than the activity observed with spores entrapped in alginate treated with 0.05 M $CaCl_2$ solution. Thus the catalytic activity was maintainable while stability of the beads increased.

Tallu (1986) added antibiotic streptomycin and chloramphenicol at concentration 167 μg/ml to ensure sterile conditions and reported that these two antibiotic had no effect upon the biotransformation process.

Best performances on batch production of 2-heptanone by submerged bioconversion process are with immobilized spores entrapped in Ca-alginate beads coated by acrylate/methacrylate copolymer in combination with ethanol as activator (Arpah, 1987; Larroche et al., 1989a). This treatment combination have been reported to give the following results: average substrate consumption rate=0.349 $\mu mol.ml^{-1}.h^{-1}$; maximal average ketone productivity =0.197 $\mu mol.\ ml^{-1}.h^{-1}$; maximal molar yield = 51%. (Arpah, 1987).

The apparent uncorrected molar yields of the biosynthesis by batch submerged bioconversion process (without taking into account the methyl ketone loss by an air stream stripping due to its height volatility) are about 45 % to 85 % depend on the treatments applied. The products loss therefore are around 65% to 15%. Several authors have mentioned that this loss due to metabolism of the methyl ketone after substrate exhaustion. (Tallu, 1986). But considering its height volatility and low solubility in the medium (phsphate buffer) catabolisme of methyl ketone apparently not occur in a submerged bioconversion process, so the loss may solely due to the stripping by air stream (Arpah, 1987). Moinas et al. (1973) observed that during ripening of natural cheeses 2-butanone and 2-pentanone are metabolized, but 2-nonanone is continuously produced whereas 2-heptanone remain at constant concentration.

Comtinuous biosynthesis in aerated stirred reactor with phosphat buffer medium

The second strategy of 2-heptanone biosythesis is continuous biosynthesis in aerated stirred reactor. It deals with extention of the course of reaction at a rationable conversion rate (Arpah, 1987; Larroche et al., 1989a). In this reaction, the volatile loss from the biotransformation medium is a very important parameter which makes product recovery a critical point for the aroma production process. In other to be able to quantify the product loss by air stream stripping, the activity coefficient (γ) of the product has to be assayed prior to experimentation in aerated stirred tank reactor (Arpah, 1987; Fichan, et al., 1999).

The experimental value of the ketone activity coefficient (γ) can be obtained experimentally through the product of $\gamma.P^o$ of the general law for vapor liquid equilibria (equation 1), where P^o is the total pressure of the system, this then may be used to calculate the corrected molar yields of the reactions and better more confirmed further by stoichiometry of the reaction (Arpah. 1987; Fichan, et al., 1999).

Prior to prolong the reaction, time courses of octanoic acid consumption and 2-heptanone production in a short time interval (about 20 h) is also valuable for estimating some kinetic parameters. Time course of a biotransformation reaction process may be separated into four phase, they are a lag phase in the beginning of the process followed by a transient state during which the reaction rate continuously increase, then a pseudo steady-state rate and finally the substrate is exhausted. Tallu (1986) showed by carried out a prduction of 2 heptanone with free-spores in an aerated stirred reactor that during the pseudo steady-state rate, the reaction is of the first order with respect to the methyl ketone with $k = 0.33\ h^{-1}$ as shown in Figure 4.

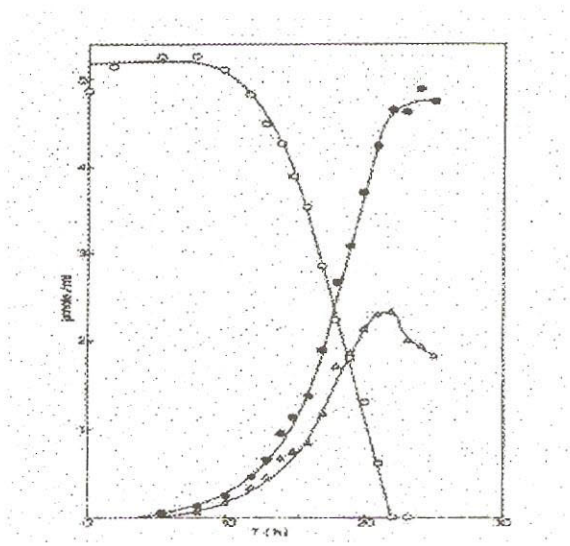

Figure 4. Time course of the reaction process in a stirred aerated tank reactor with unimmobilized spores and phosphat buffer medium, (O) octanoid acid; (Δ) 2 heptanone aqueous phase concentration; (●) total 2-heptanone produce (Arpah, 1987).

Larroche et al., (1989a) mentioned that in this kind of reactions, evolution of substrate and product concentrations exhibit a quite complex behavior, which could not be explained with simple enzymatic kinetic expressions, they include a period over which the reaction rate increased continuously, e.g. permeases of the cell wall, a transient state due to the coupled enzymes system, or a substrate inhibition. Theoretically, in this situation, a continuous production can be performed by maintaining the substrate consumption rate or ketone productivity rate at constant value near the end point of the transient state. This technique can be achieved by adjusting the substrate concentration level in the medium from fluctuation by addition of substrates (Larroche et al., 1989a; Creuly et al., 1992)

Determination of 2-Heptanone Loss by Stripping in Aerated Stirred Reactor

The general law for vapor liquid equilibria is given by the expression (Arpah. 1987; Fichan, et al., 1999):

$$y = (\gamma).(x).(P^o/P) \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where y and x are the molar fraction of the solute in the vapor and the liquid phase, respectively, γ the activity coefficient of the solute (2-heptanone), P^o the vapor pressure at the temperature of the solution and, P the total pressure in the system.

When air is bubbled through an aqueous phase of the solution (which is the case in aerated stirred reactor), the material balance may be written as (Arpah, 1987; Larroche et al., 1988):

$$-G.y = V\,(dC/dt) \quad \ldots\ldots\ldots\ldots\ldots (2)$$

where G is the molar air flow rate; y the molar fraction of the solute in the air stream; V the volume of the aqueous phase and C the solute concentration in the liquid phase. For a dilute solution, the molar fraction of the solution in the liquid phase x may be expressed as C/C_{H2O} and, with V considered as constant, equation (2) is readily integrated to give equation (3):

$$-G.(\gamma).(x).(P^o/P) = V\,(dC/dt) \text{ ..subst. of (1) to (2)}$$

$$\text{because } x = C/C_{H2O}$$

$$-G.(\gamma).(C/C_{H2O}).(P^o/P) = V\,(dC/dt)$$

$$dC/C = (\gamma.\, P^o.G/C_{H2O}.P.V).dt$$

$$\ln C/Co = (\gamma.\, P^o.G/C_{H2O}.P.V).t \quad \ldots\ldots (3)$$

The measurement of the value ln C/Co experimentally, therefore gives the value of (γ. P^o) from the slope of the curve ln C/Co (Figure 5) against t.

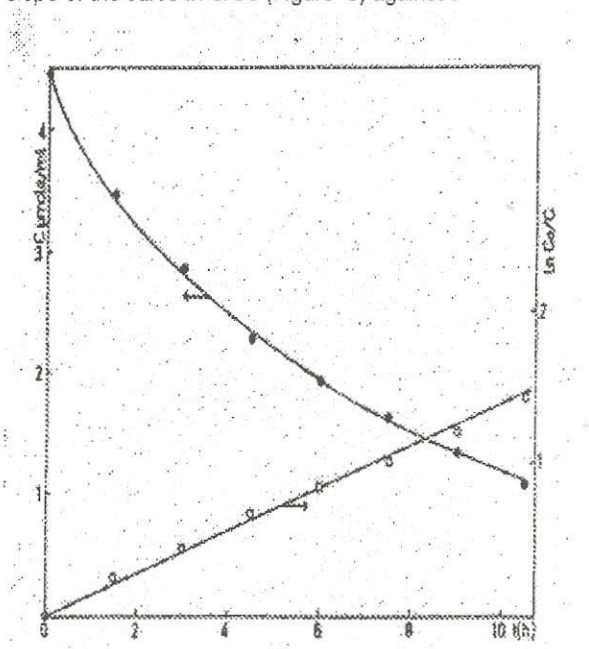

Figure 5. 2-Heptanone loss by stripping by an air stream, (●) residual 2-heptanone concentration in the liquid medium, O = ln C/Co (Arpah, 1987).

Arpah (1987) and Larroche et al., (1989a) carried out an experiment in the aerated stirred reactor filled with phosphate buffer (where the initial methyl ketone concentration Co = 5 μmol/ml) reported the average value for (γ. P^o) was 10.567 ±681 with P^o expressed as mmHg. This very high value makes it possible for 2-heptanone to be very easily stripped by the air flow rate needed for the bioconversion process. Grivel et al., (1999b) reported the same phenomenon for production of β-ionone using *Aspergillus niger*. It is possible that during production of aroma compounds all the product formed are stripped by air, especially when the reaction rate is low while the value of (γ. P^o) is high.

The estimation of total 2-heptanone produced (iincluding the volatile loss from the biotransformation medium) at time t may then be related conveniently to the aqueous phase and expressed as effective solute concentration (Arpah, 1987; Larroche et al., 1988).

$$C_{eff} = C + \otimes \int_0^t ((G.y)/V)\, dt \quad \ldots\ldots\ldots\ldots\ldots (4)$$

Assuming that G.y is constant between two sampling time Δt. Eqn (4) may be rewritten as:

$$C_{eff} = C + \int_{0}^{t} ((G.y)/V)\, dt \quad \text{.....(5)}$$

These equations can be used further to calculate the corrected molar yields of the reactions and productivity. Application of these equations allow to quantify the aroma loss due to air stream stripping during biosynthesis. By applying the equations, Arpah (1987) and Larroche et al. (1989a) reported that the molar yields of 2 heptanone bioconversion from octanoid acid by *P. roqueforti* is indeed equal to 100%. It was also already mention on the previous part that several earlier authors suggested that these losses are due to metabolism of 2-heptanone by the spores after substrate exhaustion.

Stoichiometry of the biosynthesis

Stoichiometry of 2-heptanone biosynthesis (Figure 2) is well understood, but technical informations on the verification of this biosynthesis, especially in case of utilization of spores as biocatalyst are seldom reported. Theoretically, there are other experimentations that may be carried out in order to verify whether this loss is solely by air stream stripping or by spores metabolism activity e.g. stoichiometry evaluation of the biosynthesis based on respiratory activity (Larroche et al., 1989a).

The respiratory activity of *P. roqueforti* spores during methyl ketone formation from fatty acids were mainly based on oxygen consumption (Lawrence, 1966; Lawrence and Bailey, 1970). But the point is that several authors have observed and reported an excess on oxygen consumption with comparison to the stoichiometric quantity needed for biotransformation process (Larroche et al., 1989a). This excess being related to endogenous metabolism (Tallu, 1986). Arpah (1987) showed an analogous feature occurred for carbon dioxide evolution when a biotransformation using alginate/Eudragit-entrapped spores were performed in aerated stirred reactor with phosphate buffer medium.

Larroche (1988) postulated that this CO_2 production (the excess) may be linked to endogenous metabolism, which may be considered constant throughout the reaction when there were no germination activity of the spores occurred during the processes. Consequently by determining both the CO_2 formed from substrate synthesis and the CO_2 from endogeneous activity it is possible to verify the metabolism of 2-heptanone before and after substrate exhaustion using the stoichiometry of the biosynthesis:

$$CH_3\text{-}(CH_2)_6\text{-}COOH + \tfrac{1}{2}\, O_2 \rightarrow CH_3\text{—}(CH_2)_4\text{—}C(=O)\text{—}CH_3 + CO_2$$

By substracting the total CO_2 formed by the CO_2 from endogeneous activity, Arpah (1987) and Larroche et al. (1988) were able to confirm that the true yield of the biosynthesis is 100% and that 2–heptanone is the sole methyl ketone synthesized from octanoic acids. The curves of the experiment designed for verification of the stoichiometry of this biosynthesis is shown in Figure 6, indicated that the total CO_2 production continue after the substrate is exhausted at a constant rate equal to 0.208 $\mu mol.\ ml^{-1}.h^{-1}$. This CO_2 production (after the substrates were exhausted) was due to endogeneous activity. The curves also indicated (Figure 6) that the curve of the "true" CO_2-evolved linked to the biosynthesis and the the curve of substrate consumed are superpose perfectly. Thus verified that 2–heptanone is the sole methyl ketone synthesized from octanoic acids and emphasizes the important of volatile (2-heptanone) loss due to stripping by the air stream required for the reaction.

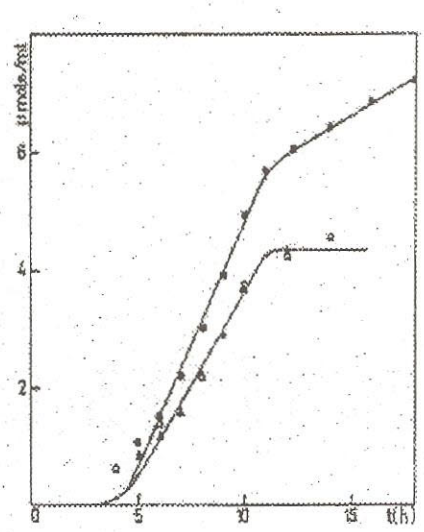

Figure 6. Time course of the total CO_2 evolution (●), CO_2 related to the reaction (Δ) and substrate consumed (O). during a biotransformation using alginate/Eudragit-entrapped spores performed in aerated stirred reactor with phosphate buffer medium (Arpah, 1987).

Continuous biosynthesis in a non-buffered medium

It is evident that the mechanical agitation of the stirred reactor, suitable for continuous process, give higher shear stress than those observed in the Erlemeyer flasks mounted on water bath shaker used for batch production of 2-heptanone by submerged bioconversion process (Larroche and Gros, 1997). Consequently, this (mechanical agitation) leads to more alginate beads damage in the reactor, making them unusable for prolonged or continuous

process. Larroche et al., (1989a) mentioned that at the end of the reaction performed in stirred aerated reactor with phosphate buffer medium, the alginate beads were completely disrupted, rendering increasement of turbidity in the medium. Larroche et al., (1989a) explained further that phosphate buffer aggrave disintegration of the alginate beads.

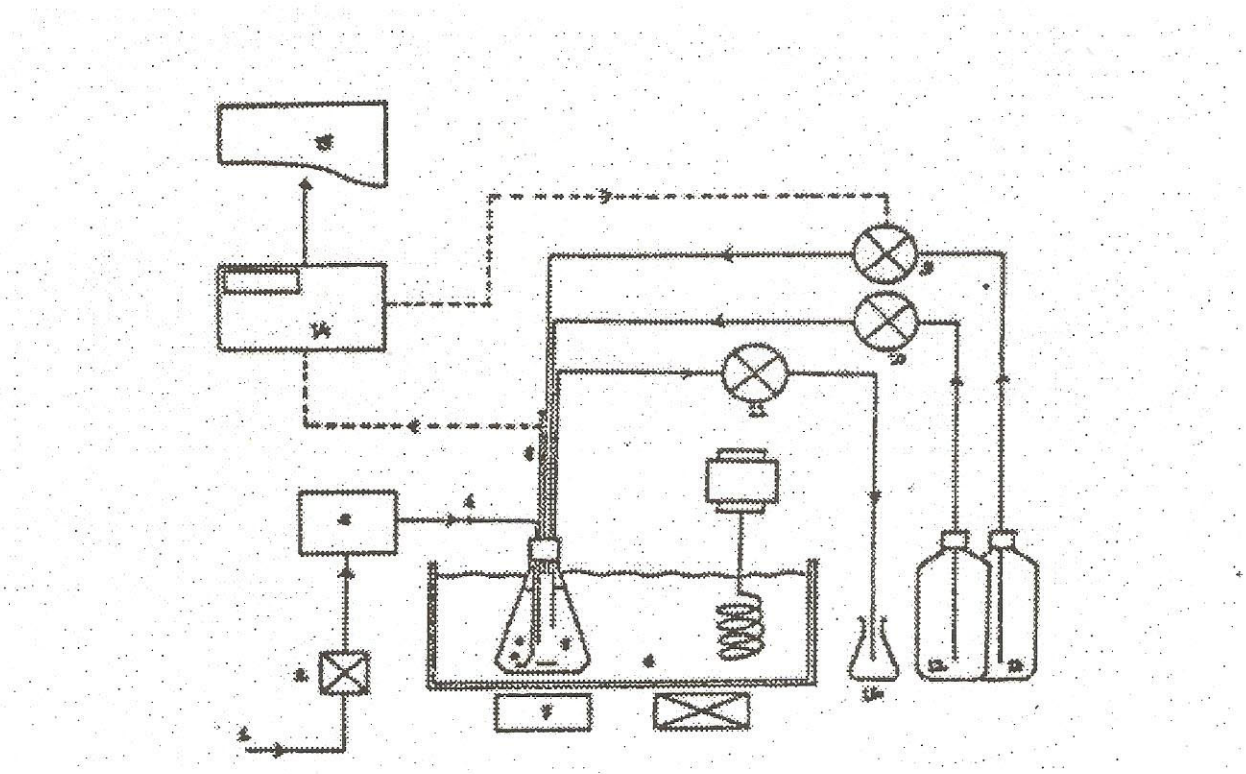

Figure 7. The "Tailor made" stirred aerated reactor designed for reaction purpose in the non-buffered medium (Arpah, 1987).

1. air stream entering valve
2. glass flow regulator
3. air flow meter
4. air filter
5. reaction medium (wide-neck, 500 ml Erlemeyer flask)
6. thermostated waterbath
7. magnetic stirrer
8. pH electrode

9, 10, 11. peristaltic pumps

12. octanoic acid solution
13. chlorohydric acid solution
14. pH recorder
15. pH print-out
16. effluent receiver

Methods depelopped to over come this kind of problem during biosynthesis that have been reported, including for example: maintaining the pH at 5.5 without the presence of phosphate buffer By using HCl solution addition in a non-buffered medium to maintain the pH at 5.5 (pH optium for the spores), Arpah (1987) reported that a better stability of the beads in the medium could be achieved. Furthermore this kind of reaction medium gave no effect to biocatalytic activity of the spores, provided that the time course of substrate and product evolution in non-buffered medium (pH maintained at constant value 5.5 by HCl addition) is similar when the medium is buffered (Arpah, 1987; Creuly et al., 1992). The advantage, of course, is that there are no noticeable bead breakdown is seen at the end of the biotransformation reaction carried and prolonged to 150 h, necessary for a continuous type production regime (Arpah, 1987). However commercially available aerated stirred reactors are not always suitable to fit this experiment because the lack of entrance pumps required for carrying the entering and discarding liquid.

Equally suitable, however, is with a "tailor made" aerated stirred reactor designed to perform those continuous reactions. But several considerations have to be taken into account such as the pH of the non-buffered medium should be able to be maintained at constant level and monitored by an pH-electrode, for example by continues addition of chlorohydric acid while recording the pH value at the same time. The volume of the reaction should also be made constant by controlling the effluents at the same magnitude as the entering liquids. A design adapted from Arpah (1987), is shown in Figure 7, which allows the addition of chlorohydric acid solution, thus maintain the pH at constant level, and allow the volume of the reaction to be assume constant throughout the biotransformation, thus permits a monitoring of substrate concentration in aqueous phase continuously.

The substrate concentration may be calculated according to the relation (6).

$$S = So - (N_i'V_1''/\alpha V_2) \quad \ldots\ldots\ldots\ldots\ldots (6)$$

Where: S is the residual substrate concentration at time t; So, the initial substrate concentration; N_i', the normality of the added HCl solution; V_1'', the volume of HCl added during the course of the reaction; V_2, the reaction medium volume.

Equation (6) can be derived based on the dissociation fraction of octanoic acid (α) in the medium after equilibrium is established, and the materials balance during the reaction. The material balance equations developed consist of total material balance, material balance of the substrate, material balance of 2-heptanone and material balance of chlorohydric acid. (the equations are not shown).

Larroche et al., (1989a) compared the two methods of substrate determination,.i.e, analytical method and calculation using equation (6), and reported that they were in good agreement. Thus, the latter method is simple and allows continues monitoring of the reaction process. Moreover, by printing out the pH value of the electrode permits an early intervention on the disturbances, that may come out from the peristaltic pumps debit. These pumps govern the entering and the discarding liquid, thus maintain the volume of the reaction which is considered constant. It may be automated by a regulator which sends an input for "on" and "off" according to the critical pH value and the critical volume of the reaction medium (Arpah, 1987).

Finally. an organic solvent may also be incorporated in to the medium, which extracted 2-heptanone continuously. However, cautions should be taken in choosing the solvent for not to decrease the catalytic activity of the spores (Creuly et al., 1992).

CONCLUSION

Both strategies seem to give results about 100 % molar yields. The first strategy is easier to perform and best fit for a short reaction purpose including, examination of the strains performances, effects of an activator or utilization of new synthetic mediums. The second strategy permits the utilization of non buffered medium, allowing a prolong usage of immobilized biocatalysts. Furthermore, it permits the extraction of 2-heptanone continuously by presence of an organic phase in the medium.

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