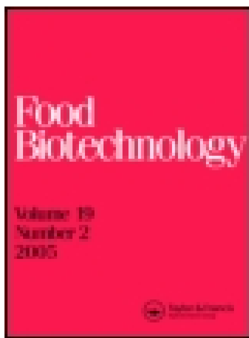

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ENZYMES AND FOOD FLAVOR - A REVIEW

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ABSTRACT

The use of enzymes in flavor generation in food technology is reviewed. In the first part, important products derived from natural macromolecules present in foods such as fats, proteins, nucleic acids and flavor precursors are discussed in terms of the enzymes involved in the reactions and the relation of the products with flavor. Enzymes that are used to eliminate natural or process induced off-flavors are also discussed. In the second part, the use of enzymes for the direct synthesis of flavoring compounds is presented.

INTRODUCTION

Flavor compounds synthesis by biotechnological processes plays nowadays an increasing role in the food industry. This is the result, among other things, of scientific advances in biological processes, making use of microorganisms or enzymes as an alternative to chemical synthesis, combined with recent developments in analytical techniques such as HPLC, GC, IR or mass spectrometry (Knorr 1987). This can be evidenced by the great number of reviews related to flavor published in the last twelve years covering a broad area: Schindler and Schmid (1982), Kempler (1983), Sharpell (1985), Gatfield (1986, 1988), Crouzet (1989), Welsh *et al.*(1989), Herráiz (1990), Cheetham (1991, 1993), Janssens *et al.*(1992), Gutierrez and Revah (1993); or concerning specific topics: lipases characteristics (Borgström and Brockman 1984) and their industrial applications (Macrae 1983,West 1988), enzymes involved in the cheese flavor biosynthesis (Kinsella and Hwang 1976, Law 1984, Seitz 1990), enzymes affecting the flavor of citrus products (Bruemmer *et al.* 1977) or tea (Jain and Takeo 1984), enzymatic aroma genesis in food (Schwimmer 1981) or biocatalysts in the natural generation of flavor (Schreier 1985) to give only a few examples. There is still more potential for this area in biotechnology since liquid cultures of plant cells may also be used as a technique to

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synthesize a wide array of chemicals (Whitaker and Evans 1987, Knorr *et al.* 1990). Research in this area for new products and bio-processes is also enhanced by a growing market and an increasing public concern for the total wholesomeness and chemical safety of food ingredients (Basset,1990). From a total world market of 6 billion dollars for the flavor and fragrance industry in 1990, food flavors account for 25%, with about 5% annual growth rate (Cheetham 1991). Flavor sales were estimated to be about US $ 675 millions in 1991 and are expected to reach US $ 376 millions in the European Community (Cheetham 1993). Most of the major companies producing aromas are carrying out research programs for developing the biotechnological production of such compounds (Dziezak 1986a). It must be also pointed out that a specific policy concerning labelling of processed foods containing natural compounds has been issued in several countries (e.g. in the EC with the directive published in July 1988 in the Official Journal of the European Community, Spinnler 1989).

On the other hand, the food industry has been strongly influenced by the increasing public awareness of the nutritional characteristics of their diet and, in particular, of the additives used in the food industry. This is shown, not only by the high number of industrial products low in fat, sodium, caffeine or cholesterol, but also in the displacement of saccharine by aspartame and the search for natural colorants or alternative antioxidants and preservatives.

Enzymes as biocatalysts offer a wide variety of possibilities for food flavor production: their specificity, whether applied via whole-cell or cell-free systems enable the production of certain chemicals difficult to synthesize; their stereoselectivity is an important advantage for the food industry where a specific optical conformation may be associated to flavor properties. Enzymes may also be used directly as food additives, not only to produce or liberate flavor from precursors, but also to correct off-flavors caused by specific compounds, naturally occurring or produced during processing (Bigelis,1992). Whitaker (1990) presents in a broad review on the prospective of enzymes in food technology in general. Enzymes involved in flavors may also be endogenous, inherent to the food or may come from microbial sources, added intentionally to foods or coming from contamination. In Table 1, the main objectives for the use of enzymes in flavor technology are presented.

In this article, different ways in which enzymes are related to flavor are reviewed, presenting examples of actual research in this area as well as potential applications. In some cases, processes and reactions known for two decades are mentioned and updated with recent advances such as whole-cell biocatalysts or reaction in organic media.

ENZYMATIC MODIFICATIONS OF MACROMOLECULES

Almost all macromolecules present in foods have an impact on flavor when hydrolyzed. There is a wide variety of enzymes available for the hydrolysis of proteins, starch and other carbohydrates, fats and nucleic acids (Shahani *et al.* 1976), so there are enormous alternatives for their transformation or for the development of processes having as a main objective the production of flavoring compounds.

1. Fats.

There are many examples in the food industry where the main flavor properties are derived from fat. As proof of the interest of scientists and companies in the application of lipolytic enzymes

TABLE 1 - Enzyme technology related to food flavor.
. Additives to enhance or produce flavor from precursors.
. Biocatalysts in processes for flavor production.
. Additives in flavor extraction processes from natural raw materials.
. Activation of endogenous enzymes to induce reactions leading to flavor production.
. Inactivation of endogenous enzymes to avoid off-flavor generation.
. Use of enzymes for the elimination of off-flavors.

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as a tool to improve cheese manufacture, one can refer to the reviews of Arnold *et al.*(1975), Kilara (1985a) and Dziezak (1986b). The basic and first step of the process is the lipase-catalyzed hydrolysis of glycerides. In some cases, the free fatty acids released are converted to the flavoring compounds by microorganisms (e.g. in the case of cheeses). Fatty acid profiles required for particular flavors are obtained with various lipases: short chain fatty acids (C4) will develop a sharp, tangy flavor tending towards rancid notes, while intermediate chain fatty acids (like C12) are associated with a soapy flavor (Nelson 1972). This is a problem for example in the piña colada beverage were the C12 fatty acid of the coconut is released by the thermostable lipase of the pineapple producing a strong soapy off-flavor in the canned beverage (Heath and Reineccius 1986). On the other hand, largest fatty acids (up to C12) are known to do not make a significant contibution to flavor.

A balance between these extremes with only small amounts of intermediate length fatty acids (C6 - C10) is important in fat flavor development: pancreatic lipases are adequate for short chain fatty acids, *Aspergillus* and *Candida spp.* for a wide range of sizes and *Penicillium roqueforti* for butyric acid release (Godfrey and Hawkins 1991). *A. oryzae* liberates C6 - C10 fatty acids, characteristic of Cheddar, so that the ripening process might be reduced from several months to only 12-72 hours. Butyric and propionic acids are characteristic of Romano, Provolone and Swiss cheese, respectively and while the former is released by lipase action, the latter is produced by *Propionibacterium* fermentation.

The rationalization of this traditional microbiological process has resulted in the development of the Enzyme Modified Cheeses (EMC), obtained by direct addition of enzymes to fresh cheese. As an application of this process, Torres and Chandan (1981) used lactic culture, yogurt culture and lipase preparations to modify flavor, texture and to reduce the ripening time of the Latin American white cheese. Revah and Lebeault (1989), working in the manufacture of blue cheese, demonstrated that controlled lipolysis results in a concentrated product, 20-30 times stronger in flavor than the mature cheese. The accelerated ripening process is currently practiced with pancreatic and microbial lipases as well as proteinases (Rabie 1989). Another application of this process has been proposed by Pannel and Olson (1991a,b), for the production of methyl ketones, major components of blue cheese flavor. In this case they used pancreatic lipase and spores of *Penicillium roqueforti* in milk-fat coated microcapsules. They reported the production of 2-pentanone, 2-heptanone, 2-nonanone and hexanoic, octanoic and decanoic acids at the level of 12 mg/g capsules in 17 days. In this process it is assumed that the spores convert the lipase-hydrolyzed fatty acids to ketones, the critical pathway being

the liberation of free fatty acids. Cheddar cheese has also been studied (Arbige *et al.* 1986): after isolating a very active lipase from *Aspergillus oryzae*, these authors carried out the accelerated ageing of the cheese by adding a combination of a lipase and a protease, obtaining a balance between flavor development and body breakdown in a reduced time. El-Soda *et al.* (1992), by using freeze-shocked mutant strains of *Lactobacillus casei* as a source of lipase, could shorten the ripening time of the Egyptian Ras cheese, minimizing the development of bitterness. As another example, Davide and Foley (1981), tried to improve sensorial properties (appearence, flavor and texture) of Cheddar type cheese with coconut oil as a substitute for milk fat by adding commercial lipase preparations. Experiments showed it was not feasible. As a conclusion, full flavored cheeses of different types may be obtained, with a specific fatty acid profile, depending on the enzyme used (Godfrey and Hawkins 1991, Kim Ha and Lindsay 1993).

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Lipases have also been used for the modification of animal fats and tallows. As an example, the production of aromas from raw material such as butter has been proposed (Seitz 1990). In 1984, a process for producing an aroma rich fat phase from butter was patented: a mixture of *P. roqueforti* cells and pancreatic lipase was used on a pilot scale (Kunz *et al.* 1984). Another process for this purpose employed extra and intra cellular enzymes of *Lactobacillus plantarum* (Reimerdes 1984). Both authors claim that the product can be added to cheeses, sausages or meat products. Chen and Pai (1991) applied this process to the hydrolysis of milk fat in reversed micelles stabilized by lecithin. They optimized this technique for parameters such as temperature, pH and molar ratio of water to surfactant and found that enzyme activity could be improved with increasing enzyme and surfactant concentrations. Garcia *et al.* (1991) carried out a selective lipolysis of glycerides from butteroil with an *Aspergillus niger* lipase in order to obtain a pleasant flavor enhancement (releasing preferably butyric acid). Luck and Hagg (1991) discussed the influence of parameters such as pH, enzyme concentration and temperature on the kinetics of lipolysis in an enzymatic/microbiological process for the production of cheese flavor from a butter emulsion. Finally, it is interesting to point out that fungal lipase produced in solid state fermentation displayed 3.3 times greater activity than in submerged culture and could be applied to hydrolyze olive oil or for a simple control of flavor profile of lipolyzed milk (Chen and McGill 1992).

Although lipases and cheeses are the most common enzymes and substrates, respectively, in relation to enzymatic flavor production from fats, there are other examples: active soya flour is added as a source of lipoxygenase - acting by hyper oxidation of linoleic acid and other polyunsatured lipids - to bleach and to improve the volatile composition of bread. It has been found that the concentrations of hexanal, hexanol, 1-penten-3 -ol, 1-pentanol and 2-heptanone are increased upon the addition of soya flour (Luning *et al.* 1991, Addo *et al.* 1993). Fatty acids are oxidized for the production of "green" flavor components, the so-called "leaf aldehydes" and "leaf alcohols", which may be obtained enzymatically through lipoxygenase and hydroperoxide lyase. However, this area is still far from practical applications due to the lack of availability of these enzymes (Gatfield 1988).

Josephson and Lindsay (1986) reported that lipoxygenase could be employed successfully in the generation of fresh fish aroma, liberating alcohols and carbonyls from polyunsatured fatty acids. They also pointed out that plant-derived lipoxygenases may be potentially used to restore this aroma in fish.

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2. Proteins.

The development of soy sauce fermentation, centuries ago, is probably one of the first processes where traditional biotechnology had a stronger impact on flavor than in preservation. More recent developments of protein hydrolysates from vegetals, soy bean, wheat or yeasts are specifically related to the production of flavor and flavor enhancers (Kilara 1985b). Although the most important commercial product - yeast extract - is produced by autolysis, which involves the activation of degradative enzymes inherently present in the yeast (Dziezak 1987, Mermelstein 1989, Nagodawithana 1992), when proteases like papain are used, glutamic acid may be obtained as a free amino acid : its perception in food is the main factor influencing flavor. Cysteine is also important in the development of meat flavor due to its participation in the Maillard reaction (Tyrrell 1990, Grosch and Zeiler-Hilgart 1992); methionine, leucine and isoleucine (in that order) are the next most reactive (Weir 1986). Different proteases have been proposed for the production of flavorings from protein hydrolysates: an immobilized protease from *Penicillium duponti* for the hydrolysis of soy protein; pronase for casein hydrolysis; pepsin and renin for pea protein and reconstituted skimmed milk respectively; pepsin for cotton seed and a variety of proteases for the protein from faba bean, as reviewed by Weir (1986).

After proteolysis it is possible to further enhance the flavor by treatment with a glutaminase (Yasuyuki *et al.* 1989). The flavor enhancing glutaminase enzyme increased the level of glutamic acid by a factor of 2.6 in a mash of *Koji* wheat treated with *Bacillus subtilis* and *Aspergillus oryzae* proteases.

A succesful approach in this context is that of "cascade hydrolysis". It consists of two or three successive enzymatic hydrolysis steps starting from an alkaline protease allowing the pH to fall or maintaining it constant. The final steps may include peptidases to hydrolyze fragments that will otherwise give bitterness to the product. Acid hydrolysis results in products such as mono and dichloro compounds that have recently given rise to concern (Godfrey 1990).

Peptidases and proteases may also be used in cheese making processes (Kilara and Iya 1985). Muir *et al.*(1992), reported that enhancement of the level of degradative enzymes in reduced-fat cheese, by addition to curd of an attenuated starter culture rich in peptidase and protease, resulted in significant improvements in both intensity of Cheddar flavor and in the mouth-coating character. In a review, Fernandez-García (1986) discussed the new trends in the accelerating process of cheese ripening. It appears that not only lipases may be helpful, but also β-galactosidase and proteases. If the use of a lactase preparation displayed an improvement in sensorial properties of cheese, proteases must be employed carefully because of the possible induction of bitter taste due to the release of hydrophobic residues (Fernandez-García *et al.* 1988). This research group also demonstrated that neutral bacterial protease could highly increase the amount of non-protein nitrogen in two kinds of Spanish cheese and therefore contribute to the acceleration of the ripening of such cheeses (Fernandez-García *et al.* 1990, 1993). It has also been reported that bacterial methioninase may be helpful in the generation of aroma when added to unripened Cheddar cheese by promoting the transformation of sulphur containing amino acids into methanethiol, one of the constituents of the typical aroma of that cheese (Lindsay and Rippe 1986).

Also, during fermentation, specific enzyme activities are important in flavor generation. In a recent publication, the importance of the combined protease and esterase activities of wine yeasts on aroma compound formation was studied (Rosi *et al.* 1989). It is probable that the advances in research concerning the specific activities involved in aroma production during microbial transformations will have an impact on traditional processes. This is already the case in the dairy industry and may also include in the near future other fermented products such as wine and beer.

3. Nucleic acids.

Besides glutamate, the most widely used flavor enhancers are 5'-ribonucleotides (5'-GMP and 5'-IMP). They can be obtained by fermentation or by nuclease- mediated hydrolysis from RNA (Nakao 1979). The nucleotides may be obtained by direct addition of the enzymes to the food or in enzyme reactors using soluble or immobilized enzymes (Benaiges *et al.* 1990, Cho and Lung 1991, Olmedo *et al.* 1993).The enzymatic process utilizes 5'-phosphodiesterase produced from *Penicillium citrinum*. It is important to use an enzyme preparation free of contaminating nuclease activities. 5'-AMP is obtained in a second enzymatic conversion from 5' AMP with adenylic deaminase. It has also been reported that a mixture of IMP and monosodium glutamate could provide a strong "meaty" taste and develop a sense of greater smoothness, body and viscosity (Bigelis 1992).

4. Hydrolysis of precursors.

As early as 1956, Hewitt reported that flavor lost by food processing could be restored by addition of an appropriate enzyme preparation (Crouzet 1977). The hypothesis was that flavor precursors, glucosinolates in that case, were still present in the food after processing, while flavor had been lost and enzymes deactivated. Upon addition of fresh enzymes from the fresh product or from a part of the plant of the processed food, the reaction giving rise to flavor compounds could be restored. This was the case with watercress and later with mustard, cabbage, string bean, onion and raspberry (Gatfield 1988).

Sulfur volatile compounds are produced by alliaceous (onion, garlic, leek,...) and cruciferous (cabbage, mustard, broccoli, horseradish,...) plants by direct action of enzymes. With the former, enzymes known as alliinases produce disulfides and related volatile substances from derivatives of cysteine (S-allyl and S-propyl cysteine sulfoxide for garlic and onion, respectively) while in the latter, thioglucosidases like sinigrinase, sinalbinase, myrosinase produce isothiocyanates, responsible of the pungent taste of these plants. In both cases, enzymes and substrates are compartmentalized, so when the tissues are disrupted, enzyme and substrate generate the flavor compound. Using this well known mechanism, horseradish aroma has been produced with myrosinase immobilized in a plug-flow reactor (Gatfield *et al.* 1983). Hanley *et al.* (1990) applied this enzyme to the hydrolysis of glucosinolates in a low water system, finding out that reverse micelles process was more efficient than aqueous buffer solution. Some of these products are toxic: although a wide variety of glucosinolates are present in cruciferous seeds, the principal one in rapeseed and crambe is progoitrin which yields goitrin, a growth depressor, after hydrolysis with myrosinase (Liener 1987).

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Hundreds of compounds from biosynthetic pathways have been identified in flavors. Some of these compounds are derived from the direct action of enzymes precursor-transforming and often play a significant role in defining the aroma. In a general model, a glycosidase is used to hydrolyze the precursor of the type carbohydrate-aglycone. For this reason, enzymes are now included in the aroma extraction techniques (Simultaneous Enzyme Catalysis Extraction: SECE) (Schwab and Schreier 1988). In Table 2, a survey concerning the kind of flavor products obtained by enzymatic extraction is presented.

During maturation, odorous monoterpenes are converted to glycosyl derivatives such as O-α-L rhamnopyranosyl-β-D-glucopyranosides and O-α-L arabinofuranosyl-β-D-glucopyranosides. In most cases, these glycosides accumulate in the fruit and can be found at a concentration greater than the free aroma constituents. However, the enzymatic treatment must be short and specific, otherwise the changes in aroma may result from side incubation effects, as endogenous enzymes like esterases, lipoxygenases, aldehyde-lyase or alcohol dehydrogenase which are active for a long period of time, as demonstrated in apple juice by Jenniskens *et al.*(1991).

For many fruits, there are now enzymatic processes proposed for an efficient release of aroma compounds. Vasserot *et al.*(1989), studied the properties of a β-glucosidase from a *Hanseniaspora* strain. They showed that this enzyme, due to its lack of substrate specificity, was highly interesting for fruit aroma liberation, especially for bound terpenols. Its activity is inhibited by glucose but activated by ethanol. Side activities of commercial enzymatic products are also used for this purpose. This is the case of arabinopyranosidase activities present in hemicellulase preparations. In a systematic study carried out by Cordonnier *et al.*(1989), in order to test the ability of 34 commercial preparations to liberate aroma from glycosidic precursors, 5 enzymes were selected due to their β-D glucopyranosidase, α-L rhamnopyranosidase and α-L arabinofuranosidase activities. These enzymes were able to liberate linalool and geraniol. It is clear that in the near future, commercial enzyme preparations will be developed for specific fruits to liberate specific aroma compounds. However, there are still some limitations to overcome, such as inhibition by glucose or by ethanol, the pH-activity profile and the thermostability.

Another interesting feature is that many precursors are also found in other parts of the plant (leaves, skin, etc.) so it might be possible to produce natural aroma from agro-industrial residues. An example of this trend is the hydrolysis of picrocrocin, the glucoside precursor of safranal, the essential volatile characteristic of the saffron aroma and odor, by β-glucosidase (Iborra *et al.* 1992). The same type of process may be possible in the near future for the production of vanilla, by treatment of the precursor, the glucovanilline present in the pod with a β-glucosidase. Pouget *et al.*(1990) showed the feasability of the process at a laboratory scale submitting vanilla pod residues to the action of *Aspergillus niger* hydrolases. This process has been developed at a pre-industrial scale (Therre 1990).

Using sensory analysis, Abbott *et al.*(1991) found that glycosidic hydrolysates contain aroma compounds that are very important in high quality Shiraz wine. For this purpose, they hydrolyzed the volatile components derived from isolates of wine and juice of Shiraz grapes, with a non specific glycosidase. By sensorial analysis, the hydrolyzed samples were found to contribute to notes such as

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TABLE 2 - Examples of aglycons involved in the flavor of fruits liberated by enzymatic treatment.

Fruit	Enzymes	Bound volatiles	Authors
Papaya	emulsin*	benzaldehyde benzyl alcohol 2- phenylethanol.	Schwab *et al.* 1989
	acid phosphatase	linalool 2,6-dimethyloct-7ene-2,3,6-triol	
Apple	emulsin*	C13 norisoprenoids	Schwab & Schreier 1988
Various fruits	endo-glucosidase tannase anthocyanase (from *A.niger*)	linalool	Shoseyov *et al.* 1988
Raspberry	pectinase	C13 norisoprenoids (ionones & damascones)	Pabst *et al.* 1991
Strawberry	pectinase	(s)-2-methil butanoic acid benzyl alcohol 2,5 dimethyl-4-hydroxy-2H-furan-3-one E-cinnamic acid benzoic acid	Wintoch *et al.* 1991
Grape	glucosidase	norisopenoids from lutein, violaxanthin, neoxanthin.	Williams *et al.* 1992
	β-glucosidase from yeasts	monoterpene alcohols	Günata *et al.* 1990
	various (fungal enzymes) almond emulsin	C-13 norisoprenoids: β-damascone, β-ionone,megastigm-diols	Sefton & Williams 1991
	fungal β-apiosidase	apiosylglucoside of terpenols	Dupin *et al.* 1992
Lulo fruit	glucosidase	(R)-linalool and other terpenoids.	Suarez *et al.* 1991
Apricot Peach Yellow plum	emulsin*	Monoterpenes C-13 norisoprenoids Marmelactones (peach)	Krammer *et al.* 1991
Vanilla	β-glucosidase hesperidinase	vanillin	Belford *et al.* 1992
Tomato	β-glucosidase pectinase	2-phenyl ethanol, benzyl alcohol, benzoic acid,shikimate type products....	Marlatt *et al.* 1992
Apricot	β-glucosidase	hexanol,2-phenylethanol, linalool, dienediols, nerol,...	Salles *et al.* 1991
Blackberry	pectinase	benzyl alcohol, 3-hexen-1-ol, benzoic acid,vitispiranes, heptanol	Humpf & Schreier 1991
African mango	β-glucosidase	8 monoterpene alcohols, 5 aldehydes, 7 esters, 5 C-13 norisoprenoids, 4 acids.	Adedeji *et al.* 1992

* Emulsin is a comercial β-glucosidase enzyme preparation.

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"earthy", "stalky" and "tobacco". Wines considered of higher quality were rated higher in these non-berry attributes. The use of this technique lead Francis *et al.*(1992) to similar conclusions with Semillon, Chardonnay and Sauvignon Blanc grapes. Enzymatic or acid hydrolysis was found to enhance intensity of the attributes "honey", "tea", "lime" or "floral" present in the neutral wine.

5. Elimination of off-flavors in foods.

If some enzymes, like lipoxygenases in soybeans, lipases in dairy products or proteases in aged cheeses, have been reported to be associated with off-flavor generation (Heath and Reineccius 1986), there is also an increasing number of enzymes proposed to eliminate off-flavors naturally occuring in foods or produced as a consequence of food processing. Some examples date back decades. This is the case of the use of a microbial naringinase (a fungal enzyme with rhamnosidase and β-glucosidase activities) to debitterize grapefruit juice or grapefruit concentrate (Chase 1974). This enzyme hydrolyzes the naringin (responsible for the bitter taste) to prunin and rhamnose. There is evidence that this process could be achieved by immobilizing the enzyme on porous glass (Manjón *et al.* 1985). Another interesting example is given by Hasegawa and Maier (1983). These authors used a limonoate dehydrogenase from *Acinetobacter* to oxidize limonin, a bitter compound of grapefruit and orange to non-bitter limonoate A-ring lactone and they showed that the process could be greatly improved by immobilizing the enzyme in acrylamide gel. In the field of alcoholic beverages, the removal of diacetyl from beer has been studied by Tolls *et al.* (1970). They used a bacterial diacetyl reductase to reduce diacetyl (responsible of unpleasant butter-like note) to 2,3 butylene glycol but, although the process was successful, it presented the drawback of requiring great amounts of NADH cofactor. A different approach exists in the addition of acetolactate decarboxylase to the fermentation broth at the end of the process. This enzyme allows the direct transformation of α-acetolactic acid to acetoin avoiding its spontaneous conversion to diacetyl (Cochet 1988). In a more recent report, the gene coding for this enzyme has already been cloned in *Saccharomyces cerevisiae* used for brewing, reducing the level of diacetyl to less than 0.01 ppm (Scott 1989). However, the brewing industry is very conservative, so it is difficult to predict if this kind of strain will reach commercialization stage.

Glucose oxidase can be helpful in removing traces of dissolved oxygen which cause oxidative rancidity and removing glucose which leads, in turn, to enzymatic browning in processed foods. As an example, Takenawa *et al.*(1990) patented a process in which this enzyme is added to hydrated soybeans in order to reduce the development of oxidation in the preparation of soya milk. This enzyme has also been used in processed eggs, dehydrated potatoes and mayonnaise (Godfrey and Reichelt 1983).

Another example of enzymatic off-flavor removal is the use of sulphydryl oxidase in ultra high temperature (UHT) milk. This enzyme oxidizes sulphydryl or thiol groups to disulfides, the former being responsible for the cooked flavor of sterilized milk (Shipe 1976, Swaisgood and Horton 1989). However, a big limitation is the lack of an abundant source of the enzyme.

Nomura *et al.*(1988, 1991) examined the decomposition of hexanal, which causes the off-flavor in food materials, by immobilized acetic acid bacteria and cell-free extract of Baker's yeast. They

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found that hexanal was converted to either hexanol or hexanoic acid under the action of ADH, aldehyde dehydrogenase and the NADH oxidase enzymes. They also reported its possible application to reduce off-flavor in deffatted soy milk, since the system was used during sixty cycles without any loss in enzyme activity. The ADH has also been studied directly in foods. Matoba *et al.*(1990) reported the reduction of n-hexanal to n-hexanol in soybean extracts with best results using NADH as cofactor. Hildebrand (1992) reported also that genetic engineering could be an interesting solution to indirectly eliminate some off-flavors from food : for example by altering the gene coding for the lipoxygenases system synthesis thereby stopping the lipoxygenase-catalyzed peroxidation of fatty acids reponsible for the formation of hexanal.

Finally, an indirect participation of enzymes in the elimination of off-flavors is the increasing use of cyclodextrins (CD) in the food industry. These are cyclic glucose oligosaccharides containing 6,7 or 8 glucose units, produced by the enzyme cyclodextrin glycosyl transferase from starch. CD's have been successfully used in the removal of the off-flavor due to naringin and bitter peptides (Pszezola 1988, Korpella *et al.* 1989).

ENZYMATIC SYNTHESIS OF FLAVOR COMPOUNDS

One of the important focusses in the flavor industry is the biosynthesis, isolation and purification of individual active chemicals using enzymes. The many advantages of enzymes in biosynthesis are widely recognized, considering their specificity (enantio and stereo selectivity), the use of mild reaction conditions and their availability (Sicsic 1987). There are parallel efforts in the enzyme research to develop new enzymes through protein engineering (Haas 1984, Leuchtenberger 1992), new ways of stabilization by immobilization or chemical modification (Shami *et al.* 1989), cofactor regeneration process in redox reactions (Duine 1991), new strategies such as extractive bioconversions (Andersson and Hahn-Hagerdal 1990) and new properties and specificities by the modification of the reaction medium (Klibanov 1986, Zaks and Klibanov 1988). In the following section we will review some examples of flavor compounds that may be produced by enzymatic processes, following the already mentioned trends in enzyme technology.

1. Lipases and esterases.

A great deal of research has been directed towards the use of lipid related enzymes - lipases mainly - (Welsh *et al.* 1989, Yamane 1991) for *in vitro* flavor synthesis. In Figure 1, the reactions in this field catalyzed by lipases are shown. Moreover, special interest has been given to using organic solvents as reaction media because in these conditions, these hydrolytic enzymes work preferably in the synthetic way (Klibanov 1986). Recently, Vulfson (1993) reviewed the application of this technique for food ingredient production.

Ester synthesis by means of lipase is an interesting alternative considering that there are many well known flavor esters in the natural aroma of fruits, traditionally obtained by extraction or by chemical synthesis. The enzymatic synthesis of more than 50 esters has been described to date by Welsh *et al.*(1989). Some of the more recent examples are reported in Table 3.

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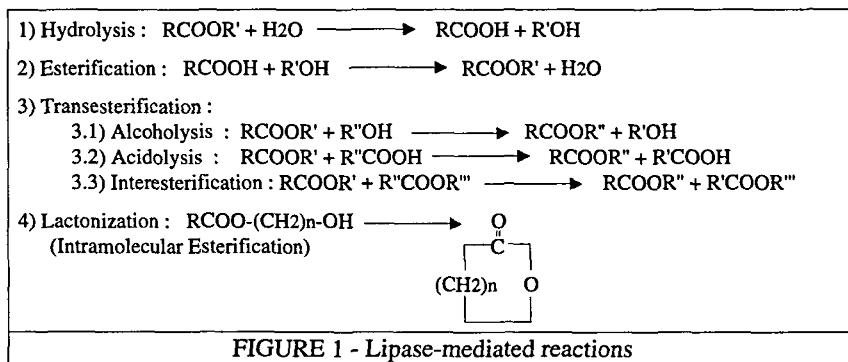

FIGURE 1 - Lipase-mediated reactions

TABLE 3 - *In vitro* lipase-mediated synthesis of flavor esters.

Substrates	Lipase source	Reaction medium	Immobilization	Authors
Esterification				
. n-butanol + butyric acid	*M. miehei*	n-hexane	no	Borzeix *et al.* 1992
. ethanol + oleic acid	*M. miehei*	organic	no	Gatfield 1988
. heptanol + oleic acid	*C. cylindracea*	-	no	Goldberg *et al.* 1990
. ethanol + butyric acid, aliphatic alcohols and acids	*M. miehei*	n-hexane	celite	Manjón *et al.* 1991
. C3-C5 aliphatic alcohols + acetic or butyric acids	*C. cylindracea*	n-hexane	no	Welsh & Williams 1989
. ethanol + acetic acid	*C. cylindracea*	n-heptane	silica gel	Gillies *et al.* 1987
. ethanol and n-butanol + butyric acid	*A. niger*, PPL* *C. cylindracea*	n-hexane	no	Welsh & Williams 1990
. C2, C4, C5 alcohols + C2, C4 acids	various (*M. miehei, C. cylindracea,...*)	hexane, octane, decane	no	Welsh *et al.* 1990
. terpene alcohols + C2, C3, C4, C6 acids	*A. niger, R. delemar, G. candidum, ...*	water	no	Iwai *et al.* 1980
. isopentanol and geraniol + C2-C4 acids	various (*A. niger, M. miehei,...*)	n-heptane	no	Langrand *et al.* 1988
. C1-C6, terpene alcohols + C2-C6 acids	various (*M. miehei, R. arrhizus,...*)	n-heptane	no	Langrand *et al.* 1990
. ethanol + free fatty acids or unhydrolyzed butterfat	*C. cylindracea*	water	no	Kanisawa 1983
. C5-C10 n-alcohols or phenyl-alkanols + dodecanoic acid	PPL*	n-heptane	no	Gerlach *et al.* 1989
. butanol + butyric acid	*M. miehei*	hexane	no	Borzeix et al. 1992
Transesterification				
. ethanol or isopropanol + triglycerides	*P. fluorescens*	excess alcohol	celite	Shaw *et al.* 1991
. nonanol + ethyl propionate	*C. cylindracea*	-	no	Goldberg *et al.* 1990
. isopentanol + ethyl acetate	*M. miehei*	n-hexane	anionic resin	Rizzi *et al.* 1992
. isopentanol or geraniol + ethyl or isopentyl ac. or but.	various (*A. niger, M. miehei,...*)	n-heptane	no	Langrand *et al.* 1988
. secondary alcohols + vinyl acetate	not given	not given	no	Holla *et al.* 1990

*PPL : Porcine Pancreatic Lipase.

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Synthetis yields have been shown to be a function of the hydrophobicity of the solvent (log P), the source of lipase and the molecular weight of alcohols and acids. In particular, Goldberg *et al.*(1990) showed that substrate polarity and water activity greatly influence the esterification and transesterification rates. Enzyme activity was shown to be highly dependent on the water molecules released during the esterification reaction, while the transesterification rate was found to be constant from the beginning of the reaction (Goldberg *et al.*1990). Generally, solvents with log P > 3.5 and a water content of less than 1% are optimal conditions for the synthesis of short-chain flavor esters (Welsh *et al.*1989, 1990, Manjón *et al.*1991). However, in many cases, enzyme stability in organic solvents is still a major problem.

Transesterification (acidolysis or solvolysis) is preferable to esterification because inhibition by the acid or the alcohol does not occur (Langrand *et al.*1988, Rizzi *et al.*1992). Moreover, this reaction did not release water molecules and hence, initial water content of the reaction medium is kept constant (Goldberg *et al.*1990). This process has been successfully applied by Shaw *et al.*(1991) for the solvolysis of triglycerides with long-chain fatty acids for the production of the corresponding esters, while Holla *et al.*(1990) carried out the transesterification of vinyl acetate for the resolution of secondary higher alcohols.

As a practical application of esterification, Kanisawa (1983) produced an ethyl ester mixture from butterfat oil and Welsh *et al.*(1989) achieved the synthesis of C3-C5 alcohols from fusel oil, concluding that controlled hydration of the enzyme increased the efficiency of the system in terms of reaction rate. Nevertheless, this parameter must be considered carefully: Gillies *et al.*(1987) pointed out that hydration of immobilized *C. cylindracea* lipase decreased the final ester concentration, although the hydrated enzyme was more stable after repeated use. In terms of strategy, microaqueous or biphasic water/solvent systems are preferable to reverse micelles as shown by Borzeix *et al.*(1992) with butyl butyrate synthesis as a model.

Ueda *et al.*(1992) studied the biosynthetic pathway of aroma esters in various fruits such as banana, melon and strawberry. They showed that the production of ethyl, butyl, isobutyl and isoamyl esters of acetate and butyrate was linked to the activity of an alcohol acyl CoA transferase - or ester synthetase - extracted from these fruits. They proved that the reactivity of the purified enzyme, employed *in vitro*, was dependent on its origin and on the substrate structure. For example, the synthetase from banana and melon reacted well with acetyl-CoA, propionyl-CoA and butyril-CoA while the enzyme from strawberry reacted better with valeryl-CoA.

L-menthol is one of the most important terpene alcohols widely used in the perfume and flavor industry. It is used in tooth paste and many other products for its mint taste and refreshing sensation. It is commonly obtained by isolation and crystallization from peppermint oil or chemically synthesized from benzoic acid. However, in this process the limiting step is the separation of L-menthol (the active compound) from DL-mixtures. There are many patents dealing with the enzymatic resolution of the mixture by the specific hydrolysis of DL-menthol esters, L-menthol being then recovered by crystallization. This process has been successfully achieved with esterases of *Saccharomyces* (Moroe *et al.*1970), bacteria (Moroe *et al.*1971), *Alginomonas* (Watanabe and Inagaki 1977, 1979). More recently, a whole cell process involving the esterase lipase of *Rhodotorula minuta* has been

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described (Omata *et al.* 1981). Moreover, these authors showed that the catalytic activity of the cells was enhanced because of its entrapment in polyurethane resin gels (estimated half life of more than 55 days) yielding L-menthol of 100% optical purity. The esterification can be carried out directly in a specific process to produce L-menthol-5-phenyl valerate using *Candida cylindracea* lipase adsorbed onto celite and entrapped in polyurethane (Cheetham 1991). The same selective esterification may be carried out for the resolution of DL-citronellol (Cheetham 1991) and DL-borneol mixtures with esterase of *Trichoderma* (Oritani and Yamashita 1976).

Enantiomeric secondary alcohols are present in fruits such as blackberry, corn, coconut or banana. As an alternative route to synthesize these compounds, lipase enantioselective esterifications have been studied in the last few years. Janssen *et al.* (1991) have shown it was possible to apply this technique successfully for the resolution of secondary alcohols by transesterification in alkyl carboxylates as solvent. They pointed out that addition of a molecular sieve to the reaction mixture greatly improved the reaction yield for the resolution of 1-phenyl ethanol. Another example is given by Gerlach *et al.* (1989) who prepared aliphatics and phenyl alkanols with high chemical and optical yields, outlining the importance of experimental conditions such as temperature, chain length of the substrate and enzyme immobilization.

Lipase of *Mucor* species has been shown to catalyze the lactonization reaction of 15-hydroxypentadecanoic and 16-hydroxyhexadecanoic acids to the corresponding macrocyclic lactones (Antczak *et al.* 1991). The reaction was achieved in an organic medium containing ether and toluene at 80 C and a conversion yield of over 30% was reached. The yield of this reaction can be increased if free water content is maintained at 0.083% and if sugar alcohols such as erythritol, arabitol or sorbitol are added before lyophilization of the enzyme (Yamane *et al.* 1990).

2. Oxidoreductases.

Oxidoreductases play major roles in determining the quality of certain food products (Whitaker 1984). Nevertheless, very few of them have been used for industrial applications, mainly due to the difficulties found in the economical production and/or regeneration of cofactors involved in the process. There are, however, some examples indicating the potential application of oxidoreductases, regenerating the cofactor by a second enzymatic reaction, mainly in a membrane reactor where the cofactor may be retained.

2.1 Alcohol dehydrogenase (ADH).

This enzyme can be extracted from horse liver, plants like the grape (Molina *et al.* 1986, 1987) or produced by microorganisms. It is able to work in both directions, for reducing ketones to aldehydes, aldehydes to alcohols or oxidizing alcohols to the corresponding aldehydes. Initially, possible applications of the ADH were reported by Ericksson (1975). The relation between the interconversion yield of aldehyde to alcohol and the sensorial note obtained was studied, showing that ADH added with NADH to milk containing polyunsaturated fats could reduce the amount of aldehydes, particularly hexanal (Ericksson *et al.* 1977). A few years later, Tamaki and Hama (1982) purified and characterized the ADH from bakers' yeast. This enzyme was found to be active for the oxidation of a wide range of aldehydes to their corresponding carboxylic acids.

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Acetaldehyde plays a significant role in the flavor of certain fruits like orange and foods like yogurt. Raymond (1984) patented an enzymatic procedure employing ADH to produce acetaldehyde from ethanol present in orange essence, in which the cofactor NAD is regenerated by use of flavin mononucleotide (FMN) in a light-catalyzed process with oxygen and catalase. Three other different methods for the production of flavor aldehydes were tested by Legoy *et al.*(1985). These authors studied the conversion of geraniol to geranial in a biphasic system (water/hexane) taking advantage of the difference in solubility of each compound in the organic phase. This system avoids the end product inhibitory effect allowing conversion yields close to 50%. It has been applied successfully to the production of citronellal, hexanal and 3-phenyl propanal. Bowen *et al.*(1986) used a similar process to study the conversion of cinnamaldehyde to cinnamyl alcohol with an immobilized yeast ADH.

Reducing enzymes (specifically secondary ADH) of acetic acid bacteria were found to transform a great number of ketones to their corresponding alcohols. Best results were found with the enzymatic system of *Gluconobacter oxydans* and *Acetobacter acetii* reducing 12 ketones to (S)- alcohols with an enantiomeric excess of more than 94% (Adlercreutz 1991) while the redox-enzymatic system of *Gluconobacter suboxydans* was found to be able to convert glucose in 5-ketogluconic acid, which is then converted by controlled heating into a monomethyl furanone, a strongly meat-flavor molecule (Cheetham 1991).

Braun and Olson (1986) attained the controlled production of acetic acid, 3-methyl-butanal and 3-methyl-butanol with cofactor recycling (NAD-NADH) with the reducing enzyme system of *Gluconobacter oxydans* and *Streptococcus lactis*. The alcohol dehydrogenase and aldehyde dehydrogenase of *G. oxydans* were shown to be able to regenerate NADH and to produce acetic acid from ethanol while the enzymatic system of *S. lactis* (transaminase, decarboxylase and ADH) was found to synthesize the aldehyde (with malty flavor and the alcohol from leucine as precursor).

2.2 Alcohol oxidase.

Benzaldehyde is the main molecule in the cherry fruit flavor and is enzymatically formed when the seeds or pits of fruits such as almonds, apricots, peaches or apples are crushed. The biotechnological production of this compound by enzymic oxidation with alcohol oxidase has been studied in the past few years (Cheetham 1993). Duff and Murray (1989), showed it was possible to use this unidirectional enzyme (only oxidation pathway) to produce benzaldehyde from benzyl alcohol. They developed a two phase whole-cell process, with *Pichia pastoris*, a methylotrophic yeast, using catalase to transform the denaturing H_2O_2 produced. The same authors (1990), demonstrated that it was possible to extend this application to the oxidation of C2-C6 aliphatics alcohols to their corresponding aldehydes. Nevertheless, Nikolova and Ward (1992), showed that the whole-cell process gave higher rates of conversion than cell-free extracts with *Saccharomyces cerevisiae*. As a novel concept in bioprocess engineering, Barzana *et al.*(1989) studied the enzymatic oxidation of ethanol in the gaseous phase with two microbial alcohol oxidases. The authors demonstrated the importance of water activity on both enzyme thermostability and activity.

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2.3 Glucose dehydrogenase and aldose reductase.

In a strategy of cofactor regeneration by means of coupling two enzymatic reactions, a process has been developed by the Denki Kagaku Kogyo company to produce gluconic acid, an acidulant, from glucose using glucose dehydrogenase requiring NADP+, the cofactor is reduced again with the same substrate, glucose, but transformed by the enzyme aldose reductase to sorbitol: the resulting concentrated syrup has sweet and acid taste (Fukui 1990).

2.4 Other oxidoreductases.

A dehydrogenase from *Pseudomonas putida* has been found to be able to convert L-menthone into L-menthol (Nakajima *et al.* 1978). This finding can be applied to the bioconversion of the essential oil (containing nearly only L-menthone) of the plant *Mentha piperita* in its immature stage in order to increase the proportion of L-menthol, the active flavoring compound. Mitsui Company has recently reported a similar process using cells of *Cellulomonas turbata*. In both cases, there is a need for a cofactor regeneration system. In 1979, Takahashi *et al.* isolated an aldehyde oxidase from bovine liver and applied it for the reduction of the green beany taste in soybean. The reaction was carried out under aerobic conditions with dissolved oxygen as the electron acceptor, without the need of cofactor. It is assumed that the loss in the intensity of green note was due to the oxidation of n-pentanal and n-hexanal.

Ionones and other related compounds including β-ionone and β-damascone are very important aroma compounds in tobacco and many fruits, formed by enzymatic degradation of carotenoids. Sode *et al.* (1989) have been able to carry out the microbial conversion of β-ionone using *A. niger* cells in organic solvents. However, using enzymes such as polyphenoloxidase, it has been possible to obtain small amounts of β-ionone, when acting on substrates like palm oil rich in carotenoids (Therre, 1990).

In the field of lactones, Maguire *et al.* (1991) achieved the reaction of lactonization of the linoleic acid employing an immobilized soybean lipoxygenase leading to a C13 macrocyclic lactone as a product. Willets *et al.* (1991), obtained the transformation of endo-bicyclo heptan-2-ols and endo-bicyclo hept-2-en-6-ol into their corresponding lactones using a coupled enzyme system (dehydrogenase from *Thermoanaerobium brockii* and monooxygenase from *Acinetobacter calcoaceticus*) with coupled regeneration of NADPH+/NADP+ cofactor.

As an indirect positive influence on the development of flavor in wine, the action of glucose oxidase to remove any remaining glucose, transforming it in gluconic acid may be cited (Heresztyn 1987).

Using formate dehydrogenase as the second enzyme for regeneration of NAD, the company Genzyme produces a wide range of optically active α-hydroxy acids via lactate dehydrogenase as first enzyme (Dunn *et al.* 1991). In the case of flavor compounds, several enzymes display potential applications. Magee *et al.* (1981) produced diacetyl (responsible for butter flavor) and acetoin by multienzymatic system consisting of milk fat coated microencapsulated cell free extracts of *Streptococcus lactis*. These authors assumed that diacetyl was synthesized through the oxidative path-

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way involving acetyl-CoA, and acetoin through the α-acetolactate route, both of them by means of oxidative enzymes.

3. Other enzymes.

The major groups of enzymes involved in the production of flavors are hydrolases or oxidoreductases. Nevertheless, other types of biocatalysts can be regarded as potentialy interesting. This is the case of a lyase: the α-terpineol dehydratase catalyzes the reaction of limonene (a by-product of the citrus industry) conversion to α-terpineol, a monoterpene of great interest. This enzyme has been isolated from *Pseudomonas gladioli* by Cadwallader *et al.*(1992). These authors showed that the isolate was composed of 2 proteins with apparent molecular weight of 94,500 and 206,500 daltons. Almosnino and Belin (1991) isolated an enzyme system from apple pomace, able to produce C6-C10 volatile aldehydes from linoleic acid through the action of two main enzymes: lipoxygenase which is reponsible of the formation of hydroperoxides from unsaturated fatty acids and hydroperoxide lyase which ensures the generation of the volatile aldehydes. They found that the amount of aroma compound could be significantly increased by adding SO_2 or vitamin C in the reaction medium.

CONCLUSIONS

Research in the areas of food technology and biotechnology has been influenced by the increasing demand of consumers for nutritious food and natural food ingredients. In this review, the impact of enzyme technology on flavor has been examined. It must be pointed out that this review mainly concerns reports in the scientific and technological literature and shows the trends in research on flavor related enzymes. It is helpfull to add that most of the actual research is far from the application to the flavor industry. Some potential applications are mentioned and they still need further research and to show economical feasability in order to reach the commercial scale. Other problems encountered are the production capacity and the enzymes stability. Genetic and protein engineering may contribute to overcome some of these drawbacks but it is important to consider the development costs including eventual toxicological studies and market sizes. Also, some sectors of the food industry are regarded as traditional and might not be opened so easily to the introduction of new products or processes.

However, some advantages of enzymatic-catalyzed synthesis (mild temperature, pressure and pH conditions, high enantio or regiospecificity, lack of contaminating by-products) are attractive for the production of flavor compounds. It may be concluded that the use of enzymes as additives for flavor production and/or modification may become an important part of enzyme technology as shown by some recent developments. The better understanding of flavor generation in foods as well as the advances in the use of enzymes in non-aqueous media also offers a great potential for this activity in the near future. This is already a fact for EMC and in general, for flavor derived from dairy products.

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