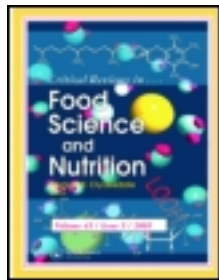


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Enzyme-Assisted Extraction of Flavorings and Colorants from Plant Materials

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From times immemorial, colorants, and flavorings have been used in foods. Color and flavor are the major attributes to the quality of a food product, affecting the appearance and acceptance of the product. As a consequence of the increased demand of natural flavoring and colorant from industries, there is a renewed interest in the research on the composition and recovery of natural food flavors and colors. Over the years, numerous procedures have been proposed for the isolation of aromatic compounds and colors from plant materials. Generally, the methods of extraction followed for aroma and pigment from plant materials are solvent extraction, hydro-distillation, steam distillation, and super critical carbon dioxide extraction. The application of enzymes in the extraction of oil from oil seeds like sunflower, corn, coconut, olives, avocado etc. are reported in literature. There is a great potential for this enzyme-based extraction technology with the selection of appropriate enzymes with optimized operating conditions. Various enzyme combinations are used to loosen the structural integrity of botanical material thereby enhancing the extraction of the desired flavor and color components. Recently enzymes have been used for the extraction of flavor and color from plant materials, as a pre-treatment of the raw material before subjecting the plant material to hydro distillation/solvent extraction. A deep knowledge of enzymes, their mode of action, conditions for optimum activity, and selection of the right type of enzymes are essential to use them effectively for extraction. Although the enzyme hydrolases such as lipases, proteases (chymotrypsin, subtilisin, thermolysin, and papain), esterases use water as a substrate for the reaction, they are also able to accept other nucleophiles such as alcohols, amines, thio-esters, and oximes. Advantages of enzyme-assisted extraction of flavor and color in some of the plant materials in comparison with conventional methods are dealt with in this review.

Keywords enzymes, extraction, volatiles, plant materials, pigments, hydro-distillation, steam distillation

INTRODUCTION

Flavor is one of the most important factors governing the selection of food we eat. The creation and utilization of flavors of high quality are of major concern in the manufacture and sale of food products. A short definition of flavor would be “that impression which is made on taste buds when a food product is consumed.” There are many plant materials which can be a good source of flavors. Among the plant materials which are rich in flavors are spices due to their essential oil contents which are enriched in flavor compounds. Flavor can be categorized as follows based on physical form and raw materials with which the flavor compound types are present:

- (i) Physical forms: Extracts, distillates, oleoresins, absolutes, fruit juices, essential oils, isolates,
- (ii) Compound type: Alcohol, acids, esters, aldehydes, ketones, lactones, ethers, sulphur, and nitrogen derivatives.

Flavor and flavor enhancers are important ingredients in the manufacture of modern convenience food products. Mostly synthetic flavors are used in processed foods and their numbers are increasing due to the scarce availability of natural flavors. Natural flavors are non-uniform in composition and flavor strength, mostly unstable, and generally expensive compared to synthetics when compared on an equal flavor strength basis. But in recent times natural flavors are gaining importance from the point of using everything natural. In ancient times only natural colors and flavors were used in foods. Due to the shortages of natural color and natural flavor, synthetic color, and flavor came into vogue. At present there is a big “No” to synthetics from the consumers due to the proven toxicological effects of some

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Table 1 Major classes of pigments and their sources

Class of pigments	Sources
1. Anthocyanins (Red to Blue)	Blue grape skin, blue berry, cherry plum, kokum fruit, hibiscus
2. Carotenoids (Yellow to Red)	Annatto seeds, paprika, alfalfa, carrot, saffron, marigold
3. Betalains (Red, Purple)	Beetroot
4. Flavones and Chalcones (Orange)	Safflower florets
5. Chlorophylls (Green)	Green plants, spinach, alfalfa
6. Miscellaneous:	
i Caramel (pale yellow to dark brown)	Modified sugar
ii Curcumin (yellow to orange)	Turmeric
iii Carminic acid (Red)	Cochineal
iv Riboflavin (yellow)	Quercitron bark

Adapted from Wallford, 1980.

of the synthetic colors. Moreover, regulating agencies do not allow any synthetic colors and flavors in foods, which are toxic and unfit for human consumption. The importance of natural colors and flavors in food products is increasing day by day. Association of color with flavors is specific because each color is associated with a particular flavor.

Color is recognized as a major factor affecting food acceptance. The consumer expects a specific food to possess a well-defined color and rejects any appreciable deviation from the normal. The change in color during the preparation of food is a useful guide to quality control and is used by many food processors as the criterion for selecting raw materials. The characteristic color of raw food is due to the natural pigments present in the plant and animal material. These can be enhanced in processed food products through the addition of food colorants. Addition of color can make up for the color losses during processing. Some of the major groups of colors are anthocyanins, carotenoids, betalins, chlorophylls, flavones, and chalcones. Some of the plant sources of pigments are kokum, roselle, chilli, marigold, beetroot, and safflower (Table 1).

As a consequence of the increased demand for natural flavors and colors by industries, there is a need for extensive collection of information on the composition of natural flavors and colors and their recovery methods. Over the years, numerous procedures have been used for the isolation of aromatic compounds and colors. A brief description of the various techniques employed for aroma and pigment recovery is given below.

EXTRACTION TECHNIQUES

Steam Distillation

Steam volatile aroma compounds are isolated from aroma rich raw materials by steam distillation. The powdered raw material is charged into the still and steam is introduced from the bottom of the still. The steam carries off the volatile compo-

nents and passes through a condenser where the volatiles get condensed and separated from water. Most of the spices such as pepper, ginger, cardamom, etc. are subjected to steam distillation to obtain essential oils, the flavorant from the spice. (Ravindran, 2000).

Hydrodistillation

The plant material is powdered and boiled along with water, when volatile aroma compounds get carried away with steam and condense along with the steam (ASTA, 1985). Volatile compounds are separated from water and dried over anhydrous sodium sulphate. Generally, this method is followed at laboratory level to screen the quality of the raw material. Hydrodistillation is not commonly practiced in industries because of the long distillation time and the resulting mass after hydrodistillation is not easily amenable for oleoresin extraction with solvents.

Solvent Extraction

Solvents like dichloromethane, dichloroethane, acetone, hexane, alcohol, etc. are used to selectively extract the aroma principles from various raw materials by column extraction either in hot or in cold conditions followed by the removal of solvents. Countercurrent extraction is also practiced. Spice oleoresins are prepared by this method (Ravindran and Madhusoodhanan, 2000).

Supercritical Fluid Extraction

Supercritical fluid extraction (SCFE) involves the use of a gas above its critical temperature and pressure. Critical temperature of the gas is the temperature above which it cannot be liquefied, no matter how low the pressure is. The critical pressure of the gas is the pressure above which it cannot be liquefied, no matter how low the temperature is. When the temperature and the pressure of the gas are above its critical temperature and pressure, the gas is said to be in supercritical state and exhibits physicochemical properties intermediate between liquid and gas. At constant reduced pressure their solvent power increases and this technique is used for the extraction of flavors and bio-active compounds from plant materials. Generally carbon dioxide gas is used as solvent for the extraction of spices. SCFE has many advantages over other methods of extraction like the absence of solvent residues and that the extract is rich in top flavor notes. Under varying operating conditions, selective extraction of a single component can also be achieved which will result in enriched fractions. SCFE is employed for difficult separation processes based on high degree of relatively low volume and high value products (Mukhopadhyay, 2000).

Enzymatic Extraction

One of the recent approaches for extracting flavors and colors is by using enzymes. Enzymes have been used for the pretreatment of the plant material prior to the conventional method for extraction of flavor and color. Enzymes are also used in various organic reactions for specific purposes such as cleavage of ester bonds, separation of racemic mixtures to obtain a single optically active compound, and cleavage of double bonds. The applications of enzymes in the extraction of oil from oil seeds like sunflower, soybean, rapeseed, corn, coconut, olives, avocado, and also for the extraction of rice bran oil etc. are well documented in the literature (Dominquez et al., 1993; Cintra et al., 1986; Burenrosto and Lopez, 1986; Hernandez et al., 2000). It has been reported that pretreatment of oilseeds with enzymes help to overcome the low extraction efficiency of the conventional methods, thus increasing the yield and quality of meal (Dominquez et al., 1995).

Enzyme-assisted aqueous extraction of oil from oil seeds is an emerging technology in the oil and fat industry. Application of aqueous extraction and enzymatic treatment for the enhancement in oil extraction from fruits and oil seeds have been published (Fullbrook, 1983; Rosentahl et al., 1996; Dominquez et al., 1994). Enzymes have been used in food industries from time immemorial in ripening of cheese, wine making, starch processing, conversion of starch to high fructose corn syrup, and for the release of ferulic acid from sugar beet pulp (Micard et al., 1994). There are reports of enzyme application for peeling of grape fruits, pomela, and orange (Rouhana, and Manneheim, 1994; Tol Soffer, Chaim, and Manneheim, 1994), flavor enhancement in vanilla pods, and transformation of glucovanillin to vanillin (Teran et al., 2001; Pouget et al., 1990). Application of commercial enzymes in food processing, their functions and sources are well documented in literature (Pomeranz, and Meloan, 1978).

Enzymes are globular proteins composed of one polypeptide chain. Their molecules are round in shape. In living cells enzymes occur as with structure having secondary tertiary or even quaternary structure. Some enzymes are found inside cells (intracellular enzymes) and some are released so that they lead a life outside the cell (extracellular enzymes). The enzyme speeds up the process of conversion of substrates into products without being consumed itself. They have an area as a pocket shaped gap in the molecule which is called the active site involved in catalytic activity. This site represents a small portion of the enzyme molecule and may involve amino acids at various sites along the primary chain of polypeptides. Normally, the substrate binds to the enzyme at an active site. Molecules which bind to the enzyme molecule at sites other than the active site may cause conformational change (distortion) in the tertiary structure of the enzyme. The substrate for the enzyme must have ready access to the active site. If the access of the substrate to the active site is impeded, then enzyme activity will be affected. Thus the tertiary structure of the enzyme is such that it produces a suitable conformation to allow access of the substrate to the

active site. The tertiary structure of the enzyme and enzyme activity are influenced by factors such as temperature, pH, ionic strength, of the environment. The specificity of action of an enzyme on a specific substrate is determined by the structure and conformation of active site (Balasubramanian et al., 1996). Enzymes catalyze specific biochemical reactions at different temperatures. The small quantity activity of the enzyme can catalyze the transformation of a large quantity of substrate into the end product. For example, sucrose can hydrolyze 10,000 times of sucrose compared to its own weight (Purohit and Mathur, 1999). An enzyme can be thought of as a molecular lock onto which only specifically shaped molecular keys—the substrates can fit. The most frequent use of enzymes is in breaking up of the substrates such as starch, cellulose, etc. Enzymes can be derived from bacteria, fungi, animal organs, or vegetable extracts and fruits. They are classified into the following types: hydrolyzing enzymes, oxidation-reduction enzymes, ligases, group transfer enzymes, desmolases, isomerizing enzymes, and carboxylation enzymes. Based on their catalytic property of catalyzing definite reactions, a particular enzyme acts on a specific substrate. For example, the enzyme lipase exerts no action on starch substrate and amylases do not act on the proteinaceous substrate. (Purohit and Mathur, 1999).

The enzymatic reactions are normally conducted at low temperatures (15°C to 45°C), which could be important in the extractions of thermolabile compounds. Above a temperature of 60°C, heat alters the enzyme molecule irreversibly which is due to molecular vibrations caused by heat which change the shape of the protein, altering the folding and internal cross-linkages in its polypeptide chains. In each case of enzyme application, one has to standardize the operational conditions viz., enzyme concentration, time of incubation, temperature of incubation, and optimum pH for the maximum activity of the enzyme.

When enzymes and substrates bind together, the shape of the enzyme molecule changes resulting in an optimum fit for the enzyme-substrate interaction. The change in the shape of the enzyme molecule will result in stress and strain on the substrate causing the bonds to break thereby promoting the reaction. When the substrate concentration is high, the addition of the enzyme can enhance the rate of reaction, till the substrate concentration becomes limiting. There is a direct proportionality between the rate and the substrate concentration until the enzyme concentration becomes limiting. Enzyme activity is defined in terms of the numbers of moles of the substrate converted or the number of moles of the product produced in unit time per unit weight of protein (micromoles/milligram/mg protein/minute). The potential use of enzymes (nearly 85%) is mainly based on their hydrolytic action, e.g., hydrolysis of high molecular weight compounds such as pectin starch, proteins, and cellulose. Almost 60% of the proteases find application in health food, dairy, pharmaceuticals, etc. To use enzymes effectively in food applications, proper knowledge about the nature of the enzyme, the source of the enzyme, active site, mode of action, optimum operational conditions like the pH, the temperature, etc. are very essential. Within the normal range, changes in

the temperature, the pH, and concentrations of the substrate and enzyme affect the rate of reaction in accordance with predictable interactions between the enzyme and the substrate molecules.

Plant materials like vanilla, pepper, mace, mustard, fenu-greek, rose, and citrus peel which have potential as a rich source of flavor have been studied for enzyme-assisted extraction of flavors. Similarly enzyme-assisted extraction of color has been studied in plant materials like marigold, safflower, grapes, paprika, tomato, alfalfa, and cherries. Details of the source of the plant materials, the enzymes used for extraction, and the effect of enzyme treatment on the yield and quality of the product obtained in comparison with the conventional methods of extraction with merits and demerits of enzyme-assisted extraction are discussed in the following sections.

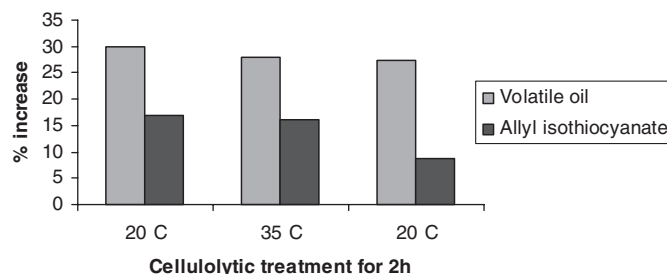
ENZYMATIC EXTRACTION OF FLAVOURS

Recent studies employing enzyme pretreatment for the extraction of flavor components from various plant materials have shown enhancement in aroma recovery. Enzymes such as cellulases, hemicellulases, and pectinases, and a combination of these have been used for the pretreatment of plant materials (Freese and Binnings, 1993; Ranali et al., 2004; Mishra et al., 2005). The major action of cellulase and hemicellulase are on cell walls. They act on cell wall components, hydrolyze them in turn, increase the permeability of the cell wall thus resulting in higher yield of the flavorant. Use of enzymes in the production of food flavoring extracts has been reported. Application of this technique for the extraction of flavors has been carried out in a very few spices like ginger, mustard, chilli, vanilla, pepper, mace, and marjoram. Details of the extraction and the enzymes used are dealt with in the following sections.

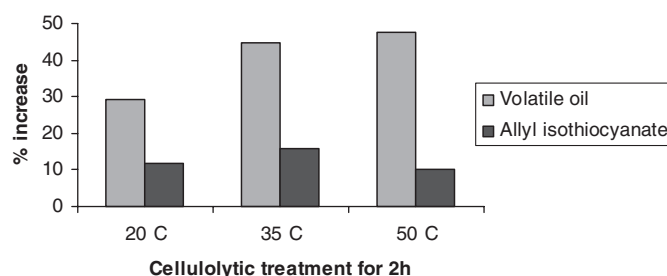
Mustard

The major component of mustard is allyl isothiocyanate which is usually produced by synthetic routes. This substance has a number of industrial, pharmaceutical, and agricultural uses. A novel method of application of cellulolytic enzymes from *Trichoderma resei* has been successfully carried out in mustard (Dobozi et al., 1988) and is found to increase the yield of mustard volatile oil considerably (20–30%). The extraction of volatile oil is based on the fact that the mustard seed contains thioglucosides (singrin in black mustard: sinalbin in white mustard). Singrin is hydrolyzed by myrosinase enzyme present in the seed itself. The products of the hydrolysis are glucose, potassium hydrogen sulphate, and allyl isothiocyanate. Allyl isothiocyanate is highly volatile and can be recovered by distillation. Mustard seeds do not yield enough volatiles upon the decomposition of singrin to make its recovery worthwhile. So, it is essential that singrin be decomposed by hydrolysis to yield allyl isothiocyanate in good amount. It is necessary to make the enzyme myrosinase available for decomposition of singrin. This is achieved by the application of cell wall breaking cellulase enzymes.

Effect of cellulolytic enzyme treatment of unground mustard expeller cake on yield of oil



Effect of cellulolytic enzyme treatment of ground mustard expeller cake on yield of oil



Adapted from Dobozi et al., 1988

Figure 1 Effect of cellulolytic treatment of mustard cake on yield of oil.

Mustard expeller cake both in ground and unground form at 100 g batch was treated with enzyme cellulase at 0.2 to 1% level v/v under optimized conditions of temperature and time to bring about the release of flavor precursors. The cellulase enzyme treatment resulted in increase (20–30%) of the volatile oil yield compared to control (without enzyme treatment) samples (Dobozi et al., 1988). Of the pretreatment conditions studied 0.2% enzyme (v/v) concentration at 35°C and pH 4.85 for 2 h was found to be most effective with the increase in volatile oil, reducing sugar and the allyl isothiocyanate contents of the unground and ground mustard expeller cake (Fig. 1). It is reported that grinding of the expeller cake enhanced the yield of allyl isothiocyanate. About 1% of the mustard expeller cake mass was recovered as volatile oil. Both titrimetric and gas-chromatographic methods indicated that the 0.2% (v/v) enzymatic treatment of the ground expeller cake at 35°C for 2 hours resulted in the highest allyl isothiocyanate yield. By splitting glycoside bonds, cellulolytic hydrolysis may lead to the liberation of volatile oil and thus increasing the yield of volatile oil content which would otherwise remain bound in the presence of thioglucosidases.

Oregano

Action of enzymes on the extraction of aroma compounds from oregano has been reported (Rokhlenko et al., 1982). The extraction was carried out twice using 50% and 18% ethanol

and an enzyme preparation containing a combination of pectin, polysaccharides, and starch hydrolyzing enzymes. The disintegration of plant tissue improved the extraction of aroma, flavor, and other components, with an increase in the yield of dry matter as well as essential oils. The sensory quality was also improved.

Pepper, Marjoram, and Mace

Production of spice extracts by application of cell wall degrading enzymes in pepper, marjoram, and mace was investigated by Freese and Binning (1993). Extracts were obtained by mixing spices (pepper, marjoram, or mace) with water, adjusting the pH with citric acid, addition of soy oil, enzymatic treatment for 2 h, and centrifugation. Various commercial cellulase, pectinase, hemicellulase, and liquid enzyme preparations were used individually or in combination. Extracts obtained were subjected to sensory and GC analysis. Enzymatic treatment of mace and marjoram did not yield satisfactory results. Pepper extracts with good sensory and compositional properties were obtained with some enzyme combinations. Best results were obtained using a combination of cellulase and pectinase enzyme preparations, but addition of hemicellulase did not improve the flavor. Pepper extracts had stable aroma for 4 weeks.

Vegetable Food Flavorings

Efforts were made to develop new technologies that could provide a thorough extraction of flavoring principles from vegetables (Tateo, 1979). The effects of de-polymerizing enzyme, hemicellulase, and influence of alcohol concentration upon the quantity and quality of the flavoring product was studied. The products thus obtained were submitted to organoleptic and analytical tests, and compared with the flavor extract that are commonly used to produce alcoholic beverages and soft drinks. Extract yields obtained were also assessed. The yield obtained by enzymatic treatments compared well with yields from static infusions.

Citrus Peel

Use of citrozyme CEO containing hemicellulolytic, pectolytic activity with high poly galactouranase activity for pretreatment of citrus peel to recover oil is reported (Coll et al., 1995). Enzyme treatment reduced emulsion viscosity and assisted in breaking of the emulsion to recover oil from the aqueous phase. Optimization of the oil recovery process (enzyme addition time, use of a buffer tank, and closed system and optimum enzyme dosage) resulted in advantages like increase in yield of essential oil, reduction in fresh water consumption, and wastewater production, wastewater being more easily biodegradable, increased centrifuge capacities, improves de-waxing of citrus oils, and had no influence on oil quality.

Wine and Must

Studies were conducted to investigate the influence of enzyme preparations with pectolytic and glycolytic activity enzymes on aroma development in Sauvignon, Pinot nero, Pinot grigio, Trebbiano, Lambrusco Sorbara, Labrusco Salamino and Cerasuolo musts, and Riesling and Pinot nero/Chardonnay wines. Aroma compounds in musts and wines are mainly present as glycosides, i.e. bound to sugars so that their full aromatic potential is not realized. The molecule bound to the sugar is called an aglycone. Due to the presence of β -glycosidases, α -l-rhamnosidase, α -L-arabinosidase, beta-d-apiosidase, and beta-glucosidase the action of enzyme combination (2-6 g/hl) significantly increased the free aglycone concentration in musts and wines (Granata, 1994). Enzyme activity was greater in finished wines than in fermenting musts suggesting an inhibitory effect of glucose during fermentation. An increase in the complexity of the aroma was observed in wines treated with enzymes.

Vanilla

Vanillin, the principal flavor compound of vanilla beans in its natural form, is one of the most expensive flavor compounds. Traditionally vanilla beans are subjected to a curing process, when the characteristic aroma and flavor are developed as a result of biochemical reactions. Curing is done by an established method known as the Mexican method wherein the pods in the sacks are dried in an oven for 36–48 h. at 60°C followed by keeping in a wooden box for 24 h to drain water. Then the pods are exposed to sunlight during the day and stored at night in a wooden box. Drying in the sun and stirring at night is repeated until the pods dry, turn dark brown, and develop an elastic consistency (Dignum et al., 2001; Muralidharan and Balagopal, 1978). Hydrolysis of glucovanillin by β -glucosidase enzyme during the curing process releases the major flavour component vanillin. The vanillin content varies with the variety. Madagascar beans are with a vanillin content of 2–3.4% whereas the Mexican variety contains 1.1–1.8%. The traditional fermentation method of curing and extraction is inefficient in terms of the amount of vanillin extracted. In view of this, enzymatic treatment of vanilla beans has been studied for improved extraction of vanillin from vanilla beans (Ramachandra Rao and Ravishankar, 2000).

Enzymatic transformation of glycosides is not very efficient during the traditional curing/fermentation and extraction process. There are reports of increase in vanillin content up to 24% in vanilla beans upon treatment with exogenous β -glycosidase enzymes. Treatment of cured vanilla beans with exogenous pectinase and β -glycosidase has resulted in a 14% increase in vanillin content (Teran et al., 2001). The soxhlet method of extraction of vanillin present in lower yields compared to the enzymatic extraction technique. Green beans were subjected to enzyme treatment for cell wall degradation and glucovanillin hydrolysis wherein glucovanillin was transformed to vanillin simultaneously. The conditions were standardized by

Table 2 Effect of enzyme treatment on vanillin extraction from vanilla beans

Enzymes used for pretreatment	Vanillin g/100 g
Control	1.12 ± 0.05
cellulase + water	1.17 ± 0.06
cellulase + ethanol	1.17 ± 0.17
viscozyme + cellulase + water	1.17 ± 0.11
cellulase + viscozyme + water	2.66 ± 0.07
cellulase + viscozyme + ethanol	2.31 ± 0.1
cellulase + viscozyme + ethanol	3.66 ± 0.04
viscozyme + water	1.52 ± 0.08

Adapted from Teran et al., 2001.

subjecting the beans to a two-step enzymatic reaction using two commercial enzyme preparations containing mainly pectinase and cellulase activities in the 47.5% aqueous ethanol at 70°C for 8 hours. The reaction was efficient in extracting 3 times more vanillin than the soxhlet method. Investigators have concluded that the enzymatic reaction may substitute the microbial process involved in fermentation prior to vanillin extraction with simultaneous hydrolysis of glucovanillin. A synergistic effect was observed between enzymatic preparations when sequential enzymatic reaction was studied. It was also observed that both enzyme preparations do not work efficiently when used together in the same reaction.

Using viscozyme, the vanillin extraction was 1.52% as against 1.2% in control samples. By enzyme application followed by ethanol extraction, the amount of vanillin further increased to 1.96% on a dry weight basis. Results obtained using two enzyme preparations sequentially were similar to the results obtained by using a single enzyme preparation containing mainly pectinase enzymes. It is reported that the order of addition of enzymes also influenced the yield. Addition of cellulase followed by pectinase enzyme preparation gave best results by increasing the extraction efficiency of vanillin by 3.13 times higher than control (without enzyme treatment). Enzyme application was found to be useful in the conversion of glucovanillin to vanillin and also in the extraction of vanillin from pods avoiding the fermentation and extraction process. Of the different enzyme combinations used for pretreatment of vanilla beans, pods + cellulase + viscozyme + ethanol treatment gave the best results with an increase in the yield of vanillin three times the control (Table 2).

Extraction of Vanillin from Extracted Vanilla Beans

The effect of hydration time and ethanol concentration on the rate of hydrolysis of extracted vanilla beans by commercial enzyme preparation has been studied (Ovando, 2005). The enzyme preparations were used to study the kinetics of residual vanillin liberation from extracted vanilla beans during pretreatment with an enzyme followed by ethanol extraction. Pretreatment with cellulolytic enzymes improved the separation of intracellular compounds (e.g. flavor compounds) from plant materials by solvent extraction. Effects of prehydration times (72 h at 25°C)

of extracted vanilla beans (byproducts of the vanilla extraction industry) and ethanol concentration (0, 5, 10, 12.5, or 15%) on the kinetics of enzymic hydrolysis of cellulose by 8 commercial cellulolytic enzyme products were investigated. Efficiency of hydrolysis was assessed by the rate of production of reducing sugars. Hydration of extracted vanilla beans improved the efficiency of hydrolysis. Treatment using Crystalzyme PML-MX at a concentration of 2.64 units/g of bean is reported to be the most efficient at ethanol concentration at less than or equal to 5%. After 48 h of prehydration and 26 h of enzymic hydrolysis with this enzyme preparation, 196.6 mg/g reducing sugars containing 15.9 mg/g glucose were liberated. Less active enzyme products, Zymafilt L-300 and Novozyme, were reported to exhibit cellulolytic activity when 10 and 15% ethanol were added respectively.

Mandarin Peel Oil

Extraction of essential oil from the flavedo part of mandarin peels by enzyme pretreatment followed by hydrodistillation and cold pressing extraction has been studied. Peels were pre-treated with xylan-degrading enzymes at varying concentrations of 0.1 to 0.3%. Essential oil was recovered from these enzyme treated samples and the yield obtained was compared with control samples. By enzyme treatment at varying concentrations, the increase in the yield of essential oil compared to the control sample was up to 15%. This increase in recovery can be attributed to rupturing of the oil sacs or glands by the enzymatic action resulting in excess release of oil. Yield of oil by hydrodistillation was slightly higher compared to cold pressing extraction. The effective pH was 4.5 and the contact time of enzymes with samples was 3 hr (Mishra et al., 2005).

Lemongrass

The effect of ensiling lemongrass and lemon eucalyptus leaves with lactic acid bacteria and cellulase, hemicellulase, and pectinase enzymes in combination is reported in anaerobic conditions and samples were analyzed on 2, 6, 10, and 36 days of storage for essential oil (Dudai et al., 2001). The extraction efficiency of essential oil from lemongrass was improved by the enzyme treatment. The level of citronellal, the main component of the essential oil of eucalyptus leaves, decreased during ensiling, whereas the levels of isopulegol and 3, 8-terpinolhydrate increased.

It can be concluded that because of their stability with ethanol, the enzyme preparations could be used in the pretreatment of plant materials that are rich in flavor compounds to improve the final extraction of valuable flavors.

ENZYME ASSISTED EXTRACTION OF COLORS

At present there is an increasing demand for colorants from natural sources. Since there is a possibility of banning of synthetic colors in view of their carcinogenic properties and

already a reduction in the number of permitted synthetic colors from 13 to 8 numbers (PFA, 2005), the demand for natural colors is evident. In Japan and European countries because of the green movement, the demand for synthetic colors has been reduced. Synthetic colors in the global market accounts for 400 million dollars. Despite processing limitations and higher costs, the global market for natural colors is estimated to be more than 939 million dollars (Epszszola, 1998). The sources of natural colors can be plants, animals, and microbes. Different methods are followed for their extraction like solvent extraction, supercritical carbon dioxide extraction, etc. Enzymatic extraction is a recent approach for color extraction from plant materials. Increase in extraction efficiency by any new approach is welcome when viewed in terms of the value added end product. Studies have been carried out with an enzymatic approach for color extraction as a pretreatment or fermentation (ensilage) combined with enzyme treatment. Some of the plant materials rich in colors which have been exploited for enzyme-assisted extraction are alfalfa, marigold, safflower, strawberry, aronia fruits, grapes, red cherries, and the results have shown positive effects. Some of the interesting features of the work carried out by researchers in this area are presented in the following section.

Safflower

Safflower is an annual herb cultivated throughout India. The seeds of this flower are extensively used in the extraction of oil. The colored florets of safflower are used for dyeing fabrics and is a source of yellow color in food and cosmetics, very similar to saffron. The pigments present in safflower responsible for the orange yellow color of the flower are carthamin, precarthamin, safflower yellow A and B (Yoon et al., 2003). Carthamin is a water-soluble pigment distributed among reddened florets of safflower. Precarthamin is the precursor of carthamin. It is a flame colored, oxygen-sensitive, and ultraviolet-labile chalconoid glycoside produced in the orange yellow region of safflower flowers. Safflower yellow pigments have the potential as cost-effective natural food colorants and it is reported to be used in juices, yogurt, desserts, and candy (Mishley et al., 1993). The pigment is not easily extractable and highly unstable (Saito and Murata, 1994).

Extraction and purification of carthamine by available techniques is very difficult. Conventionally, methanol mixtures with hydrochloric acid or with formic acid have been used in the extraction process, but these solvents seem to be inappropriate, because the products often deteriorate during preparatory operations. However, the acid method does not always degenerate all the precarthamine and overgrinding of the fresh tissues is also unfavorable, because the floret paste gradually turns red by enzymatic/non-enzymatic oxidation. In the reddened paste, carthamine is accumulated and thus to overcome these problems of extraction, an enzymatic technique has been introduced for the extraction of precarthamine from safflower florets (Saito, 1993).

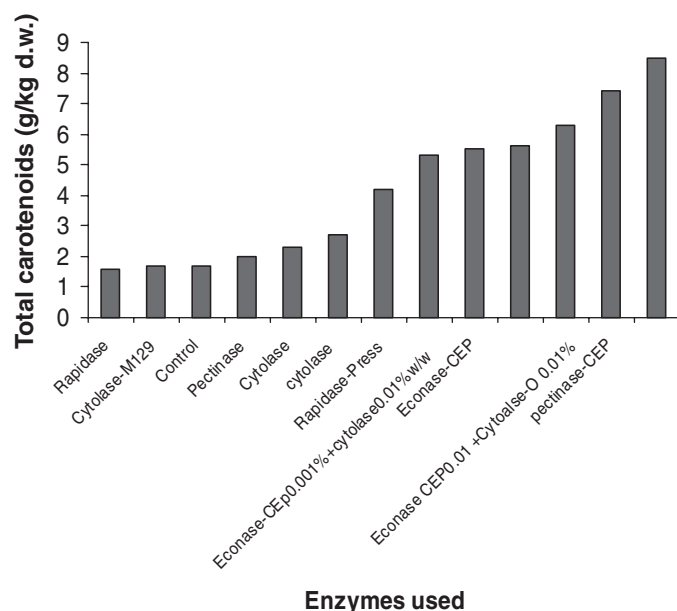
The method developed by Saito deals with treating dried and ground florets with glucosidase enzyme in aqueous medium under nitrogen and incubated for given intervals at 30°C. Treatment of dried florets of safflower with β -glucosidase yielded large quantities of orange-yellow quinoid chalcone glycosides. Enzyme hydrolyzed products obtained were purified by paper chromatography followed by countercurrent using a different solvent system. Under the conditions studied (pH 4.8, temp. 30°C, 3 h) the glucosidase activity required to extract color was calculated to be 120 mol. precarthamine $\text{ml}^{-1} \text{min}^{-1}$. The application of the enzyme was very effective for the extraction of precarthamine. Technically, this biological process has noteworthy merits like it is simple, mild, and easy to practice without the deterioration of precarthamine and there is no need to remove solvents. Thus the enzyme technique can undoubtedly replace the old acid methods or other methods in the immediate future.

Marigold

Marigold (*Tagetes erecta*) is an annual herbaceous plant with yellow to orange flowers widely grown for ornamental purposes. The flowers are rich sources of lutein and lutein esters, which have good potential as a natural food colorant (Sowbhagya et al., 2003). The major pigment lutein present in marigold belongs to the carotenoid group and has been identified in association with transtherin, a protein implicated in the transport of thryoxine and retinol. The commercial extraction of marigold pigment consists of silage, pressing, drying, hexane extraction, and saponification. The main drawback of the extraction process is the considerable loss of hexane that is diffused into the air causing an environmental problem, health risks, and economic losses (Galvin and Kirwin, 1995). To overcome all these problems, an enzyme-assisted method for extraction of pigment from marigold flowers has been worked upon (Navarette, 2004).

The effect of enzymatic treatment involving five commercial enzyme preparations on carotene extraction from fresh marigold flowers have been studied (Delgado-Vargas and Pardes-Lopez, 1997; 2002). Fresh intact flower petals were processed with enzymes, with different enzyme concentration at optimum pH. Enzymes used were of fungal origin mainly having cellulase, hemicellulase, and pectinase activities. Enzymatic slurries (0.01% to 0.1% w/w of enzymes) were prepared with Tween 80 (0.01% w/v) and sodium azide (0.01% w/v in deionized water) and added to petals at pH 5.0. The samples were kept at room temperature and mixed daily. The reaction was monitored at 2, 5, 10, 24, 48, 72, 96, and 120 h. The synergistic effect of two enzyme preparations was also studied. The total carotenoid content was determined in control and treated samples. Single enzyme at 0.1% gave a higher extraction of carotenoid compared to the action of combined enzyme treatment which implies that there was no synergistic effect of two enzymes studied (Fig. 2).

The effect of an enzyme preparation synthesized by endogenous microorganisms isolated from marigold flower has been used for the ensilage of marigold flowers. The microorganisms



Adapted from Delgado-Vergas and Pardes-Lopez, 1997

Figure 2 The carotenoid content of marigold fresh petals treated with commercial enzyme after 120 hours hydrolysis.

isolated from marigold flowers were identified as *Flavobacterium ltb*, *Acinetobacter anitratus*, and *Rhizopus nigricans* and it was found to exhibit high cellulase activity. The effect of these microorganisms on xanthophyll extraction from marigold flower (*Tagetes erecta*) has been studied (Navarette-Bolanos and Jimenez-Islas, 2003). A mixed culture of above three microorganisms in the ratio of 9.8%, 41%, and 49.25 used for the ensilaging of marigold flowers is reported to result in an increased yield of total xanthophylls extracted of 24.94 g/kg of dry weight compared to 12.92 for the control silage (without the addition of culture). Analysis of the product by HPLC indicated that the original xanthophyll profile was not altered. The enhanced extraction system appears to be very competitive compared to the traditional process and current alternatives.

An alternate extraction process for carotenoid extraction from marigold flowers has been proposed consisting of simultaneous enzymic treatment and solvent extraction (Barzana et al., 2002). The method employed the fresh flowers as raw material eliminating the inefficient silage and drying operations as well as the generation of aqueous effluents which are difficult to deal with in the traditional process. Recovery of carotenoid by this method was up to 97%.

Chilli

Chilli is a spice which is valued both for its color and pungency. Carotenoids are responsible for color and capsaicinoids for pungency. Extraction of chilli oleoresin is carried out with solvents like acetone, ethyl acetate, and hexane. An enzymatic method has been proposed for the extraction of capsaicinoids

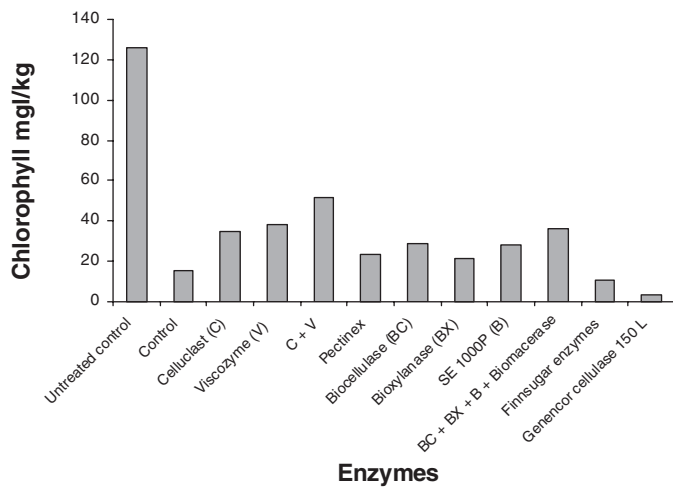
and carotenoids from chilli and the effect of enzymatic treatment on extraction yield and cell wall has been studied (Santamaria et al., 2000). A selective extraction of both color and pungency in a two-step process was proposed. A highly pungent variety of chilli called Guajillo puya and four commercial enzyme preparations containing cellulase, hemicellulase, and pectinase enzymes were selected for the study. Water and industrial alcohol (96% ethanol) were used to soften the tissue in capsicum before extraction. Dried chili powder was treated with enzyme in water at 1–5% for 5 h at pH 4.5 and 120 rpm at 1:50 (w/v) powder-to-water ratio. The paste obtained was dried in a vacuum drier at 50°C to a moisture content of 8%. The extraction was carried out with industrial alcohol from powder and paste (paste obtained after enzyme treatment). Extraction was carried out for 7 hours at 50°C with solid-to-solvent ratio of 1:50 w/v in a rotary shaker. In the second batch the solid was extracted with 96% ethanol for carotenoids. It has been reported that 80% of capsaicinoids and 73% of carotenoids extracted with industrial alcohol as extraction solvent. Single enzyme at 0.1% gave a higher extraction of carotenoids than the addition of a combined enzyme treatment, which implicated that there was no synergistic effect of two enzymes on carotenoid extraction. Histological studies have shown that capsaicinoids are inside the vesicle formed by the endoplasmic reticulum that migrate to cell wall periphery and are secreted to the cell walls into subcuticular cavity. It can be concluded from histological studies that the factors which hinder the increase in the yield of oil are seeds and the complexity of cell walls.

Capsicum cell walls are cellulosic but most of the cell walls are heavily lignified. The tissue also contains high levels of cutin, which in aqueous media reduces the accessibility of enzymes to the cell wall material. With enzyme pretreatment followed by ethanol extraction a 11% increase in carotenoid yield and 7% increase in capsaicinoids extraction is compared to control (chilli powder without enzyme pretreatment). An enzymatic pretreatment with pectinases in the final extraction increases to 24% (Santamaria, 2000). A two-step treatment coupled with enzyme pretreatment is recommended by the investigators as a significant economical alternative application to the existing industrial process.

Chilli and marigold oleoresin have been hydrolyzed using commercial lipase enzyme preparation to get pigment enriched fraction from oleoresin samples. Treatment of chilli oleoresin with lipase enzyme from *Candida antarctica* resulted in 69% release of capsanthin while lipase treatment of marigold oleoresin resulted in 44% lutein from marigold oleoresin (Zorn et al., 2003). Enzyme application can be an efficient replacement for the saponification procedure followed to achieve the same effect.

Alfalfa

Alfalfa is an interesting material for extraction, as it contains highly valuable cell contents such as protein, chlorophyll,



Adapted from Santamaria et al., 2000

Figure 3 Effect of enzyme pretreatment on chlorophyll recovery from alfalfa.

xanthophylls, and beta-carotene. A novel method of extraction of the pigments from alfalfa has been suggested involving simultaneous lactic acid fermentation with enzymatic cell wall hydrolysis (ENLAC) (Weinberg et al., 1990). The basis of the ENLAC mechanism is that the lactic acid fermentation helps to break the hemicellulose-lignin covalent bond in plant cell walls by partial hydrolysis of the linking L-arabinose side chains. This action would open access to hemicellulase and other enzymes for some efficient cell wall hydrolysis. Based on this principle alfalfa was treated with enzymes and ensiling process for the extraction of pigments. For enzyme ensilage process of extraction, alfalfa was chopped into a 1 inch size, minced thoroughly with 0.5% lactose to ensure adequate fermentation and with other enzyme preparations containing the enzyme cellulases, hemicellulases, pectinases, β -glucanase, xylanase, and amyloglucosidase at 1% level w/w on wet basis. In another batch, alfalfa was treated with enzymes at 40°C for 254 hours without ensiling and in yet another experiment, alfalfa was treated with 1% pectolytic + cellulolytic in the presence of 2 or 4% lactic acid in order to test the possibility of the synergetic effect of lactic acid and enzymes without ensiling.

It is reported that a significant increase in the recovery of chlorophyll, protein, and beta carotene was obtained by ENLAC process. Enzymes at 1.0% level sufficiently enhanced the bacterial flora present in alfalfa to provide epiphytic optimal conditions for ensiling and cell wall degradation in the ENLAC process, thus increasing the recovery of chlorophyll and protein content (Fig. 3). The most effective enzyme treatment was cellulase combined with hemicellulases and pectinases. The other commercial preparations containing similar enzymes has not given satisfactory results except for a particular brand which may be because the particular ratio of these enzymes is necessary to bring about the enhanced extraction of chlorophyll and protein. It can be concluded by this study that sources of enzymes and the preparations of enzyme ingredients and the

cocktail of cellulases, hemicellulases, and pectinases are critical for cell wall degradation in the ENLAC process (Weinberg, 1990). This method has a potential for the development of a milder extraction due to the increased permeability of the plant cell wall.

Cherries

Pretreatment of sweet cherries (pietroase napoleon variety) with purified enzyme preparation 0.1% for 2 h at 45°C followed by pressing in a laboratory hydraulic press at 200 atmosphere was found to improve pigment extraction compared to non-enzymatic extraction methods (Baschia et al., 1972).

Red Grapes

Semi-industrial scale trials (70 kg grapes/batch) with red wine making grapes of the cv. Marzemino, Schiava, Sangiovese, Corvina, and Rondinella were conducted to assess the effects of added pectinase enzymes at a concentration of 5 g/l on phenolic compound concentration in the wines. Addition of pectinase enzymes increased and accelerated the extraction of anthocyanins and tannins (Nicolini and Mattivi, 1997). Concentration of these compounds in wine was increased by 10–30% by the addition of enzymes with an increase more for tannins. Similar results were obtained for the 3 enzyme preparations used. Characteristic varietal patterns of polyphenols were not significantly influenced by pectinase enzyme treatments. Studies on the addition of two commercial pectolytic enzymes during mashing on sensory properties of port wines was carried out (Bakker et al., 1999). Addition of enzymes is reported to result in enhanced color extraction during wine making. Wine samples made with pectolytic enzymes treatment was found to retain better color, aroma, and flavor than the control sample (wine made without enzyme treatment).

The possibility of enhancing the extraction efficiency of red pigments and other phenolic compounds by the addition of pectic enzymes to mash was investigated. Commercial enzyme preparations 8 numbers viz., Gammapect AWP, Vinozyme G, Trenolin Rot, Ovoprs, Gammapect W2L, Vonoflow, Renolin Rouge, Gammapect W701 were used for the study. The effect of the enzyme treatment was evaluated in terms of anthocyanin content, turbidity, filtration, and sedimentation rates. Maximum release of red pigments in the control sample was achieved in 7 days while in treated samples it was observed after 4–5 days. The most effective preparations were Trenolin Rot, Vinozym G, and Gammapect W2L. A ten-fold increase in filtration and a 2–3 fold increase in the sedimentation rate were achieved. With the use of pectic enzymes, wine of higher sensory quality can be produced more economically in a shorter time.

Studies on the effects of rapidase pectolytic enzyme preparation on the treatment of grape must at 5 g/l on the color of

Shiraz red wine is reported by Clare (2002). Color intensity, hue, tristimulus value, total phenolics, and ethanol produced were monitored. Treated wines showed faster and higher color and phenolics extraction at the beginning of fermentation. Treated wines showed 22% higher intensity compared to control wines after 3 days of fermentation and 7.5% more after 6 days of fermentation and the increase in total yield was 9%. The result of the above study could be significant when applied at an industrial scale in a commercial winery.

In another study carried out by Niccolini and Mattivi (1997), wine making grapes of the cv. Tinto were crushed and the mashes were treated with commercial pectolytic enzyme preparations 1 h before yeast inoculation and fermented by the conventional method. The wines were analyzed for color and turbidity at the end of wine making and at 6 months intervals during 2 years of storage. A new condensed anthocyanin occurred during the first 6 months of storage in enzyme treated wines and its concentration remained constant during the storage period which is associated with better quality of the treated wines. Turbidity was less in the treated wines compared to the control wines.

Grape Pulp

Treatment of pulped Cabernet sauvignon grapes with Irgazyme M-10, a commercial enzyme preparation, gave better extraction of anthocyanins and leucoanthocyanins than the untreated control samples (Mandzhukov and Velichkov, 1979). Variations in contact time of enzyme preparation with grape pulp between 1 and 5 h and the enzyme concentration of 0.003 to 0.01% at a constant temperature of 30°C and at constant sulphur dioxide concentration of 150 mg/l was studied by the investigators (Mandzhukov and Velichkov, 1979). It has been reported that increasing temperature had a beneficial effect on the maceration process and that increasing contact time had a major effect on the extraction rate of the pigments studied, sulphur dioxide increased the extraction rate only of the anthocyanins and concentration of enzyme preparation concentration had a variable effect. It was found that abundant recycling of the must was important. The wine obtained with the enzyme preparation was of high sensory quality.

Grape Residue

A clean, economic, and efficient method for obtaining anthocyanin-rich extracts from the residue left the following vinification of 3 *Vitis vinifera* grape var. (Cabernet Sauvignon, Ribier, and Carmenere) from Central Chile has been reported. The method, which involved using an active pectolytic enzyme preparation in an aqueous medium, reduced the time required for maceration, setting, and filtration. Enzymic complexes studied were Pectinex BE3-L (pectinesterase, pectinlyase, hemicellulase, and cellulase from *Aspergillus niger*), Vinozyme EC

(pectinase and cellulase from *A. niger* and *Trichoderma longibrachiatum*), and Vinozym G (pectinlyase, polygalacturonase, hemicellulase, and cellulase from *A. niger*). Optimal extraction results were obtained after 2 h of treatment of Ribier grape skin with Vinozyme EC for 2 h gave optimum extraction (Munoz, 2004).

Strawberry

Effect of different milling treatments (hammer mill with screen sizes 1/2–1 inch, milling rates (100–800 rev/min) and pre-press pulp treatments with commercial enzymes viz., pectinases Rohapect D5 B-20 VR: Cellulase 2240: experimental protease EL 57–59) on the extraction of juice and color from frozen strawberries were studied (Flores and Heatherbell, 1984). The optimum pre-press milling conditions and enzyme treatments to release free run juice (FRJ) determined on a laboratory scale were incorporated into pilot plant trials using a Willmes bag press. The nature of the fruit (fresh or frozen), milling speed, and sieve size were found to affect the release of FRJ. Optimum milling conditions for fruits thawed for 3 h at 20 to 23°C were 180 rev/min with a 3/4 inch sieve. Prepress pulp treated with pectinase D5s increased the yield of FRJ from 28% (in untreated pulp) to 56%. However, when press juice was added to FRJ, the total juice yield of 84–87% were obtained with all enzyme treatments. Enzyme treatment increased the anthocyanin extraction by 50%. The amount of pigment extracted varied with the enzyme used and it was maximum with a 1:1 combination of pectinase and D5 protease. All juices had low color (4.1–11.8%) indicating that most of the color is due to monomeric anthocyanins.

Aronia

Dry crushed aronia fruits were preliminarily hydrolyzed using cellulase and pectinase enzymes (0.4% initial raw material mass, 3.68 U of cellulase activity, and 0.82 U of pectinase activity per 1 g of material) and a 5-fold of water containing sulphur dioxide at 50°C for 2 h. Pigment was extracted with 40% ethanol and 2% extract of Trilon in 40% ethanol at 50°C for 20 min. This method of pigment extraction was markedly faster and 25% more efficient than the conventional method which consisted of 8–10 h extraction using 6-fold sulphur of 45% ethanol at room temperature (Kuzmens and Doronin, 1995).

Tomato Products

Extraction of food quality carotenes from tomato paste, puree, and cake using enzymic hydrolysis of initial material using pectophoetidium and celloviridin (0.2–0.5% on initial material mass) has been reported (Dubodel et al., 1995). Enzymic

hydrolysis removed 90% of the nonpigment mass. The product was centrifuged and the coagulate washed with ethanol. This was followed by a two-stage extraction (with ethanol and vegetable oil in the ratio 1:1 and 1:3 for the first and the second extraction, material cooled, and fat phase containing carotene was withdrawn. The colorant obtained was found to be stable for 3 months in the presence of a chelating compound.

Lycopene and other carotenoids were estimated in tomato pomaces during juice manufacture together with the possibility of their extraction from pomace by enzyme treatment. Frozen tomatoes were heated for 2 min at 75°C for 2 min at 95°C and the juice extracted and analyzed for concentration of carotenoids (lycopene, beta-carotene, and lutein) in tomato juices mash and pomaces. In addition, 6 kg batches of tomato pomace from 75°C treated and 95°C were mixed with water and enzyme Rohamet R max (6g) which is a mixture of pectolytic, cellulase and hemicellulase enzymes. Manufacture of tomato juice resulted in 7–27% of tomato carotenoids remaining in pomace. Enzyme treatment of pomace released 38% of the carotenoids from the pomace. The results of the study showed that enzymatic treatment will yield more than 300 mg extractable lycopene from pomace obtained by processing of 100 kg of tomatoes (Dubodel, 1995).

Black Currants

Effect of extensive doses (1000 nkat/g) of various commercial cell wall degrading enzymes on the pressability of wild European bilberries and blackcurrants (cv. Ojebyn) was investigated, with particular emphasis on improving the yield and maximizing the extraction of anthocyanins into the fruit juices (Buchert et al., 2005). Commercial enzyme preparations included: Econase CE; Pectinex Smash; Pectinex BE-3L; Pectinex Ultra SP-L; and Biopectinase CCM. The effect of the enzymic treatment on extraction was more pronounced in blackcurrants compared to bilberries. After the enzymic treatment bilberry juice yields were 116–118%; the highest blackcurrant juice yields were 133–135%. The most efficient enzyme preparation to increase the anthocyanin extraction in bilberry juice was Pectinex BE 3Ls, in blackcurrant juice it was Pectinex Ultra SP-L, and Biopectinase CCM. Results indicated that the enzyme product used during pressing strongly affected both the juice and anthocyanin yields of bilberries and blackcurrants.

Black Currant Juice Press Residue

Enzyme-assisted extraction of phenols and anthocyanins from blackcurrant pomace left over after juice extraction has been reported by Ladbo and Meyer (2001). The study has revealed that release of antioxidant plant phenols and anthocyanin in berry juice processing and from press residues can be optimized via enzyme catalysis. A decrease in particle sizes from 500–1000 μm to less than 125 μm increased the phenol yield. Black currant pomace devoid of seeds gave significant yields

of phenols. Thus there is a potential for the development of tailored enzyme treatment for maximizing antioxidant potency and phenolics in fruit juices.

Carrot

Enzymatic process for the enhanced juice yield from carrot has been optimized. Production of lycopene rich carrot juice from the lycopene rich carrot cv. Nutri-Red. has been reported (Tevini, 2005). Carrots contained approx. 72 mg lycopene / kg and 28 mg beta-carotene/kg. Trials with conventional manufacture of juice from blanched frozen carrots gave the juice with a lycopene content of approx. 57 mg/l. Enzyme treatment of the carrot tissue showed that treatment with a combination of pectinase, cellulase, cellobiase, and pectin lyase increased the lycopene yield by approx. 50%. Large-scale trials with mechanical comminution systems (hammer milling and the Supraton process) gave a lycopene yield of approx. 60 mg/l juice. Treatment of the pressed carrot tissue with enzymes gave an additional juice fraction which contained only 7 mg lycopene/l.

Effects of sodium chloride, acetic acid, and enzymic treatments on the extractability of carrot carotenes were investigated. Carrots were cut longitudinally and treated with sodium chloride (0.6 and 1.2 M) and acetic acid (1.33, 2.67, and 4%) solutions/enzymes (carbohydrases, lipases, and proteinases), either alone or in combination. Results showed that, acetic acid treatment at 4% increased the extractability of alpha- and beta-carotenes up to 99.8 and 94.6% respectively compared to the untreated samples. This increase was directly proportional to the acid concentration. An increase in extractability was also observed with sodium chloride, although the increases were not as high as in the previous case, with values of 49 and 41.4% for alpha- and beta-carotenes, respectively, at 0.6 M concentration. Changes in microstructure and extractability revealed that the enzyme treatments have broken some carotene complexes and interactions altering the carbohydrate matrix structure, resulting in increased carotene extractability. It is concluded that pickling with 4% acetic acid is a good method for increasing the extractability of alpha- and beta-carotenes from carrot.

In another study a process for the increased juice extraction from carrot has been optimized with parameters viz., enzyme, enzyme concentration, and temperature by Sharma, (2005). Enzyme concentration from 50–650 mg/kg of grated carrot, the pectolytic and cellulolytic enzyme ratio 3:7 to 7:3 and incubation time from 30–150 min and a temperature of 25–65°C have been studied on recovery juice from grated carrot. Treatment of grated carrot with a mixture of crude enzyme from *Aspergillus foetidus* and crude cellulolytic enzyme from *trichoderma resi* prior to extraction resulted in increased juice recovery. An enzyme concentration of 210.7 mg/kg grated carrot, a pectolytic and cellulolytic enzyme ratio of 3.84: 6.16, an incubation time of 110 min, and an incubation temperature of 47°C were found

to be optimum with and increase in the yield of juice of 74% with a viscosity of 1.07 cp.

Carrot Spent

Approximately one-third of the raw material used to produce carrot juice, remains as pomace. This is commonly used as feed or fertilizer, but is actually a valuable source of carotenes. Thus, a process for recovery of a carotene-rich functional food ingredient from carrot pomace was developed and optimized (Stoll et al., 2003). The process involved, fine grinding a suspension of carrot pomace in water using a colloid mill and subjecting the comminuted pomace to enzymic hydrolysis, homogenizing and concentrating the carrot pomace hydrolysate. For the enzymic hydrolysis, Pectinex Ultra SP-L (PU; pectinase with hemicellulolytic activities) was combined with either Cellubrix L or Cytolase CL (CE and CY, respectively; cellulolytic activity) were tested at various incubation temperatures. Viscosity was reduced at similar rates at 35, 40, and 45°C when PU was combined with CY. This combination of enzymes was most effective at 50°C, but at 60°C, partial inactivation occurred. In contrast, the reaction temperature had no marked effect on viscosity after digestion with PU and CE. Increasing PU proportions continuously reduced viscosity for 1 h only and in the absence of cellulases, PU was unable to degrade cell walls any further after the solubilization of protopectin. Combinations of PU and CY were more effective than either enzyme alone and a 1:1 ratio was most effective at reducing viscosity. 1500 ppm was found to be the optimum enzyme concentration and 4 as the optimum pH. Addition of 1000 ppm Ca²⁺ markedly accelerated viscosity reduction within 1 h under these conditions. A mesh size of 0.5 mm was chosen over 0.8 mm mesh for finishing as it gave better particle size distribution. The total carotene content of the hydrolysate obtained by the optimized method was 64 mg/kg.

Blueberry Press Cake

Juice processing generates wastes in press cake residue consisting of seeds, stems, and skins. Blueberry press cake contains high amounts of anthocyanins and polyphenols, and is therefore a potential source of natural colorings and nutraceuticals. Effectiveness of temperature, sulphur dioxide, citric acid, and 9 industrial juice-processing enzymes (pectinases, cellulases, hemicellulases) for the production of extracts from cv. Rubel blueberries and blueberry skins rich in anthocyanins and polyphenols were evaluated individually and in combination. Enzyme treatment had little effect on total monomeric anthocyanins and on total phenols recovery. Various combinations of heat, sulphur dioxide, and citric acid yielded extracts with higher concentrations of anthocyanins and total polyphenols compared to untreated control. Distribution of anthocyanins and polyphenols was also investigated. Anthocyanins were present almost exclusively in skins, and polyphenols were present mostly in

skins with lesser amounts in flesh and seeds. Skins exhibited the highest antioxidative activity. All components contained the same individual anthocyanins but in varying amounts. Cinnamic acid derivatives and flavonol-glycosides were found in skins and seeds, whereas flesh contained only cinnamic acids (Lee and Wrolstand, 2004).

Olives

Extraction of oil in three virgin olive varieties (caroleo, Dritta, coratina, and Leccino) was carried out by using a new commercial enzyme Bolivia consisting of pectinases and hemicellulases (Ranalli et al., 2004). Enzyme formulation was added to olive pastes at a concentration of 600 U/kg. Compared to control olive oil samples, enzyme-assisted extracted oils were characterized by higher amounts of major individual phenols (free + aglycons), total phenols, tocopherols, pleasant volatiles, green (chlorophylls and pheophytins) and yellow (carotenoids), lipochromes, beta-carotene, major xanthophylls (lutein, violoxanthin, and neoxanthin), higher aliphatic and triterpene alcohols, phytol, higher values of integral color index, and higher sensory, bitter spicy, and green fruit scorings with lower values of lightness and turbidity.

Effects of three new enzyme preparations cytolase O, Max-oliva, Bioliva during mechanical olive oil extraction of 3 major varieties Dritta, caroleo, and coratina were studied (Ranalli et al., 2003). Use of the above three enzyme formulations increased both yield and quality of the oil with higher concentrations of the natural antioxidants, volatiles, and tocopherols. Application of these 3 enzyme formulations can be adopted by the olive oil producers to obtain oil in higher yield and quality.

Plant Carotenoids

Extraction of carotenoid pigments from orange peel, sweet potatoes, and carrots were assessed using different combinations of commercial enzymes (*Aspergillus niger*, cellulase, pectinase, and polygalactouranase in the concentration of 1–20 ml/100 g pectinase) cellulase (1–10 g substrate and extraction time up to 24 hours (Cinar, 2005). Maximum carotenoid yield was achieved in orange peel by 5 ml pectinase/100 g and cellulase 0.1/100 g for 12 h. Comparable values for sweet potato were 5 ml pectinase/100 g, 1 g cellulase/100 g for 18 h while for carrots, extraction time was more significant than the enzyme concentration. It is reported that higher yields of natural colorants from all the three commodities could be achieved at a lower enzyme concentration by extending the extraction time which results in lowering the cost of enzyme application.

PATENTS ON ENZYME ASSISTED EXTRACTION OF FLAVORS AND COLORS

Flavors

Spices

Enzymatic pretreatment of spices like fresh ginger, garlic, and cloves for the extraction of volatile oil has been worked out. It is reported that enzymatic treatment resulted in increase in the yield of volatile oil (50%) was obtained (Shamala et al., 2003).

Ginger

A process for the preparation of liquid flavor bases from fruit (especially hard to liquefy tropical fruits) and tubers especially ginger has been patented (Brouard-Fenie, 1998). The plant material is liquefied by treatment with an enzyme preparation containing pectinase, cellulase, hemicellulase, and amylo glucosidase. The enzyme reaction is reported to be carried out in an atmosphere of carbon dioxide. The flavor bases prepared by this method can find application in the food industry.

Rose

A process for the production of rose oil by the extraction of fresh rose flower or wastes using enzymes has been patented (Rumyantseva, 1981). Rose flower/wastes are treated in an aqueous medium at a ratio of sample to water 1:1.5 to 3 with enzyme preparation from trichoderma or Geotrichum containing mainly β -glucosidases and polysaccharidases either separately or in combination at a concentration of 0.1 to 0.5% on raw material basis for 2–4 h at a temperature range of 40–45°C followed by hydro-distillation to recover the oil.

Fenugreek

Flavorant is prepared by hydrolyzing fenugreek (*Trigonella foenum*) seeds with enzymes and heating the hydrolyzate to inactivate the enzymes, centrifuging the heated hydrolyzate to separate liquid phase from the residue, and concentrating the liquid phase (Blank Imre et al., 2000). The concentrated liquid phase i.e. flavorant can be dried to get the flavorant in powdered form.

Vanilla

A process has been patented for the production of natural vanilla flavor using enzyme and also for the method of enzymatic extraction of glucovanillin and ethanol soluble aroma compounds (Mane and Zucca, 1993). The enzymatic transformation of vanillin from green pods without the curing step has also been patented (Burnerie, 1998).

Garlic

Crushing the fresh garlic and treating with cellulolytic enzymes followed by steam distillation to obtain garlic volatile oil is reported (Tomoyuki, 1999; Kenkyusho, 1993). It is reported that the garlic oil thus obtained has been used in formulations used for controlling the withering of plants.

Colors

Plant Material

Extraction of pigment from plant materials in general has been patented (Ronald, 1998). The process consists of treating the shredded plant material with an enzyme which breaks down the plant cellular walls releasing the carotenoids from the cell. The enzyme preparation used were cellulase, pectinase, hemicellulase, or a mixture of these enzymes.

Paprika Oleoresin

A high quality color concentrate is obtained from paprika oleoresin by treating the oleoresin with lipase enzyme followed by extraction with an organic solvent with aqueous alkali wherein the fatty acid and odorous ingredient are removed. High yield of stable color concentrate without chilli odor is obtained which finds application in food, pharmaceutical, and cosmetics purposes (Nippon Terpeien, 1987; 1988).

Chilli Oleoresin

Preparation of chilli oleoresin with improved color and pungency by treating the chilli powder with cellulolytic enzymes has been reported (Sampathu et al., 2003). It is reported that treating chilli powder with a mixture of enzymes consisting of cellulase, hemicellulase, pectinase, arabinase, β -glucanase and xylanase incubating, followed by extraction with a binary solvent mixture which on evaporation resulted in oleoresin with an enhanced color and capsaicin content.

CONCLUSIONS

Application of enzymes for flavor and color extraction from plant materials is a new area, which requires more intense research inputs to establish itself as a promising technique. Enzymes-assisted pigment extraction has been worked upon for alfalfa (chlorophyll), chilli (carotenoids), safflower (pre-carthamine), marigold (lutein), strawberry and grapes (anthocyanin), and tomato products (lycopene) using enzyme preparations containing cellulase, hemicellulase, pectinase, and glycosidase. Enzyme pretreatment of mustard expeller cake resulted in enhanced oil recovery. Aqueous pepper extracts with good sensory quality and aroma stable for four weeks could be obtained by the application of combination of cellulase and

Table 3 Application of enzymes for the extraction of flavorant

Plant material	Compound extracted	Enzyme Used	% increase over conventional method	Reference
Mustard	Volatile oil	Cellulase	50	Dobozi et al., 1988
Oregano	Volatile oil	Pectinase + starch hydrolysing enzymes	—	Rokhlenko et al., 1982
Citrus peel	Volatile oil	Hemicellulase + pectinase	—	Coll et al., 1995
Mandarin peel	Volatile oil	Xylan degrading enzymes	15	Mishra et al., 2005
Vanilla	Vanillin	β -glucosidase, pectinase	14-24	Mane and Zucca, 1993 Teran et al., 2001 Pouget, 1990
Rose	Oil	β -glucosidases and carbohydrases 0.1–0.5%	—	Rumyantseva, 1981
Fresh ginger, garlic, cloves	Volatile oil	Cellulase, hemicellulase	30%	Shamala et al., 2003
Wine and must	Aroma Comps	Pectolytic and glycolytic enzymes	—	Granata, 1994

pectinase enzyme preparations. Flavorant preparation from ginger and fenugreek by the application of enzymes has been reported. The work carried out on the above lines has been summarized in Tables 3 and 4.

Application of enzymes for complete extraction of colors and flavors without the use of solvents could be an attractive proposal. Application of enzymes in solvents could be tried for the enhanced extraction of value-added cell constituents like major non-volatile impact component from spices. Advantages of enzyme pretreatment is the reduction in extraction time, minimal usage of solvents, and a product with increased yield and quality. A limitation of this method could be the cost of the en-

zymes. This could be overcome by balancing the concentration of enzyme preparations and tailor-made enzyme preparations for specific reactions. In some commercial applications, crude enzyme preparations can be used which can reduce the cost of the enzyme preparation. The increased yield of value-added products (volatile oils) obtained by the enzyme pretreatments can balance the increased cost of using enzymes. Knowledge of the cell wall compositions of the raw material to be treated helps in the selection of the enzyme and the concentration to be used. Enzymes have been used safely in a wide variety of foods for centuries. The biodiversity of enzymes is providing the food industry with a wide range of functionalities.

Table 4 Application of enzymes for the extraction of colorant

Source	Colour	Enzyme used	% Increase in yield over conventional method	References
Safflower	Carthamin	β -glucosidase	—	Koshi Saito, 1993
Marigold	Lutein	Cellulase, hemicellulase pectinase 0.01–0.1% w/w	2–5 fold increase	Delgadovergas, 1997, 2002
Chilli	carotenoids and capsaicin	Cellulase, hemicellulase, pectinase	carotenoid-11 Capsaicin-7	Santamaria et al., 2000 Sampathu et al., 2006
Alfalfa	chlorophyll	Cellulases, hemicellulase, β -glucanases, xylanases, amyloglucosidase 1%	—	Weinberg et al., 1990
Red grapes	anthocyanins	Pectinases	10–30	Bakker et al., 1999 Nicoloni, and Mattivi, 1997
Strawberry	anthocyanins	Pectinase + protease (1:1)	28	Flores and Heatherbell, 1984
Dry aronia fruits	anthocyanins	Cellulase + Pectinase	25	Kuzmens and Doronin, 1995
Blueberry press cake	Anthocyanins	Cellulase	—	Lee and Wrostrand, 2004
Carrot	Carotenes	Pectinase, cellulase	41–49	Tevini, 2005 Sharma, 2005
Carrot spent	Carotenes	Pectinase + hemicellulase	—	Stall et al., 2003
Tomato	Lycopene	Pectinase, cellulase	20	Dubodel et al., 1995
Olives	Chlorophyll carotenoids	Pectinase + hemicellulase	—	Ranalli, 2003; 2004
Blackcurrant Press cake	Anthocyanin	Cellulase + hemicellulase + Pectinase	—	Landbo and Meyer 2001

FUTURE OUTLOOK

In case of herbs, which are quite sensitive to heat, the drying and extraction of flavor from fresh herbs is time consuming and cumbersome. The enzymatic extraction offers a very good choice of extraction of herbs, which needs to be exploited.

New enzymes with added functionalities can be designed by biotechnology. There is a wide scope for further research in the line for the preparation and evaluation of tailor-made enzymes for the extraction of value added products from the plant materials not exploited so far. Enzyme cocktails obtained by genetical engineering can be highly efficient (with enhanced functionality of the enzyme) requiring low doses for the required effects, thus reducing the cost of enzymes used in the process.

The prospect of enzyme mediated flavor and color extraction from plant materials appear to have a high potential and are promising in terms of future technology.

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REFERENCES

- ASTA. (1997). *Official Analytical Methods of American Spice Trade Association*, 4th Edition, Engelwood Cliffs, New Jersey.
- Bakkar, J., Bell Worthy, S. J., Reader, H. P. and Watkins, S. J. (1999). Effect of enzymes during vinification on colour and sensory properties of port wines. *American Journal of Enology and Viticulture*. **50**(30):271–276.
- Balasubramanian, D., Bryce, C. F. A., Green, J. and Jayaraman, K. (1996). *Concepts in Biotechnology*. Universities Press (India) Limited.
- Barzana, E., Rubio, D., Santamaria, R. I., Garcia, Correa., Garcia, F., Ridaura Saaz, V. E. and Lopez, M. A. (2002). Enzyme mediated solvent extraction of carotenoids from marigold flower (*Tagetes erecta* L), *J. Agric. Food Chem.* **50**(16):4491–4496.
- Baschia, M., Segal, B. and Toma. (1972). Effect of pectolytic enzyme preparation on flavones and anthocyanins of sweet cherries, *Industrial alimentaria*. **23**(12):683–684.
- Blank, I., Jaeger, D., Zurbruggen and Beat, D. (2000). Flavourant prepared from fenugreek (*Trigonella foenum graecum*) seeds US Patent 6,013,289.
- Brouard-Fenie. 1988. Process for production for flavour base. US Patent 2,6A1,19.
- Buchert, J., Koponen, J. M., Suutarinen, M., Mustaranta, A., Lille, M., Torronen, R. and Poutanen, K. (2005). Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and blackcurrant juices. *J. Sci. Food and Agric.* **85**(15):2548–2556.
- Buenrostro, M. and Lopez-Munguia. (1986). Enzymatic extraction of avocado oil. *Biotechnol. Lett.* **8**:505–506.
- Burnerie, P. M. (1998). Process of the production of natural vanilla extracts by enzymatic processing of green vanilla pods and extract thereby obtained. US Patent 5,705,205.
- Cinar I. (2005). Effect of cellulase and pectinase concentration on the colour yield of enzyme extracted plant carotenoids. *Process Biochemistry* **40**(2):945–949.
- Cintra, O., Lopez-Munguia, A. and Vernon, J. (1986). Coconut oil extraction by a new enzymatic process. *J. Food Sci.*, **51**:695–697.
- Clare, S. S., Skurray, G. and Theaud, L. (2002). Effect of a pectolytic enzyme on the colour of red wine. *Australian Grape Power & Winemaker*. **456**:29–35.
- Coll, L., D. Saura., Ruiz, M. P. and Canovas, J. A. (1995). Viscometric control in the enzymatic extraction of citrus peel oils. *Food Control*. **6**(3):143–146.
- Delgado-Vargas, F. and Pardes-Lopez., O. (1997). Effects of enzymatic treatments on carotenoid extraction from Marigold flowers, (*Tagetes erecta*). *Food Chem.* **58**(3):255–258.
- Delgado-Vergas, F. and Pardes-Lopez, G. (2002). Enzyme mediated solvent extraction of carotenoids from marigold flower (*Tagetes erecta*). *Agric. Food Chem.* **50**(16):4491–4496.
- Dignum, M. J. W., Kerler, J. and Vespoorte. (2001). Vanilla production: Technological chemical and biosynthetic aspects. *Food Reviews International*. **17**(2):199–219.
- Dobozi, S., Halasz, A., Kozma-Kovacs, E. and Szacks, G. (1988). Enhancement of mustard oil yield by cellulolytic pretreatment. *Applied Microbiology & Biotechnol.* **29**:39–43.
- Dominquez, H., Nunez, M. J. and Loma, Y. M. (1993). Oil extractability from enzymatically treated soybean and sunflower, range of operational variables. *Food Chem.* **46**:277–284.
- Dominquez, H., Nunez, M. J. and Loma, Y. M. (1994). Enzymatic treatment to enhance oil extraction from fruits and oil seeds: a review. *Food Chem.* **49**: 271–286.
- Dominquez, H., Nunez, M. J. and Loma, J. M. (1995). Enzyme assisted hexane extraction of soyabean oil. *Food Chem.* **54**(2):223–231.
- Dubodel, N. P., Spravink, E. V. and Sikorskaya, W. L. (1995). Isolation of food dye from tomato products. *Pischevaya Promyshlennost* **1**:16.
- Dudai, N., Weinber, Z. G., Larkov, O., Ravid, U., Ashbell, G. and Putievsky, E. (2001). Changes in essential oil during enzyme-assisted ensiling of lemon-grass (*Cymbopogon citrates* Stapf.) and eucalyptus (*Eucalyptus citriodora* Hook). *Journal Agric. Food Chem.* **49**(5):2262–2266.
- Espyszczola, D. (1998). Natural colors: pigments of Imagination. *Food Technology*. **52**(6):70–76.
- Flores, J. and Heatherbell, D. (1984). Optimising enzyme and prepress mash treatment for colour and juice extraction from strawberries. *Fluessiges – Obst*. **51**(7):320–324.
- Freese, S. and Binning, R. (1993). Enzymatic extraction of spice aromas. *Gordian* **93**(11):171–175.
- Fullbrook, P. D. (1983). The use of enzymes in the processing of oil seeds. *J. Am. Oil Chem. Soc.* **60**:476–478.
- Galvin, J. B. and Kirwin, C. G. (1995). Risk assessment of hydrocarbon solvents. *INFORM* **6**(8):951–952.
- Granata, A. (1994). Experiments with new enzymic preparations for aroma extraction. *Vignevini* **21**(7):36–39.
- Hernandez, N., Rodriguez, M. E., Gonzalez, F. and Lopez, M. A. (2000). Enzymatic treatment of rice bran to improve processing. *J. Am. Oil Chem. Soc.* **77**(2):177–180.
- Kenkyusho, R. (1993). Prevent treat filariasis dog administer garlic process powder granule. Patent No. XP-002287412.
- Kuzmens, A. B. and Doronin, A. F. (1995). Extraction of food quality pigment from aronia. *Pischevaya Promyshlennost* **1**:21–22.
- Ladbo, A. K. and Meyer, A. S. (2001). Enzyme – assisted extraction of antioxidant phenols from black currant juice press residues (*Ribes nigrum*). *J. Agric. Food Chem.* **49**(7):3169–3177.
- Lee, J. and Wrolstad, R. E. (2004). Extraction of anthocyanins and polyphenolics from blueberry processing waste. *J. Food Sci.* **69**(7):C564–C573.
- Mamatha Mukhopadhyay. (2000). Natural extracts using super critical carbon dioxide. Pp. 184–192, CRC Press, Boca Raton, FL.
- Mane, J. and Zucca, J. (1993). A process for production of natural vanilla flavour by treatment of vanilla pods and vanilla flavour so produced. French Patent PNFR 2691880AI.

- Mandzhukov-B. and Velichkov, A. (1979). Maceration capacity of the enzyme preparation Irgazym M-10 on grape pulp, *Khranitelna Promishlnost* (Bulgarian) **28**(10):23–25.
- Micard, V., Renard, C. M. G. C. and Thibault, J. (1994). Enzymatic release of ferulic acid from sugar beet pulp, *Food Sci. & Technol.* **27**(59):402–420.
- Mishra, D., Shukla, A. K., Dixit, A. K. and Singh, K. (2005). Aqueous enzymatic extraction of oil from mandarin peels. *J. Oleo Sci.* **54**(6):355–359.
- Muralidharan, A. and Balagopal, C. (1978). Studies on curing vanilla. *Indian Spices*, **10**:3–4.
- Munoz, O., Sepulveda, M. and Schwartz, M. (2004). Effects of enzymatic treatment on anthocyanic pigments from grapes skin from Chilean wine. *Food-Chem.* **87**(4):487–490.
- Navarette-Bolanos, J. L., Jimenez-Islas, H., Bottelo-Alvarez, E., Rico-Martinez, R. and Parades Lopez. (2004). Improving xanthophylls extraction from marigold flower using cellulolytic enzymes. *J. Agric. Food Chem.* **52**(11):3394–3398.
- Navarette Bolanas, J. L. and Jimenez-Islas, H. (2003). Mixed culture optimization for marigold flower ensilage via experimental design and response surface methodology. *J. Agric. Food Chem.* **51**:2206–2211.
- Nicolini, G. and Mattivi, F. (1997). Red grapes vinification with exogenous pectolytic enzymes. *Enotechnico*. **33**(3):65–71.
- Nippon Terpenen, K. K. (1988). Concentration of high quality coloring preparation from paprika by heating with lipase and extracting with paraffin hydrocarbon. Japanese Patent No. 88,030,346.
- Nippon Terpenen. (1987). Concentration of paprika dye stuff preparation under treating with lipase, then fractionally extracting with paraffin organic solvent. Japanese Patent No. 62,115,067.
- Ovando, S. L., Waliszewski, K. N. and Pardo, V. T. (2005). The effect of hydration time and ethanol concentration on the rate of hydrolysis of extracted vanilla beans by commercial cellulase preparations. *International J. Food Sci. & Technol.* **40**(9):1011–1018.
- Prevention of Food Adulteration Act*, 4th Edition, International Law Book Company, New Delhi, 2008.
- Pomeranz, Y. and Meloan, E. C. (1978). Enzymatic methods. In: *Food analysis – Theory and Practice*. Pp. 472–475. AVI Publishing Company, Inc. USA.
- Pouget, M. P., Pourrat, A. and Pourrat, H. (1990). Recovery of flavour enhancers from vanilla pod residues by fermentation. *Food Sci & Technol.* **23**:1–3.
- Purohit, S. S. and Mathur, S. K. (1999). *Biotechnology-Fundamentals and Applications*, Agro Botanica, Annis Offset Printers, New Delhi, India.
- Ramachandra Rao and Ravishankar, G. A. (2000). Vanilla flavour: production by conventional and biotechnological routes. *J. Sci. Food & Agric.* **80**:289–304.
- Ravindran, P. N. and Madhusoodanan, L. J. (2002). *Cardamom: The genus Ellectoria*. Taylor & Francis Inc., New York, 232–236.
- Ranalli, A., Lucera, L., Contento, S., Simone, N. and Re, P. del. (2004). Bioactive constituents, flavours and aroma of virgin oils obtained by processing olives with a natural enzyme extract. *European Journal of Lipid Science and Technology*. **106**(3):187–197.
- Ranalli, A., Malfatti, A., Pollastri, L., Contento and Lucera, L. (2003). Analytical quality and genuiness of enzyme extracted virgin olive oil. *Journal of Food Quality*. **26**(2):149–64.
- Rkhlenko, S. S., Grebeshova, R. N., Lutskaya, B. P. and Slavutskaya, N. I. (1982). Production of aroma compounds from plant raw materials. *Fermennaya-I-Spirtovaya-Promyshlennost, (Russian)* **3**:11–12.
- Ronard, T. (1998). Extraction of pigment from plant material. US Patent 5,830,78.
- Rosenthal, A., Pyle, D. L. and Niranjana, K. (1996). Aqueous and enzymatic process for edible oil extraction : review. *Enzyme and Microbial Technol.* **19**:402–420.
- Rouhana, A. and Manneheim, C. H. (1994). Enzymatic peeling of grape fruits. *Food Sci. Technol.* **27**:103–107.
- Rumyantseva. (1981). Process for producing rose oil. US Patent 4,301,251.
- Saito, K. (1993). A new enzymatic method for the extraction of precarthamine from dyer's saffron florets. *Zeitschrift fur Lebensmittel-Untersuchung und Forschung* **197**:34–36.
- Saito, K. and Murata, T. (1994). The influence of thermal treatment on the stability of safflower yellow B. *Food Chem.* **51**:307–310.
- Santamaria, R., Reyes, Duarte, M. D., Barzana, E., Fernando, D., Gama, F. M., Mota, M. and Lopez-Munguia, A. (2000). Selective enzyme-mediated extraction of capsaicinoids and carotenoids from Chilli Guajillo Puya (*capsicum annum L.*) using ethanol as solvent., *J. Agric. Food Chem.* **48**:3063–3067.
- Sampathu, S. R., Madhava Naidu, M., Sowbhagya, H. B., Pura Naik, J. and Krishnamurthy, N. (2006). A process for making chilli oleoresin of improved quality, US Patent No. 7097867.
- Shamala, T. R., Manonmani, H., Venkateswaran, G., Sowbhagya, H. B., Sampathu, S. R. and Joseph, R. (2003). An enzymatic process for the preparation of spice oil WO2995063953.
- Sharma, A. K., Sarkar, B. C. and Sharma, H. K. (2005). Optimization of enzymatic process parameters for increased juice yield from carrot (*Daucus carota L.*) using response surface methodology. *European Food Research and Technology*. **221**(1–2):106–112.
- Sowbhagya, H. B., Sampathu, S. R. and Krishnamurthy, N. (2002). Natural colourant from marigold-chemistry & Technology. *Food Reviews International* **20**(1):33–50.
- Stoll, T., Schweiggert, U., Schieber, A. and Carle, R. (2003). Process for the recovery of a carotene rich functional food ingredient from carrot pomace by enzymatic liquification. *Innovative Food Science and Emerging Technologies*. **4**(4):415–423.
- Tateo, F. (1979). Uses of enzymes in the production of food flavouring extracts. *Enzyme & Microbial Technol.* **1**(4):282–286.
- Teran, R. F., Amador, P. I. and Munguia, L. A. (2001). Enzymatic extraction and transformation of glucovanillin to vanillin from vanilla green pods. *J. Agric. Food chem.* **49**(11):5207–5209.
- Tevini, M., Delong, H., Heene, E. and Schmitt, A. (2005). New type of carrot juice made from lycopene containing carrots. *Flussig-Obst.* **72**(4):176–177.
- Tol Soffer, Chaim and Manneheim, C. H. (1994). Enzymatic peeling of orange and Pomelo, *Food Sci. Technol.* **27**:245–248.
- Tomoyuki, N. (1999). Control of plant withering and activating agent. Patent No. 112222410.
- Walford, J. (1980). *Historical developments of food colorants*, In: *Developments in Food Colors* Vol. 1. Applied Science Publisher, Ltd. London. 1980, 193–194.
- Weinberg, Z. G., Szkacs, G., Linden, J. C. and Tengerdy, R. P. (1990). Recovery of protein and chlorophyll from alfalfa by simultaneous lactic acid fermentation and enzyme hydrolysis (ENLAC), *Enzyme Microbial Technol.* **12**:921–925.
- Yoon, J. M., Cho, M. H., Park, J. E., Kim, Y. H., Hahn, T. R. and Paik, Y. S. (2003). Thermal stability of the pigments Hydroxysafflor Yellow A, Safflor Yellow B, and precarthamine from Safflower. *J. Food Sci.* **68**(3):839–843.
- Zorn, H., Breithaupt, E. D., Jackenberg, M., Schwack, W. and Berger, R. G. (2003). Enzymatic hydrolysis of carotenoid esters of marigold flowers (*Tagetes erecta L.*) and red paprika (*capsicum annum L.*) by commercial Lipases and plearotus sapidus extracellular lipase. *Enzyme and Microbial Technol.* **32**(5):623–628.