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Module – 26: Enzyme Application for Fruits and Vegetables, Brewing and Wine Processing

26.1 Enzyme application for Fruits and Vegetables Processing

Enzymes play a major role in the quality of fresh fruits and vegetables. Enzymes are very important for growth and ripening of fruits and vegetables and are active after harvesting and during storage. While most of the enzymes present in plant tissues are important for the maintenance of metabolism, some have also undesirable effects on colour, texture, flavour, odour, and nutritional value.

Processors wishing to produce clear concentrated fruit juices must overcome many challenges, including handling different kinds of fruits in the same factory, and managing fluctuating quantities of fruit with variable compositions and textures depending on their ripening stage. Dealing with these factors requires efficient and reliable tools, such as equipment and enzymes. Enzymes are processing aids used worldwide for fruit processing, particularly for the production of clear fruit juice and concentrate. They offer numerous advantages:

- **Economic**: Enzymes increase the overall productivity of the processing plant. The juice remains stable, long term, without additives or preservatives, storage volumes are reduced and shipping weight reduced.
- **Quality**: Fast juice processing with enzymes lowers the risk of microbial spoilage, reduces oxidation and improves juice and concentrates shelf life. Pectin hydrolysis of fruit cell walls weakens cells and vacuoles and thereby maximizes extraction of their components such as the red colour from berries (anthocyanins), aromas and antioxidants of phenolic type known for their positive effect on human health, particularly heart disease prevention.
- **Sustainability**: The use of enzymes has a positive effect on sustainable production. They lower energy consumption (electricity, steam and water), reduce waste flow by maximizing fruit use and reduce dependency on chemicals used in equipment cleaning products.

Vegetable juices are most commonly made from tomato, carrot and red beet. Vegetable Juice is one commercial example, made mainly from tomatoes and the juices of other additional vegetables like beets, celery, carrots, lettuce, parsley, watercress and spinach. Consumer demand for vegetable juices is increasing and fruit and vegetable juice combinations are becoming more popular. Numerous products came on the market. These new drinks are cloudy and pulpy. It may be easy to produce tomato juice without enzymes, but this is not the case for carrot, leek or

cabbage. In fruit, pectin is the major component to break down in order to facilitate juice extraction. But vegetables also have a high content of dietary ligno-cellulosic fibres, which hinder juice extraction. Vegetable juice processing therefore requires more cellulases in addition to pectinases to reduce viscosity sufficiently for juice extraction using a decanter.

Rapidase is recommended for vegetable juice extraction. It contains pectinases and cellulases. After blanching (to limit oxidation), crushed vegetables are cooled to 50^{o}C and mixed with enzymes. After 1–2 h, the juice is extracted using a decanter, pasteurized and possibly concentrated. The juice's carotene content is not decreased by enzymes, and the cloud is stable for the duration of the juice's shelf life.

26.1.1 New Developments in the Application of Enzymes in Fruit and Vegetable Processing

In recent years many new developments have been observed in the application of enzymes in fruit and vegetable processing. Several examples are summarized below.

26.1.1.1 Citrus peeling

The first step in the preparation of citrus juices is the peeling of the fruits. This is a mechanical process requiring energy. Pectinases are used to soften the peel by disruption of the albedo and thereby facilitate a significant reduction in energy costs. Current industrial practice is starting with pectinase treatment of whole fruit, followed by a vacuum infusion treatment with a pectinase solution like Rapidase Intense and Peelzyme containing pectinesterase and polygalacturonase; thereafter the peel can be removed easily (1–2 hours) and the enzyme solution can be recycled.

26.1.1.2 Whole fruits

Processing of whole fruit or fruit parts requires several precautions to safeguard the firmness of the fruits. Pectins consist of very complex structures giving strength to fruits but are sensitive to mechanical pressure (shear), heating (chemical hydrolysis), pasteurization, storage (polymer dehydration),and osmotic pressure. Moreover, most pectinase preparations consist of multiple enzymes leading to weakening of the pectin polymers.

For example, demethylation by pectin methyl esterase (PME) exposes the homogalacturonan backbone, which will be further degraded by enzymes like polygalacturonase (PG) and rhamnogalacturonase (RG), causing physical weakening of fruits. The Firm Fruit concept is based on the use of a PG and RG-free PME in combination with calcium, which binds to the freed pectic acid *in situ* to form insoluble calcium pectates.

Other developments are the inhibition of PG by plant-born PG inhibitors thereby preventing PG activity in the fungal enzyme mixtures.

26.1.1.3 Immobilized enzymes

Traditionally, enzymes in the fruit and vegetable processing industry are applied as liquids or powders, while enzymes in pharma are often immobilized. Although it adds an additional step (and thus costs) in the preparation of the enzyme, improved characteristics like a lower pH optimum and increased half-life (through recycling) can turn this into an attractive opportunity. First examples are shown for polygalacturonase and tannase.

26.1.1.4 Preventing haze formation

There are several ways to clarify extracted juices. In fruit juices, this is traditionally done with bentonite, silica gel, or gelatine followed by filtration. Although these methods do work, they remove a considerable amount of the antioxidant phenolics, which can form haze-causing interactions with the proteins present. The addition of gallic acid in combination with various proteases also reduced the haze formation drastically, but retained much more of the beneficial phenolics. The best-performing enzyme is Enzeco Fungal Acid Protease produced from *A. niger*, which reduces only 12% of the phenolics, while the traditional methods reduce up to 30%.

26.1.1.5 Preventing cloud loss

To produce cloudy juices, the process from extraction to pasteurization must be fast to minimize the effect of plant endogenous enzymes like pectinases and oxidases. Besides active inhibition of the plant enzymes by virtue of their inhibitors, α-amylases and gluco amylases can be added to reduce the starch content, as dissolved starch retrograde during cooled storage and can form precipitates.

26.1.2 Future Scope

Enzymes, enzyme production and enzyme applications are fine-tuned by using the latest technologies like directed evolution, codon optimization, and specialty products (like clever enzyme mixtures), respectively. This will lead to further improvements in taste, shelf life, and nutritional value of the derived products. Moreover, after adaptation of the processing conditions to these new enzymes and/or enzyme mixtures, the sustainability of the fruit and vegetable industry will be improved as the yields will be higher, while the waste streams and the net energy consumption will reduce.

26.2 Enzyme Application for Brewing

Enzymes from the raw materials used in brewing play an extremely important role in the process, but due to natural variations, these enzyme levels can vary significantly in the raw materials. Therefore enzymes allow more tolerance in the quality of the raw materials and the conditions of the brewing process.

26.2.1 Enzymes in malting

During the malting process the enzymes native to the barley (amylases, glucanases, proteases and hemicellulases) are activated. As a result the malted barley is made more amenable to milling and starch/carbohydrate extraction.

Apart from the endogenous enzymes present in malt (and barley), no exogenous enzymes are applied in the malting process. The key target of the maltster is to generate a well-modified, homogeneous malt. The modification relates to the enzymatic conversion of the endosperm. As the enzymes are released through the aleurone layer, it will be clear that the modification gradually moves on through the endosperm as germination time progresses. The maltster has to find an optimal limit for the extent the modification (germination) proceeds, as this occurs at the expense of valuable sugars for the germinating kernel and thus of the yield of this process.

26.2.2 Enzymes in mashing

During the malting process some enzymes have already been active in degrading the various polymeric substrates, such as starch, glucan, protein and many more low molecular weight components (e.g. oil). However, particularly in case of under modified malt or low quality heterogeneous malt, exogenous (added) enzymes such as proteases, amylases and glucanases can help to increase the degradation of these polymeric substrates.

The most important benefit of using exogenous enzymes is that they give the brewer the required tolerance in using his malt, particularly as the mashing process is a more homogenous process than malting.

26.2.3 Protein converting enzymes

Mashing usually starts with the degradation of malt protein (about 10% w/w in malt) by means of proteases and peptidases, as a result of their lower thermo stability compared to the other enzymes involved. Therefore lower temperatures (e.g. 45-55°C) are usually applied at this stage. These enzymes are referred to respectively as exo- and endo-protease and produce, respectively, free amino acids and peptides (oligomeric amino acids), and smaller proteins (thereby making them more soluble). This process step is also referred to as 'the proteolytic stand'. During the malting process some protein degradation has already taken place, but particularly in the case of under modified malt (or low quality heterogeneous malt), exogenous

enzymes can help to increase the free amino nitrogen (FAN), which is required during fermentation as the nitrogen source for the yeast. However, some care should be taken in applying excessive protein degradation, as this can cause excessive colour formation (through the Maillard reaction) and may affect the foam potential of the final beer.

26.2.4 Cell wall degrading enzymes

Degradation of cell walls is carried out by glucanases. The main problem with incomplete cell wall degradation, as is the case with heterogeneous and/or under modified malt, is the negative impact these polysaccharides can have on the mash filtration (lautering) and beer filtration process steps, and also on the colloidal stability (haze) of the final beer. The most important polysaccharide to degrade is β-glucan. This polymer contributes most to the viscosity of wort, and in beer it has the tendency to form insoluble complexes. Xylan is usually already sufficiently degraded in the malt. Any residual xylan will also be degraded by the side activities present in commercial glucanase preparations. Barley (malt) glucan consists mainly of β 1–4 glucose units, interspersed with β 1-3 linkages. The commercial β-glucanases contain activities to degrade this type of glucan and are therefore called β 1-3, 1-4 glucanase.

26.2.5 Starch-converting enzymes

Complete starch conversion is carried out by a range of amylolytic enzymes. Amylose consists of glucose units linked through a 1-4 linkages, while amylopectin also has a 1-6 linkages, making it into a branched polymer. The amylolytic enzymes in malt are α-amylase , β-amylase and limit dextrinase. From microbial origin, the enzymes pullulanase and amylo glucosidase can be added. α-Amylase splits the polymer in an 'endo-' manner, and therefore leaves a considerable amount of branched maltodextrins from amylopectin. α-amylase splits only maltose units from the (non-reducing) end of the polymer (exo-activity). It can act on both amylose and amylopectin, but stops if it encounters an α 1-6 link. While β-amylase is less stable than α -amylase, limit dextnnase is even less stable.

26.2.6 Enzymes in lautering/mash filtration

Lautering and other forms of mash filtration are usually performed at so-called 'mashing-off' temperatures of 75–78°C. Almost all malt enzymes are already inactivated at these temperatures. Only a small amount of residual α-amylase activity will be present where a relatively low mash filtration temperature is used.

If large quantities of adjunct or under modified malt are used, starch may be released late in the mashing cycle. The amylolytic enzymes only show a limited amount of activity at this stage during mashing; this could result in starch polymers being carried over into the filtered wort, especially if using sparging water at temperatures above 75°C (where α-amylase is rapidly inactivated).

Lower sparging temperatures could minimize these problems but would have a negative effect on wort viscosity. Addition of thermo stable exogenous α-amylase during mashing can reduce this wort-starch problem. At the same time it allows the brewer to use higher sparging temperatures with the accompanying benefits.

26.2.7 Enzymes in fermentation

Exogenous enzymes are used in fermentation to help prevent difficulties occurring later in the process. In case of beer filtration problems, β-glucanase can be added to the fermenter (or during maturation), degrading residual glucans which would otherwise cause the filters to block. Another area for enzyme application during fermentation is to reduce haze problems in the final beer. After analyzing the type of haze material (which can be done using enzymes), fungal α -amylase or β-glucanase (for respectively starch or glucan haze) can be applied.

26.2.8 Low calorie beer production

Since the malt-derived limit-dextrinase is not very temperature stable, a large amount of non-fermentable dextrins will be present in finished beer, as brewer's yeast is not able to convert these branched malto-dextrins. Together with other beer components such as proteins, these dextrins are responsible for the mouth feel and fullness of the beer and also contribute heavily to its caloric value. Low calorie beer can be produced with the application of exogenous amyloglucosidase during fermentation, which will degrade dextrins to fermentable sugars. In this way normal alcohol/low dextrin beer can be made from wort containing a reduced amount of extract.

One step further is the production of low alcohol or non-alcohol low calorie beer where the application of exogenous amyloglucosidase is combined with the fermentation of low extract-containing wort or with removal of alcohol, for example by vacuum distillation.

26.2.9 Enzymes in maturation

26.2.9.1 Enzymes to correct problems (filtration and beer haze)

Exogenous enzymes can correct incomplete degradation of starches and glucans causing beer filtration problems or haze problems in the final beer. Exogenous enzymes can also be used during maturation. The point of addition is best upon transfer to the maturation tank, so that the mixing of the enzyme is controlled.

26.2.9.2 Enhanced maturation with acetolactate decarboxylase (ALDC)

A number of conversions resulting in flavour changes are taking place during maturation. One of the aims of maturation is to 're-absorb' diacetyl, which is considered a (buttery) off-flavour in lager beer. This component is produced by the yeast during the main fermentation.

The spontaneous conversion of the precursor α-acetolacate into diacetyl is particularly slow. This reaction can be speeded up by raising the temperature at the end of the primary fermentation, but this will also increase the rate of other reactions, some of which are undesirable. Therefore a microbial enzyme *(Bacillus)* was developed to convert α -acetolactate into acetoin, before it can be converted into diacetyl. This enzyme is called α-acetolactate decarboxylase (ALDC).

26.2.9.3 Chill proofing enzyme

If no preventive actions are taken, beer will loose its clarity quite quickly. Apart from the earlier mentioned glucan hazes (permanent haze), so called 'chill haze' can play a major role in beer. The chill haze particles usually develop through the complexing of proteins and polyphenols (also called tannins). This process is reversible upon heating and cooling, hence the name 'chill haze'. Therefore strategies have been developed to increase the colloidal stability.

The enzymatic approach is to degrade the haze-forming proteins so the protein-polyphenol complexes will not be able to aggregate over time and the beer thus remains clear. Typically, the enzyme selected for this purpose is papain.

26.2.10 Future developments

One of the major concerns in brewing has been how to control beer flavor stability. Nowadays there are many methods for controlling colloidal stability of beer (chill proofing) which has led to colloidal stability in excess of a year. However strategies controlling flavour stability are still scarce. One of the ideas in the past was to apply an oxygen scavenging enzyme, such as glucose oxidase, into the bottle of beer. Although technically possible, the idea of having an active enzyme in the final beer was not very appealing. Alternative ways have been since developed to limit oxygen in the bottle with improved packaging technology

26.3 Enzyme Application for Wine Processing

The production of wine is a biotechnological process marked with tradition. However, research into winemaking is providing an understanding of the biochemical reactions that occur in grape juice during its transformation into wine. Enzymes play a definitive role in the process of winemaking. Indeed, wine can be considered the product of enzymatic transformation of the grape juice. Enzyme activity is required as early as possible in the pre-fermentation stage. The enzymes originate not only from the grape itself, but also from yeast sand fungi and other microorganisms. Moreover, the action of such endogenous enzymes is nowadays reinforced and extended by the use of exogenous industrial enzymes. Enzymes coming from adventitious microorganisms infecting the grapes may also play a role. The applications of industrial enzymes are discussed bellow.

26.3.1 Applications of enzymes

Enzyme preparations are used throughout the whole winemaking process:

- On the grapes (weakening, maceration)
- In the must and the press wine (clarification and sedimentation)
- In the young wine at the end of the fermentation

Enzymes are added to the process in different ways. In all cases solutions are made of the enzyme (granular enzyme preparations are also readily soluble).

Actual addition proceeds via:

- Spraying the enzyme solution on the grapes
- Dosing the solution via a pump system directly into the inlet of the press
- Adding the solution to the tank before clarification.

Due to their specific character and action, enzymes release molecules that play a role in the taste characteristics of wine (colour, aroma, structure) or break down molecules that limit the effect and efficiency of technological treatments, like pressing, clarification and filtration. Better control of fermentation processes and enzyme production has lead to the possibility of purifying enzymes, for example, excluding the presence of cinnamyl esterase.

Simultaneous use of bentonite and enzymes should be avoided because the enzyme will be inhibited due to specific adsorption to the bentonite and the bentonite will become less effective due to blocking of the active sites by enzyme protein. Bentonite treatment should preferably take place after the enzyme treatment. Addition of bentonite will help in flocculation of enzymatically hydrolyzed pectins.

The activity and efficiency of an enzyme can vary enormously, depending on temperature and pH. Must pectinases can be used in the temperature range of 10–55°C. Below 10°C the enzyme dosage should be increased. Above 55°Cthe enzyme will be inactivated. β-Glucanase can only be used above 15°C and requires a longer incubation time. Enzyme dosages should be also increased at low pH values.

Enzymes are not inhibited by sulfur dioxide (SO_2) at levels that are acceptable in wine. Inhibition by polyphenols may occur in red wines. Increasing the enzyme dosage can counteract the effect. Alcohol up to a level of 14% (w/v) has no negative influence on enzyme action. In fact it has been reported that alcohol has an activating effect on β-glucanases used for aroma liberation.

26.3.2 Enzymes for pressing and maceration

Increased understanding of the way enzymes act on the polysaccharides of the grape cell walls, such as arabinans, galactans, arabinogalactans and arabinoxylans, makes it possible to optimize the usage of enzymes in winemaking.

The mechanism of action of pectolytic enzyme preparations, usually called pectinases. As far as is currently known, the enzyme activities involved in hydrolyzing pectic substances are pectin esterase, polygalacturonase, pectin lyase, rhamnogalacturonase, rhamnogalacturonan acetylesterase, arabinase and galactanase.

Other enzyme activities are of hemicellulase and cellulase type and are normally present in varying amounts in pectinase preparations. The combined action of all these enzymes leads to a partial hydrolysis and solubilization of acid and neutral polysaccharides present in the pectocellulose wall and middle lamella of the grape cells. These structures are responsible for the characteristic stiffness of the berries. The result of this hydrolysis is the release of juice, aromas and colour in a selective and controlled way. The remaining cell wall fragments are solubilized in the colloidal wine solution.

26.3.3 Enzyme preparations for aroma liberation

The varietal character of (white) wines is especially defined by the presence of aromatic molecules, among which mono terpene alcohols play a well-defined role. These compounds are found in grapes as free, volatile, odourous molecules, and as non-volatile glycosidic precursors, also known as 'bound terpenes'. In many grape varieties the amount of bound terpenes can be higher than the amount of free aromatic terpenols. Consequently, the distinctive character of wines could be increased by releasing the glycosidically-bound terpenes.

Enzyme preparations containing glycosidase activity were developed to improve the aromatic profile of certain wines. These enzyme preparations are added at the end of the

alcoholic fermentation and during 'retapping' of wines (pouring into another vessel), which are not treated with bentonite, in order to prevent enzyme inhibition. The optimal temperature for this enzyme treatment should be above 15°C and an incubation time of several weeks to one month is required. The aroma development has to be controlled by tasting, and the enzyme action is finally inhibited by addition of bentonite. Small amounts of this compound (20 g/hl) are usually sufficient to block all enzyme activity.

Since the mechanism of hydrolysis of terpenyl glycosides has been elucidated, various different fungal enzyme preparations have been developed capable of enhancing wine aroma. *Aspergillus* spp. Pectinase and glucanase preparations have good results on wine making conditions. These enzymes can be used in fermented juice as soon as glucose has been consumed by the yeast, or in young wines. In this way the variety in aroma and bouquet of certain wines can be improved.

26.3.4 Enzymes for colour extraction

The extraction of phenolic compounds generally occurs with the maceration of the mash during alcoholic fermentation and depends on the variety and quality of the grapes and on technological parameters such as crashing, maceration time, temperature and pumping. Even though colour extraction trials are difficult to conduct because of the heterogeneity of the mash and the influence of processing factors, good results have been obtained on an industrial scale using enzymatic treatments. Improved anthocyanin extraction was obtained by enzyme addition at the beginning of maceration. However, more fundamental research is required to understand all mechanisms involved in colour extraction fully.

26.3.5 Other enzymes used in winemaking

26.3.5.1 Urease

An enzyme preparation containing the enzyme urease was developed in Japan. Urease catalyses the hydrolysis of urea to carbon dioxide and ammonia. This limits the formation of ethyl carbamate. This molecule, suspected to be carcinogenic and mutagenic at high dose rates, is predominantly urea. The enzyme is produced by *Lactobacillus fermentum.*

26.3.5.2 Lysozyme

Microbial stabilization of wines is accomplished by the addition of sulfur dioxide. Stabilization is guaranteed by lowering the sulfur dioxide level in combination with treatment with lysozyme. Lysozyme is derived from egg white protein and is used in the pharmaceutical and food industries.

The enzyme has the ability to break down the cell walls of lactic bacteria and its activity increases with increasing pH, Lysozyme treatment is recommended for:

- Blocking the malolactic fermentation in white wines
- Stabilizing red wines after fermentation
- Treatment of problematic end stages of alcoholic fermentation
- Preventing premature malolactic fermentation in wines

26.3.6 New developments

Recent investigations have stimulated interest in another group of enzymes, oxidases. In particular, a polyphenol oxidase originating from the fungus *Myceliophtera thermophile* and produced by *Aspergillus oryzae* has been developed for application in the wine industry.

26.3.6.1 Cork cleaning

This application deals with the corks used for closing wine bottles. The enzyme removes phenolic compounds (which can give an off-flavour to the wines) and is applied during cleaning of the corks. This enzyme treatment significantly reduces the amount of water-extractable total polyphenols in comparison with crude, unwashed corks. It is comparable with chlorine washing, but has the advantage that the level of trichloro anisol (TCA) is reduced instead of increased, as is the case with chlorine treatment.

26.3.6.2 Colour stabilization

Removal of phenolic compounds to prevent oxidation, a haze and flavour change is usually undertaken with fining agents such as gelatin or polyvinyl pyrrolidone. Treatment with enzymes such as laccases, tannases and peroxidases has been investigated for juice or wine stabilization. Fungal laccase has ability to react with a wide range of phenolic compounds. Laccase treatment can enhance the effect of conventional fining treatments.