BIOPROCESSING OF GRAPE POMACE

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DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

25/11/99

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SUMMARY

Grape pomace is the primary by-product that is generated during grape juice processing and wine production. It consists of fibrous materials that include the processed skins, stems and seeds. Beneficial in many ways, it can be used as compost, animal feed, food fibre, or a source of tartrates and citric acid. Stricter environmental regulations, however, necessitated efforts to rethink methods of recycling pomace into useful products to reduce the removal and operating costs. Due to the potential use of bio-ethanol, this study was aimed at evaluating the potential recovery of ethanol from grape pomace. We therefore investigated the potential of microbial hydrolysis and fermentation of grape pomace to explore the economic benefits of bioprocessing.

Naturally occurring microorganisms associated with pomace were isolated and their ability to degrade pure polysaccharides was evaluated. The results indicated nine yeast, eight bacterial, one actinomycete and three fungal species displaying varying levels and types of enzyme activity for the hydrolysis of polysaccharides. Only positive yeast isolates were further identified as they are known for their ability to ferment sugars to alcohol. Since grape pomace consists of the four major polysaccharides in nature, i.e. cellulose, hemicellulose, pectin and starch, proper hydrolysis thereof could make the monosaccharides available to microorganisms for alcoholic fermentation. The extent of hydrolysis, types of degradation products and ethanol production were determined.

The yeast *Pichia rhodanensis*, when compared to two recombinant strains of *Saccharomyces cerevisiae* containing several genes for saccharolytic enzymes, showed particularly promising results with the absorbency assays. However, this assay method did not provide any information on the specific enzyme activities or the type of sugars released. Methods to determine an organisms' ability to degrade pomace polysaccharides other than by the sugars released and the ethanol produced, are required. The economic viability of hydrolysing pomace with *P. rhodanensis* and the industrial application thereof should be further evaluated and optimised. If a cost-effective biodegradation process can be

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developed, it could then be applied to other agricultural by-products and contribute to ethanol production for commercial interest.

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OPSOMMING

Stingels, sade en doppe is die primêre afvalprodukte wat gedurende die prosessering van druiwesap en wyn gegenereer word. Hierdie veselagtige materiaal word gunstig as kompos, dierevoeding, vesel, of as 'n bron van sitroensuur en wynsteensuur aangewend. Druiwe-afval veroorsaak egter omgewingsbesoedeling en strenger maatreëls noodsaak alternatiewe metodes om dit in bruikbare produkte te omskep om sodoende die verwydering en bestuurskoste te verminder. Die potensiële gebruik van bio-ethanol noodsaak hierdie studie om druiwe-afval vir die herwinning van etanol te evalueer. Die mikrobiese hidrolise en fermentasie van druiwe-afval is dus ondersoek om die ekonomiese voordele van bio-prosessering te ondersoek.

Die mikroorganismes wat natuurlik met druiwe-afval geassosieerd is, is geïsoleer en hul vermoë om suiwer polisakkariede te hidroliseer is evalueer. Resultate het nege gis, agt bakterieë, een aktinomiseet en drie fungi spesies aangedui met uiteenlopende ensiem tipes en aktiwiteite vir die hidrolise van polisakkariede. Slegs die positiewe gis-isolate is verder geïdentifiseer, aangesien giste bekend is vir hul vermoë om suikers na alkohol te fermenteer. Druiwe-afval bestaan uit die vier vernaamde polisakkariede in die natuur, naamlik sellulose, hemisellulose, pektien en stysel. Die volledige hidrolise van hierdie polisakkaride kan die monosakkariede aan mikroorganismes beskikbaar stel vir moontlike alkoholiese fermentasie. Die mate van hidrolise, die soort afbraakprodukte en die etanol produksie is bepaal.

Die gis *Pichia rhodanensis* het belowende resultate met die absorbansie toetse getoon, in vergelyking met die rekombinante rasse van *Saccharomyces cerevisiae* wat verskeie ensiemgene vir polisakkaried hidrolise bevat. Hierdie toets het egter geen inligting oor die spesifieke ensiem aktiwiteite of tipe suikers wat vrygestel is, voorsien nie. Metodes om ‘n organismes se vermoë om polisakkariede in druiwe-afval te hidroliseer buiten die vrygestelde suikers en etanol produksie word benodig. Die ekonomiese prosessering van die druiwe-afval met *P. rhodanensis* en die industriële toepassing moet verder evalueer en optimiseer word. Indien ‘n koste-effektiewe biologiese proseseringsproses ontwikkel kan

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word kan dit ook op ander landbou afvalprodukte toegepas word en tot die produksie van etanol vir kommersiële gebruik bydra.

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BIOGRAPHICAL SKETCH

Lundi Joy Issel was born in Worcester, South Africa, on the 27th of April 1976. She matriculated at the Esselen Park Secondary School in 1993. Lundi enrolled at the University of Stellenbosch in 1994 and obtained a B.Sc. degree in Microbiology and Biochemistry in 1996, as well as a Hons. B.Sc. degree in Microbiology in 1997. In 1998, she enrolled for a M.Sc. degree (Microbiology) at the same university and was awarded the master's equity scholarship from the Foundation for Research Development (FRD).

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This thesis is dedicated to my mother and Zurial-Pablo, for always listening to the thoughts and feelings behind my words.

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INTRODUCTION

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INTRODUCTION

Large amounts of plant by-products are discarded by several agricultural activities and result in the occupancy of potential valuable space and environmental pollution (Amerine *et al.*, 1972). Large amounts of grape pomace, consisting of processed skins, seeds and stems, is produced annually by the wine industry (Hang, 1988). Many factors contribute to the quantity and quality of the grape that could be in turn beneficial or detrimental to the pomace. The most important factors contributing to grape pomace quality are the structure of the vine and grape, external environmental and nutritional factors, the microorganisms present during growth, changes in the biochemical content of the grape, harvesting and processing (Zoecklein *et al.*, 1995).

Grape pomace consists in part of structurally complex polysaccharides as well as residual levels of sucrose, fructose and traces of other reducing sugars. The polysaccharides cellulose, hemicellulose and pectin are closely packed together in structures to provide form and support to the plant cell (Glazer and Nikaido, 1995). Starch is stored and used as an energy reserve in the plant cells (Mullins *et al.*, 1992; Weaver, 1976). These polysaccharides provide some protection, contributing to the plant's resistance to degradation by invading microorganisms (Warren, 1996).

The complete degradation of plant cells is required in some industries, especially when large amounts of agricultural waste are being generated. Various types of degradation can be used such as enzymatic, physical, chemical or microbial methods. Since microorganisms are predominantly responsible for the degradation of polysaccharides in nature, they have proven to be potential sources of hydrolytic enzymes (Glazer and Nikaido, 1995; Prescott *et al.*, 1993). Although the yeast *Saccharomyces cerevisiae* is well known for its ability to produce ethanol from various fermentable sugars, it is not able to hydrolyse complex polysaccharides (Glazer and Nikaido, 1995). The genes encoding some of the enzymes required for the hydrolysis of polysaccharides have been cloned and expressed in *S. cerevisiae* to degrade polysaccharides to mono- and disaccharides (Pretorius, 1997). The ability of two recombinant strains to hydrolyse purified cellulose

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was previously demonstrated in synthetic media, and were evaluated in this study for their ability to hydrolyse grape pomace.

The use of the natural microbial populations for the bioconversion of plant material has steadily increased in economic and environmental demand and some instances has replaced the use of the purified commercial enzymes in the industry (Kubicek *et al.*, 1993; Pretorius, 1997). The aim of this study was therefore to isolate organisms that occur naturally on grape pomace and to evaluate their ability to hydrolyse the polysaccharides present in the pomace and utilise the resulting sugars as fermentable substrates. This could be beneficial to the treatment of the wastes and lead to new applications in the wine industry that could more efficiently meet the continuing demand for ethanol. The following research strategies were employed:

1. Isolate microorganisms that occur naturally on the grape pomace and evaluate their ability to hydrolyse the polysaccharides present in grape pomace.
2. Evaluate the ability of existing recombinant strains of *S. cerevisiae* to hydrolyse polysaccharides present in pomace.
3. Characterisation of the successful yeast candidates capable of hydrolysis.
4. Evaluate and optimise the production of ethanol from grape pomace.

The thesis begins with a literature review to set the scene for grape pomace as a potential for waste product recovery and to discuss the various factors, especially the chemical and microbial factors, contributing to the quantity and quality of the pomace. This is followed by the experimental work that is presented as a research article to be submitted for publication. The research on the bioprocessing of grape pomace was presented at various local and international meetings, i.e. the 2nd International Congress of the Federation of African Societies of Biochemistry and Molecular Biology and 15th Congress of the South African Society of Biology at Potchefstroom in 1998, and the 19th International Conference on Yeast Genetics and Molecular Biology in Italy, 1999.

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LITERATURE REVIEW

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SETTING THE SCENE FOR GRAPE POMACE

THE HISTORY OF THE GRAPE

The growing of grapes and the making of wine come to us out of the abyss of antiquity and have a prominent place in the history of civilisation. The ancients gave an importance to wine that greatly exceeded its role as a beverage. They regarded wine as a gift from the Gods that possessed mystical importance. Many centuries ago the need for sacramental wine flourished in the Christian community and led to an association between grape growing and the Church. Due to the traditions of viticulture and the mythology of wine, the grapevine has a privileged position among cultivated plants (Mullins *et al.*, 1992; Vedprakash *et al.*, 1995).

Archaeologists found fossil leaves and grape seeds that revealed that the grape has been a food of man from earliest times. It is therefore thought that the vine originated between 6000 BC and 4000 BC in Asia Minor, in the north Persian areas near the Caspian Sea, and thereafter spread to other regions (Weaver, 1976; Winkler *et al.*, 1974). The history of winemaking can, however, be traced to the time of the Pharaohs in Egypt. It is therefore most likely that with the development of village settlements, the history of viticulture (grape growing) and enology (the art and science of winemaking) began (Mullins *et al.*, 1992). The grapevine has long been known for its juicy, sweet fruit that can be eaten, but is commonly used for the fermentation of wine. Many million acres of land throughout the world (cultivated on all continents except Antarctica) are planted with the grapevine. The area used for the cultivation of the grapevine is still increasing, as it is the world's most widely grown fruit plant.

Several names have been proposed for the grapevine family, but the accepted family designation under the International Code of Botanical Nomenclature is *Vitaceae* (Vedprakash *et al.*, 1995). *Vitaceae* are mostly woody or herbaceous tree-climbing plants or shrubs with tree-climbing-like stems and is primarily inter-tropical in its distribution. The *Vitaceae* family comprises of ten genera of which only the *Vitis* class is economically suitable for cultivation (KWV, 1975). The genus *Vitis* contains about 60 species of which *Vitis vinifera* (the grape of antiquity often mentioned in the Bible)

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is the most important and the only species of European origin (Peynaud and Ribéreau-Gayon, 1971; Winkler *et al.*, 1974). *V. vinifera* is subdivided into many varieties, bearing black or green grapes, and is used in most vineyards for the production of grapes and good quality wines (Vedprakash *et al.*, 1995).

Grapes can be divided into five main classes depending on their purpose. The table varieties utilised for food and decorative purposes, wine grapes (the majority are grown for this purpose) and the dried or raisin grapes where seedlessness is preferred are mainly used by mankind. The other two classes are the juice grapes (manufacturing of sweet unfermented juice) and canning grapes (only seedless grapes used as fruit salad or fruit cocktail) (Winkler *et al.,* 1974; Weaver, 1976). Grapes therefore have many uses: fresh fruits, dried fruit (raisins), fresh grape juice, concentrated grape juice, table wines, sparkling wines, champagne, fortified wines (sherry, port) and distilled liquors from wine (cognac, armagnac, brandy) (Vedprakash *et al.*, 1995). In addition, there are several industrial applications of grapes and grape products, including grape-seed oil and ethanol production. However, more than 80% of the world's grape crop is used for wine production (Mullins *et al.*, 1992).

Since the earliest times, factors such as climate, location and soil have greatly influenced viticulture and can have a significant effect on the colour, flavour and composition of wine (KWV, 1975). The grape grower has the option to select from the general types of varieties which one may be best adapted to its climate, grow them properly, control the size of the crop and harvest at the optimum stage. Other factors such as crop hazards, mineral nutrition and the type of processing used in the wineries should also be considered to produce the best quality and quantity of wine.

Constitution and Development of the Grape

Structure of the vine

The grapevine (*Vitis vinifera*) is a perennial, deciduous creeper (KWV, 1975; Mullins *et al.*, 1992) that is severely pruned to reduce bunch number and increase fruit size and fruit quality. The biological character of this complex plant includes vigour, regenerative capability, stress tolerance and longevity. The vine has developed separate

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parts with specified functions that can be divided into the underground portion which is the root system, and the portion above ground that include the trunk, arms and shoots (Vedprakash *et al*., 1995; Weaver, 1976). The shoots consist mainly of stems, leaves and flowers that produce the seeds and the fruits, whereas the trunk, roots, shoots and leaves maintain viability (Figure 1).

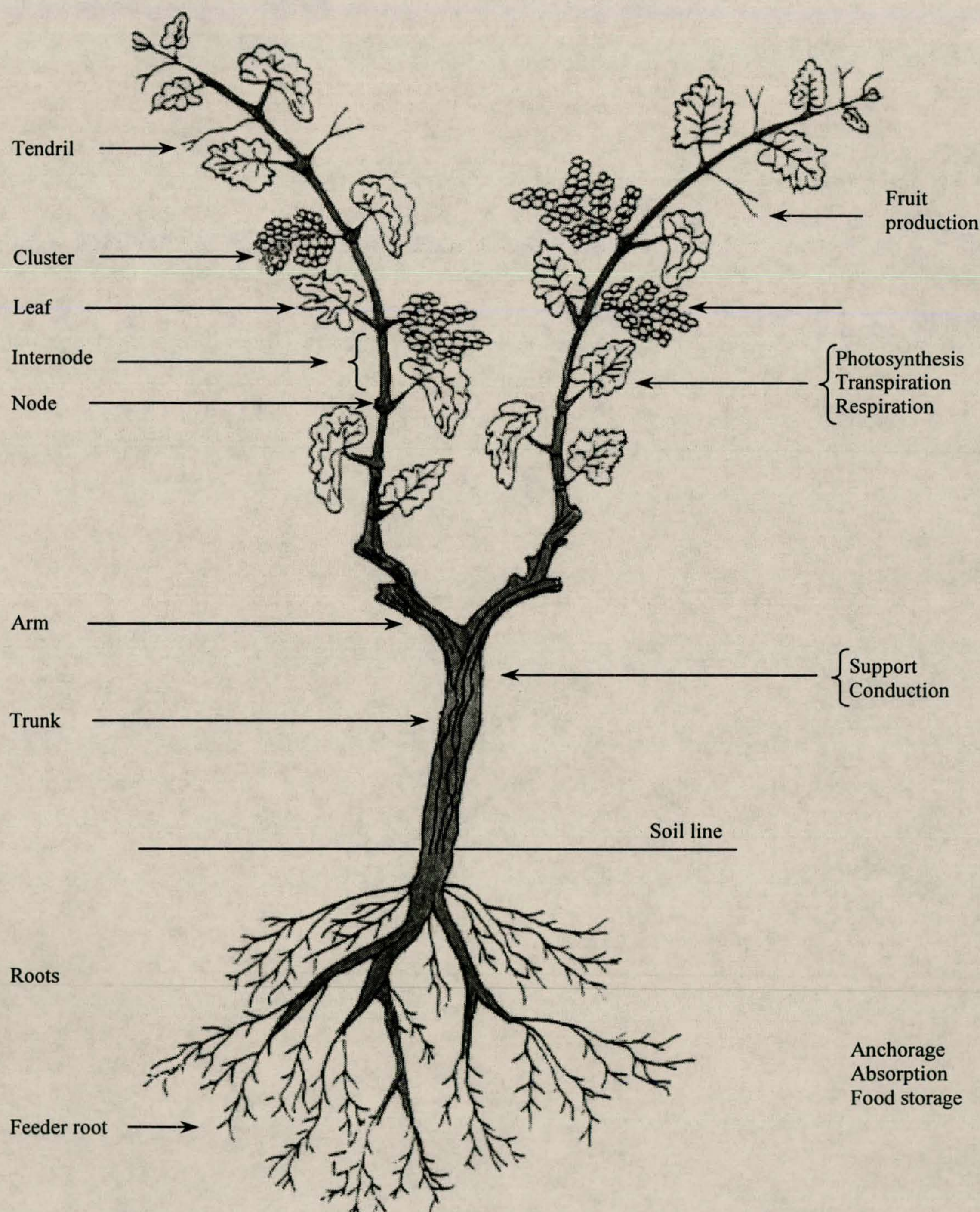

Figure 1. Diagrammatic illustration showing important structures and functions of a grapevine (Weaver, 1976).

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Roots of the grapevine often penetrates to a depth of three meters or more and spread laterally in the soil and the finest roots, the feeder roots, greatly increase the absorption region of the roots (Vedprakash *et al.*, 1995). The character of the system includes the ability to regenerate new roots and to store organic nutrients that include amino acids and it must cope with water stress, water-logging, ionic imbalance and toxic ions (Mullins *et al.*, 1992). The root is therefore a major component in terms of absorption of water and mineral nutrients, storage of reserves and anchorage (Winkler *et al.*, 1974).

The stem of the vine supports the leaves and the upper vine parts and is the connecting link between the top of the vine and the roots. It conveys nutrients and water absorbed by the roots from the soil to the plant (KWV, 1975; Vedprakash *et al.*, 1995), as well as food materials from the foliage to the roots (Weaver, 1976). The grape cluster also consists of stems (rachis, branches, pedicles) on which the berries are borne and differs in variety, length of parts, toughness and adherence to the berries. This stem structure consists of 2 to 6 percent of the total weight when the grape matures.

The shoot is the succulent stem with leaves and serves as the bearer of the flowers and the fruit (Vedprakash *et al.*, 1995; Weaver, 1976). Leaves develop just behind the growth points on young roots from a bud. The primary functions of the leaf are photosynthesis and transpiration (Mullins *et al.*, 1992; Weaver, 1976). The leaves play an important role in feeding the vine and also protect the grapes against the heat of the sun (KWV, 1975).

The flower and the fruit comprise the reproductive parts of the vine. Flowers occur in bunches and when fully grown, pollen is released onto the stigmas and fertilisation takes place in the ovary (KWV, 1975; Mullins *et al.*, 1992). This results in the fruit of the vine, the berry, consisting of 2.5-5% stalk and 95-97.5% berry in weight (Frumkin, 1996). The berry consists of skin, pulp and seeds as will be discussed in the following sections.

ANATOMY OF THE BERRY

Each berry (Figure 2) consists of a thin and elastic epicarp (the skin), a juicy and fleshy mesocarp (the pulp), and an endocarp, indistinguishable from the pulp, that surrounds the carpels containing the seeds (Jackson, 1994; Peynaud and Ribéreau-Gayon, 1971).

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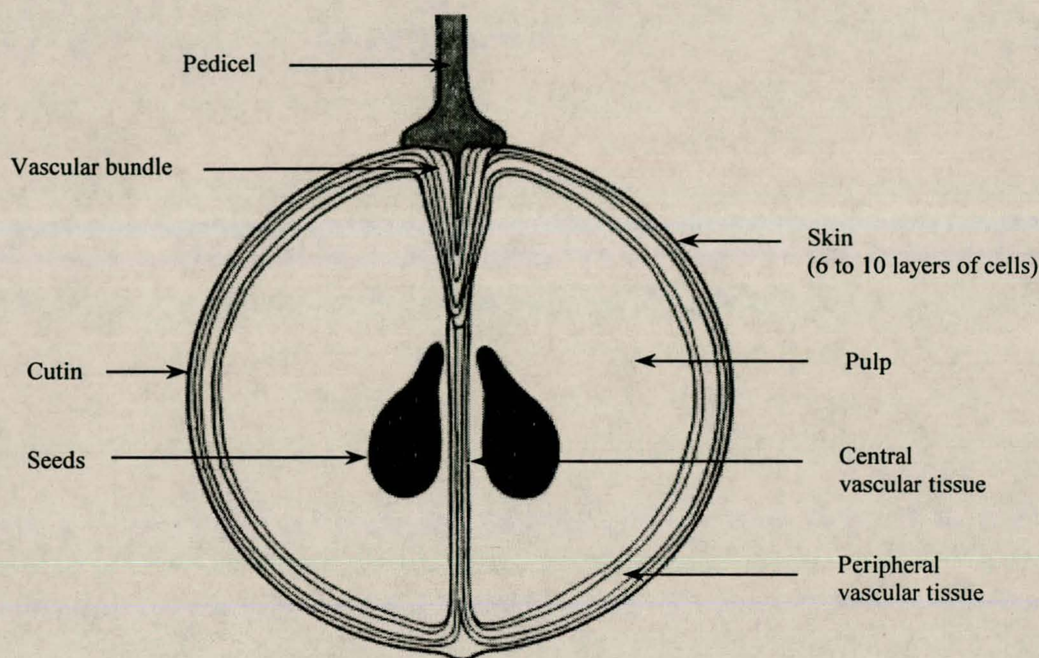

Figure 2. Cross-section of the grape berry (Peynaud and Ribéreau-Gayon, 1971).

The skin consists of an epiderm and several underlying layers of cells and comprises about 5 to 12% of the native grape cluster (Lavee and Nir, 1986; Winkler *et al.*, 1974). It is composed of 6 to 10 layers of small, thick-walled cells and consists of 78-80% water and 1-2% tannin (Frumkin, 1996). The most characteristic components of the skin are the yellow and red pigments, leucoanthocyanins, and other compounds largely responsible for the flavour, aroma and colouring of the fruit (Vedprakash *et al.*, 1995).

The pulp consists of 25-30 layers of cells with large vacuoles containing the cell sap (Lavee and Nir, 1986) and accounts for 80-90% of the crushed grape (Vedprakash *et al.*, 1995; Weaver, 1976). Inside the berry there are 10 to 12 vascular bundles that supply the nutrients and translocate the sap through the stem, peduncle and pedicle into the berry. These bundles divide into peripheral bundles that branch out within the pulp, and into axial bundles that feed the seeds and the endocarp (Peynaud and Ribéreau-Gayon, 1971). The pulp contains 78-80% water, 10-25% sugar, acids, mineral matters, pectins and heterogeneous matters (Frumkin, 1996).

The seeds consists of 0-5% of the weight of crushed grapes. The grape berry normally contains four seeds, originating from the four ovules of the ovary. The seed number

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may, however, vary between zero and four per berry (Lavee and Nir, 1986; Vedprakash *et al*., 1995) due to the absence or abortion of one or several ovules. The development of the seeds influences the size and composition of the grapes (Winkler *et al*., 1974), because the seeds produce gibberellins and certain hormones that diffuse into the flesh of the berry and stimulate growth (Hulme, 1970). If the number of seeds presence increases, the weight of the berry will be heavier, the sugar content will lower, and the acidity levels will increase (Peynaud and Ribéreau-Gayon, 1971; Weaver, 1976). The seeds or pips do not play roles in the vinification process unless they are actually crushed. The pips contain 36-40% water, 10-12% oils and 7-8% tannin (Frumkin, 1996; Vedprakash *et al*., 1995).

LIFE CYCLE OF THE VINE

DEVELOPMENT OF THE VINE

The grapevine has a fairly predictable cycle of growth that can conveniently be divided into dormancy, bud break, bloom, fruit-set, veriason, fruit maturation and leaf fall (Figure 3) (Mullins *et al*., 1992).

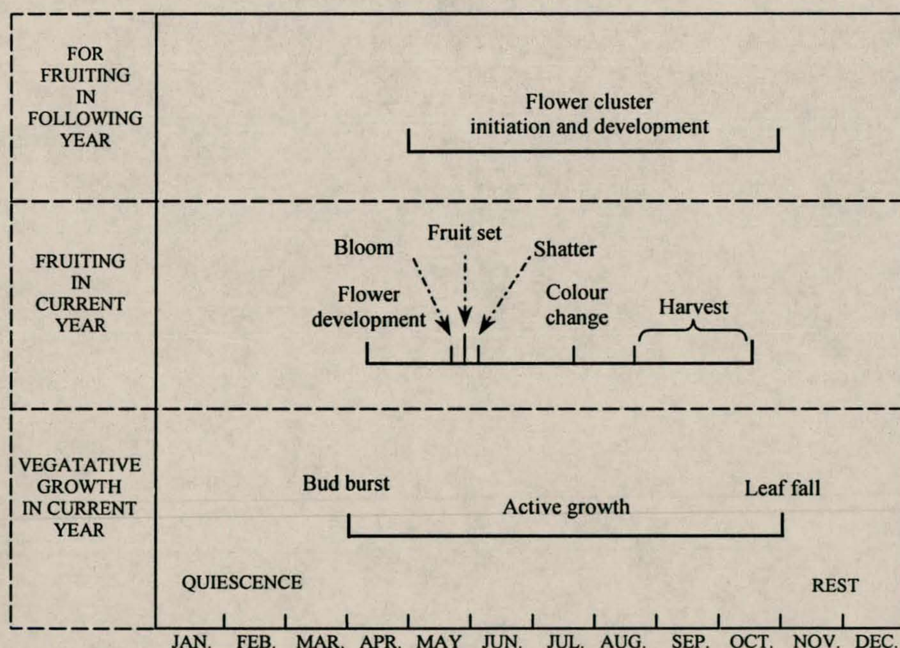

Figure 3. Calendar showing the stages in the growth of a grapevine in an average year in the Northern Hemisphere (Weaver, 1976).

The dormant season commences after the vine has shed its leaves and the daily temperature reaches about 10°C (Mullins *et al*., 1992; Winkler *et al*., 1974). In this

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period, the starch that is synthesised by the shoots of the previous season, is converted to sugars and protects the vine against low temperature injuring (Weaver, 1976). The low temperature and unfavourable external conditions prevent the outgrowth of buds in the vineyard. **Bud break** usually occurs when the daily mean maximum temperature exceeds 10°C. The buds begin to swell and green shoots come out into view; this is known as bud break. The shoots grow in length and thickness, and leaves and new buds develop (Weaver, 1976). After the third or fourth leaf is visible, bunches are present in the new buds.

Bloom is initiated when the calyptras fall from the flower and pollination occurs. The new berries that become clearly visible and remain on the cluster after bloom (transformation of flowers into fruits) are referred to as the fruit-set stage or "nouaison" (Weaver, 1976). Fruit maturation or **"veraison"** occurs when the shoot growth stops and the new bud develops and ripens (Peynaud and Ribéreau-Gayon, 1971). Colour changes are the first noticeable sign as the berries tend to lose their chlorophyll to result in yellow or dark grapes. As the berry swells, the firmness decreases and the cell wall becomes more elastic and translucent (Weaver, 1976).

The grape continues to swell (**fruit maturation**) and the entire vine begins to build up reserves. After shoot growth slows down, carbohydrates accumulate. As the rate of shoot growth almost stops, berry growth slows down and carbohydrate accumulation increases. Carbohydrates (mostly starch) and mineral nutrients are stored in the berries as reserves that are usually utilised at night and after the leaves fall in the autumn (dormant season) (Peynaud and Ribéreau-Gayon, 1971). The vegetative cycle ends with the **fall of leaves**. This takes place after harvest and the shoots colour turns brown due to the lignification of starch (Peynaud and Ribéreau-Gayon, 1971).

Development of the grape berry ("Veraison")

The reproductive biology of the grape is of fundamental importance to man. The agricultural and horticultural industries depend upon the production of flowers, fruits and seeds, therefore, the flowering and fruiting of the grape are the key processes to ensure the transfer of genetic material from one generation to the next (Marshall and Grace, 1992).

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The growth and development of the grape can be interpreted by means of its structure and composition as it is a fleshy fruit. The degree of development of the innermost layer of the pericarp – the endocarp – remains membranous. The growth of the fruit is measured by the fresh and dry weight and is sampled at intervals during the period of growth. Measuring attributes such as diameter and volume cannot be accounted for due to varieties in the size of the berries (Bollard, 1970). Growth rate characteristics also differ remarkably between seasons and between the different varieties, but grape growth normally follows a double sigmoidal type of curve (Mullins *et al.*, 1992; Winkler *et al.*, 1974). The three growth phases are frequently designated as periods I, II and III. These growth periods from anthesis to maturity, is approximately 100 days (Figure 4) (Lavee and Nir, 1986; Weaver, 1976).

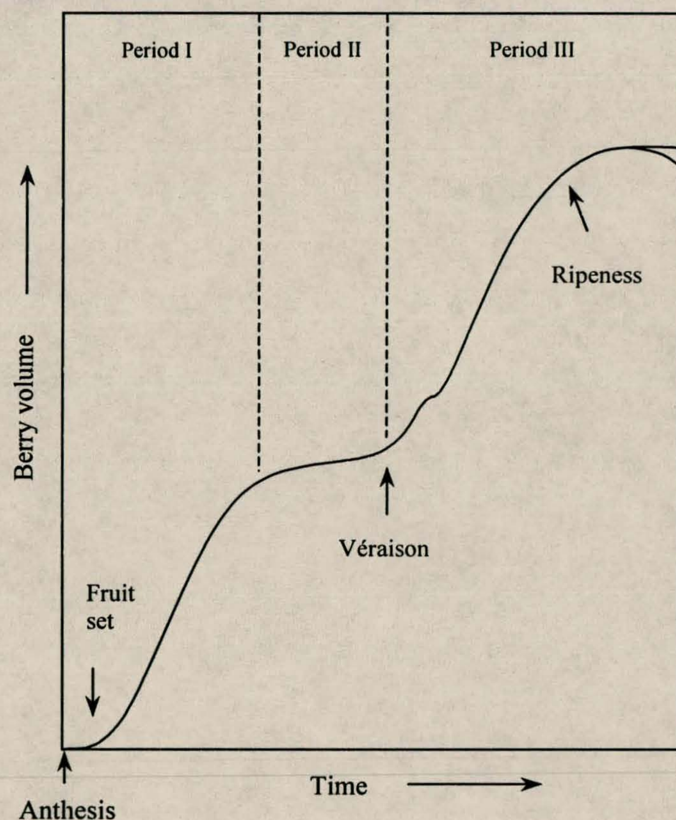

Figure 4. Diagram of the growth curve of a grape berry, showing the extent of the three stages and the location of veraison (Weaver, 1976).

Period I usually last 5 to 7 weeks in most grape varieties. During this initial period of growth, the pericarp and seed increase in size and weight (Mullins *et al.*, 1992). Rapid cell division, followed by rapid cell enlargement occurs in the pericarp and the endocarp

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and seed reaches almost full size (Lavee and Nir, 1986). The nucellus and integument grow rapidly and cease growth at the same time as the mesocarp. Little development of the embryo occurs during this period. The berries are characterised by rapid acid accumulation that accounts for the low pH in the flesh and remain green and hard (firm). Glucose is present in larger amounts than fructose and predominates during the growth of the berry. Glucose is used in growth (production of new tissues in vegetative parts), the production of other food materials or accumulates as a source of food for the vine (Winkler *et al.*, 1974).

Period II generally lasts 2 to 4 weeks (Winkler *et al.*, 1974). The overall growth rate has slowed down markedly due to the retardation in mesocarp growth and there is an initial rapid hardening of the endocarp. The embryo develops rapidly and may, depending on the species, reach maximum size during this period. The berries reach their highest level of acidity, begin to accumulate sugar, lose their chlorophyll, but remain a hard green organ until the end of peroid II when the colour begins to fade (Lavee and Nir, 1986; Mullins *et al.*, 1992; Weaver, 1976).

Period III is a period of final swell in which growth in size and weight resumes a rate approaching that of period I (Weaver, 1976). There is usually an increase in both cell size and amount of intercellular space in the flesh. Ripening of the fruit occurs towards the end of this period. The period is characterised by the softening of texture, a decrease in acid accumulation, aroma development, and the changing of skin colour in red and black varieties (Peynaud and Ribéreau-Gayon, 1971). The storage of hexoses in grape intensifies in the third phase and the concentration of glucose and fructose in the fruit juice increases and reach equal proportions. This period normally lasts 5 to 8 weeks (Winkler *et al.*, 1974).

Overripe stage: The juice of the berry becomes more concentrated due to the sugar increase, acidity decrease and the evaporation of water (Peynaud and Ribéreau-Gayon, 1971). The berries begin to use up all their reserves, and due to the discontinuation of sugar accumulation, shrivel and shatter, and finally dry and raisin (Lavee and Nir, 1986). During this stage, the sweet taste of fructose predominates, making the grapes more alluring for the fungi and insects for easy attack. Allowing the grapes to become over-ripe can, however, produce specialised wines of high quality (Weaver, 1976).

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FACTORS AFFECTING THE GROWTH OF THE GRAPE

Several factors may influence the composition and the quality of the grape during the growth to the harvest to the wineries. These include environmental and viticultural factors, such as the macro-, meso- and micro-climate, the genotype, vine management, soil and water conditions and the competition of pests and diseases (Figure 5) (Vedprakash *et al.*, 1995; Zoeklein *et al.*, 1995). The type of harvest, shipping and packaging methods, as well as the processing methods used in the wineries, contribute to the quality (the size, colour and texture) and chemical composition of the grape. Pruning, trimming, manuring and soil improvements are in the hands of the grower. Other factors, such as the variety and rootstock, the age of the vine, the nature and porosity of the soil, climatic conditions, etc., cannot be manipulated by the grower once the vines have been planted (Peynaud and Ribéreau-Gayon, 1971; Weaver, 1976).

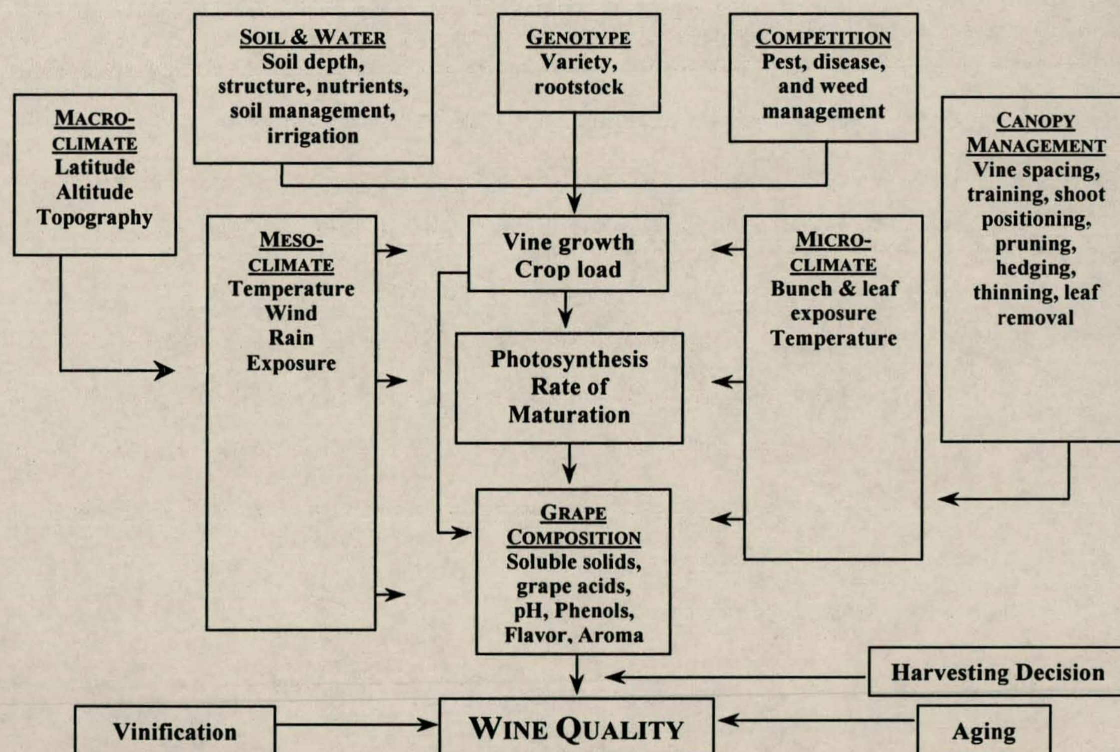

Figure 5. Environmental and viticultural factors that influence grape composition and wine quality (Zoecklein *et al.*, 1995).

EXTERNAL FACTORS

The grape flowers at a particular season of the year, implying that flowering is influenced and perhaps controlled by changes in the environment, specifically the

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temperature. The vine has certain needs that include an ideal climate and ideal soil types which constitute the two most important external factors that determine the choice of grape variety to be planted (KWV, 1975). Climate affects both the vine survival and wine quality. It influences the rates of change in the constituents of the fruit during the development and the composition at maturity. The climate can be further divided into three types that include the macro- or regional, meso- or site, and micro- or grapevine canopy climate (Zoecklein *et al.*, 1995).

The macro-climate that influences the vine include the height above sea level, high mountain ranges, distance from the equator and the distance to the sea or large lakes. The grapevine can be grown between the 28th and the 48th parallels in both hemispheres and from sea level to an elevation of 3000 feet (Peynaud and Ribéreau-Gayon, 1971). These climatic variations greatly affect the distribution of the vineyards in the different continents. Temperature is the most important meso- and microclimatic factor and is essential to the growth, yield and ripening of the vine (KWV, 1975). The grape is a highly adaptable species, but requires long, warm-to-hot, dry summers and cool winters. The vine grows optimally at temperatures of $\pm 18°C$. High temperatures or temperatures below $-27°C$ usually result in the damage of the vine and susceptibility to certain pests that may flourish. Temperature varies, however, form one area to the next or from farm to farm, and result in the differences observed in grape and wine quality. Other factors such as rainfall, moisture, frost, hail, snow, the amount of sunshine and the prevailing winds have much more limited effects on the vine (Mullins *et al.*, 1992; Zoecklein *et al.*, 1995).

Rain is beneficial to viticulture during the dormant period, but otherwise may result in poor berry-set, the rotting of fruit and the difficulty to control diseases and pests (Winkler *et al.*, 1974). The amount and time of rainfall definitely restrict the production of certain grapes (for example raisins) to certain areas. The amount of sunlight is usually determined by the presence of plant cover. Grapevines do not tolerate shade, as the production of fruit requires full sunlight. Increasing the shade cause flowers to fall and partial shade result in the inhibition of fruit bud formation. This is usually due to nearby planted trees which could result in the reduction in the effectiveness in leaf area (McGrew, 1994). The leaves are therefore the major cause of variation in production of berries. Exposure of the berries to sunlight are generally associated with soluble solids,

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titratable acidity, pH, malate concentrations, amount of proline, potassium, arginine and phenols (Zoecklein *et al*., 1995). Strong winds may cause the dehydration of the vines and may cause damage by stripping the vine of its shoots and bunches (KWV, 1975).

The nature of the soil is also of great importance in determining the choice of grape variety to be planted. The most significant factors include the depth, texture, water and nutrient supply (Jackson, 1994; Weaver, 1976). The grape is highly adaptable to a wide range of soil types and is commercially grown throughout the world. It is, however, preferred to avoid very shallow soil, heavy clays, poorly drained soil, and soil that has high concentrations of alkali salts and other toxic material (Winkler *et al*., 1974). The best grape results usually occur with deeper, more fertile soils, but too much water in the soils (caused by heavy rainfall) can influence the size and flavour of the grape (KWV, 1975).

BIOLOGICAL FACTORS

The vine is the source of organic matter and is literally covered with microorganisms. A diverse spectrum of organisms are associated with the leaves, stems, flowers, seeds and roots of the vine and may have a direct or indirect influence on the vine (Winkler *et al*., 1974). These can be spoilage organisms that are inhabitants of the grapes, vineyards or the winery equipment. Some of these organisms can be classified according to the part of the vine they "attack" or by the disease they cause (Weaver, 1976). Some may have little or no impact on the final product or could even contribute to better grape and wine quality. Some spoilage organisms infect the vineyard and change the characteristics of the grape. Others might be seen as crop hazards in that they could be detrimental to the grapes or be the cause of undesirable substances that could lead to the damage of the wine quality. Fungi, bacteria, viruses and insects are the smaller organisms, but larger vineyard pests (fruit flies, rodents) can inflict severe damage on vines and/or fruits (Table 1) (Weaver, 1976).

Although numerous diseases are present, the symptoms exhibited by the grape vary with the type of vine being attacked (Weaver, 1976). Some grapevines can, however, be symptomless carriers of a disease that could affect vineyard productivity devastatingly. These diseases can either be of local importance (limited to one farm) or be serious problems in all grape-growing areas. Diseases are sometimes difficult to control once

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they become firmly established in the vine and grape-producing area. Diseases that infect the grapevine are not transmissible by pollen or seed, but by grafting. The common diseases and different pests are listed in Table 1, and only the most important viral, bacterial and fungal diseases will be discussed in view of the symptoms they cause.

Table 1. The common and scientific names of diseases that infect the grapevine (Mullins *et al.*, 1992; Nel, 1983; Weaver, 1976; Winkler *et al.*, 1974).

Type of disease	Common name	Scientific name
Viral diseases	Fan leaf	Grapevine fanleaf virus
	Leaf roll	Unknown
	Corky bark	Unknown
	Yellow vein	
	Yellow speckle	
Bacterial diseases	Pierce's disease	*Xylella fastidiosa*
	Crown gall or Black knot	*Agrobacterium tumefaciens*
	Bacterial blight	*Xanthomona ampelina*
Fungal diseases	Armillaria root rot	*Armillaria mellea*
	Eutypia Dieback	*Eutypia armeniaceae*
	Black measles	Unknown
	Collar rot	Unknown
	Esca	Unknown
	Dead-arm	*Phomopsis viticola*
	Verticillium wilt	*Verticillium dahliae*
	Powdery mildew	*Uncinula necator*
	Downy mildew	*Plasmopora viticola*
	Botrytis bunch rot and blight	*Botrytis cinerea*
	Phomopsis cane and leaf spot	*Phomopsis viticola*
	Woodrot	*Eutypia armeniaceae*
	Anthracnose	*Elisnoë ampelina*
Mite	European red mite	*Panonychus ulmi*
	Erinose mite & vine bud mite	*Colomerus vitis*
	Common red spider	*Tetranychus cinnabarinus*
Insects	Fruit fly	*Ceratitus capitata*
	Phylloxera	*Phylloxera vitifoliae*
	Mealybug	*Planococcus ficus*
		Pseudococcus obscurus
	Snout beatles	*Eremnus cerealis*
		Eremnus setulosus
		Phlyctinus callosus
	Scale insects	
	Bollworm	*Heliothis armigera*
	Fruit-piercing moths	*Servodes partita*
	Black rot	*Guignardia bidwellii*
Others	Rootknot Nematodes	*Meloidogyne spp.*
	Lesion Nematodes	*Pratylenchus spp.*
	Birds	
	Snails	

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VIRAL DISEASES

Fanleaf refers to infected vines where the veins are spread in the leaves and resembles a fan. It is caused by the nepovirus grapevine fanleaf virus (GFLV) and is transmitted from plant to plant. Abnormal shoot morphology (double nodes, shorter internodes), yellow discoloration of the stems and leaves, and inflorescence (yellowing along the main veins in mature leaves) are all external symptoms of the disease (Mullins *et al.*, 1992). The grapevines that are infected with this virus produce small bunches and berries that have contrasting colour effects (Weaver, 1976). Fruit-set and fruit ripening is irregular and the productive life of the vineyard is reduced (Winkler *et al.*, 1974).

Leafroll viral disease is widespread and is found wherever grapes are grown. The leaves characteristically simply roll inwards. The disease is not fatal, but result in the reduction of fruit quality and a decrease in productivity and vine vigor. It also delays the ripening and decrease in pigment synthesis (colouring) of the fruit (Mullins *et al.*, 1992). The affected vines also show a reduction in sugar accumulation and an increase in the fruit's acidity (Weaver, 1976). The virus is readily transmitted from infected to healthy vines as it is air-borne.

BACTERIAL DISEASES

Pierce's disease is caused by the Gram-negative bacterium *Xylella fastidios* and is destructive to the grapevine. The characteristic symptoms include dwarfing of new shoots, delaying of shoot growth, drying of leaves, uneven maturing and premature colouring of fruit clusters that may shrivel and die (Mullins *et al.*, 1992; Winkler *et al.*, 1974). Depending on the age and variety of the diseased vine, it dies within two or more years (Weaver, 1976).

Crown gall or black knot disease is caused by the Gram-negative bacterium *Agrobacterium tumifaciens* (Nel, 1983; Weaver, 1976). This soil-inhabiting bacteria infects the vine through wounds, cracks and cuttings at any stage of its lifetime, usually when rain splashes the soil with bacteria (Winkler *et al.*, 1974). The bacterium that penetrates a root secretes a substance that stimulates plant cell growth. The symptoms therefore include abnormal cell enlargements which leads to the development of overgrowths or galls usually near the soil surface and is associated with root damage (Nel, 1983). The young galls are soft, cream or green in colour, and darken to brown as

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they age with the surface becoming open textured and hard (Winkler *et al.*, 1974). The disease can, however, spread to other parts of the vine and cause secondary tumours or growths to develop (Weaver, 1976).

FUNGAL DISEASES

Fungi are both beneficial and harmful organisms in the environment and have the ability to degrade complex organic matter to simple organic and inorganic molecules. They are also the major cause of plant diseases as over 5000 species can attack valuable crops. Fungi, especially the wine yeast *Saccharomyces cerevisiae*, are essential to the wine industry for the production of wine. Common fungi involved in vineyard spoilage include *Penicillium*, *Aspergillus*, *Mucor*, *Rhizopus* and *Botrytis* (Amerine *et al.*, 1972; Zoecklein *et al.*, 1995)

Fungi have the ability to affect different plant parts and cause various diseases in vines. They are sometimes difficult to group, as some may affect several different tissues (Weaver, 1976). The groups consist of soil fungi, fungi attacking the trunk, fungi attacking green shoots, leaves and immature fruit, or fruit fungi. The ripe berry is therefore subject to attack by several types of fungi that rapidly spread over the surface of the fruit in hot, humid regions. Attack is particularly severe in compressed bunches of thin-skinned berries (Peynaud and Ribéreau-Gayon, 1971).

The most widely distributed and important fungal disease of grapevines is **powdery mildew**, or Oidium, caused by the fungus *Unicula necator* (Nel, 1983; Weaver, 1976). This fungus attacks all green portions of the vine and symptoms include a greyish-white powdery growth on the surface of the infected tissues (Vedprakash *et al.*, 1995). When the covering powder is rubbed off, brown to black weblike discolourations are noticeable. Infected leaves tend to curl and wither and some have dark stains on their surfaces. Young infected berries usually drop off and the bigger berries also indicate symptoms of discolourations. Berries also tend to crack and split and result in a reduced yield (Nel, 1983; Weaver, 1976; Winkler *et al.*, 1974).

An easy identifiable fungal disease that is a serious problem in all vineyards is **Botrytis rot**. This rot, caused by *Botrytis cinerea*, is very dependent upon weather conditions such as rainfall or heavy dew. Penetration of the berry and growth of the fungus

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loosens the berry skin and it usually slips off when the skin is slightly pressed over the infected area (Nel, 1983; Winkler *et al.*, 1974). Symptoms include discolouration of the grape skin in white cultivars to light-brown. In later stages the surfaces of the grapes are darker grey and look almost like raisins (Vedprakash *et al.*, 1995). This disease results in the rotting of fruit, the loss of juice and wines with off-flavours (Mullins *et al.*, 1992; Weaver, 1976). The mould growth on grapes is, however, excepted in the production of sweet wines where *Botrytis* produces an overmaturation termed "noble rot" (Zoecklein *et al.*, 1995)

BIOCHEMISTRY OF GRAPES

Over the centuries a number of plant species have been selected and cultivated by man because he found their fruits pleasant to eat (Bollard, 1970). The grape has been valued as a complex fruit consisting of many compounds such as pectic substances, organic and amino acids, phenolics, pigments, vitamins, lipids, proteins, enzymes, volatiles, sugars and other carbohydrates (Table 2) (Amerine *et al.*, 1972). The relative amounts of the different constituents of the grape vary considerably with the different species, external conditions during their development and with production purposes; whether it will be used as table, wine, juice, raisin, or canning grapes. The relative concentrations of these components play an important role in the chemical composition, pH, aroma, attractive flavours, appearance and texture of the different berries.

Table 2. The chemical composition of grapes (Amerine *et al.*, 1972; Vedprakash *et al.*, 1995).

Organic and Inorganic Components	% juice by volume
Water	70-85
Carbohydrates	15-25
Dextrose (glucose)	8-13
Levulose (fructose)	7-12
Pentoses	0.08-0.20
Pectin	0.01-0.10
Inositol	0.02-0.08
Organic acids	0.3-1.5
Tartaric	0.2-1.0
Malic	0.1-0.8
Citric	0.01-0.05
Tannins	0.01-0.10
Nitrogenous Compounds	0.03-0.17
Mineral Compounds	0.3-0.5

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It is generally assumed that many developing fruits containing chlorophyll can assimilate CO_2 in the light for the photosynthetic production of carbohydrates in the grape (Bollard, 1970). The leaves are the main sites for the synthesis of sugars (Lavee and Nir, 1986) by means of light received by green leaves, carbon dioxide from the air plus water and minerals from the soil (Figure 6) (McGrew, 1994). Some of the synthesised sugars are required by living cells throughout the vine to maintain life processes, whereas some are used to develop new growth of stems, leaves and roots. Some sugars are stored in the stems and roots to re-establish growth after the winter dormancy, and the rest are available in the form of the fruit (Marshall and Grace, 1992).

$$6CO_2 + 12H_2O \xrightarrow{\text{light + chlorophyll}} C_6H_{12}O_6 + 6O_2 + 6H_2O$$

Figure 6. The overall equation for photosynthesis: the synthesis of sugars in the plant cell (McGrew, 1994).

The flavour compounds in fruit are divided into two groups; volatile compounds (e.g. alcohol) and non-volatile compounds (e.g. sugars and pectin) (Hulme, 1970; Jackson, 1994). Since the flavour is fundamentally the balance between sugar and acid in the fruit, it is apparent that the sugar content varies widely in different fruits. Sugar content of fruits from a particular species may also vary considerably with the variety. Large quantities of sugars accumulate in the fruit during the final month of fruit development (McGrew, 1994), which takes precedence over all other demands for sugar in the growth of the vine. An increase in the size of the crop therefore causes a decrease in the amount of sugar available for maintenance, new growth and reserves. Not only are these vital operations reduced in an oversized crop, but there is also not enough sugar to develop good fruit quality. Therefore, when the sugar content is low, the acid content is high, the colour poor and maturity delayed (Peynaud and Ribéreau-Gayon, 1971).

The first sugar produced by photosynthesis is known to be sucrose that is the most abundant disaccharide composed of glucose and fructose sub-units (Peynaud and Ribéreau-Gayon, 1971). There are, however, only traces of sucrose to be found in the grape berries as it is translocated or hydrolysed after photosynthesis (Winkler *et al.*, 1974; Zoecklein *et al.*, 1995). The sucrose concentration in the ripe grape berry is therefore less than 0.1% of the fresh weight (Lavee and Nir, 1986). The major

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carbohydrate compounds in the ripe grapes are mixtures of glucose and fructose found in equal quantities (Vedprakash *et al.*, 1995) that can amount to about 150-250 g/l of juice. In unripe grapes, glucose accounts for 85% of the sugar content (Peynaud and Ribéreau-Gayon, 1971).

The biochemical characteristics of the flesh of fruits vary between the different growth periods. In developing grapes during period I (Figure 4), the flesh has a low pH and accumulates acids, whereas it becomes less acid and accumulates reducing sugars during period III, among other changes (Bollard, 1970). Therefore, during the growth of the vine and as the fruit matures, the concentration of sugars increase and the organic acids decreases as the acids are converted to sugars (Figure 7) (Lavee and Nir, 1986). The organic acids dominating in the berries are tartrate and malate and to a lesser extent, citrate (Peynaud and Ribéreau-Gayon, 1971). There is, however, a distinct difference between the acid content of the outer layers (skin) and the inner, softer areas (pulp) of the grape, and between black and green varieties. In hot regions, black grapes absorb more of the sun's rays and lose acid more rapidly.

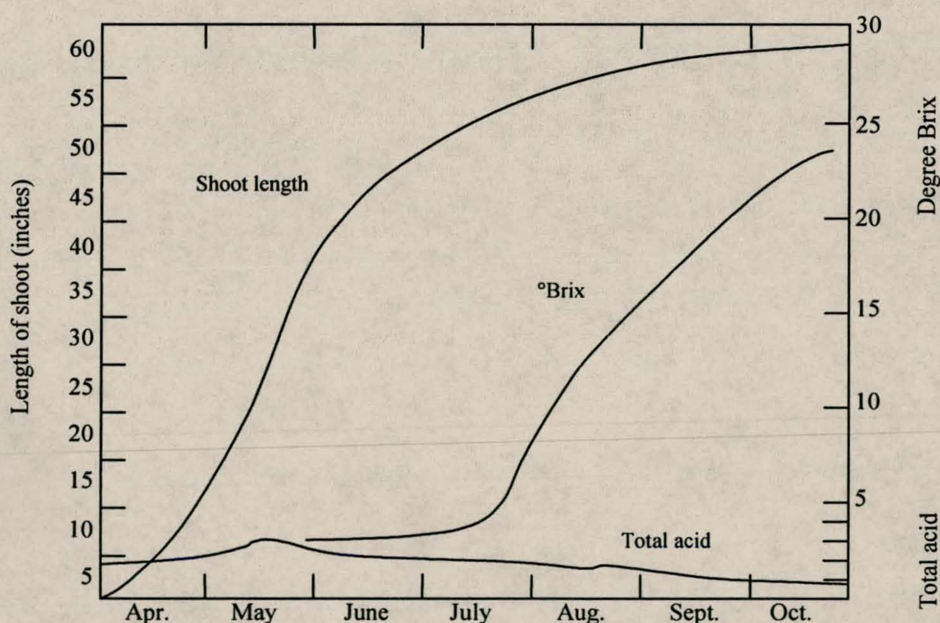

Figure 7. Diagrammatic presentations of shoot growth, level of °Brix [soluble solids per 100 g juice measured as a (% wt/wt: g/100 g) of sugar to water (Zoecklein *et al.*, 1995)], and total acids of berries during the growth season of the grape in the Nothern Hemisphere (Weaver, 1976).

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The texture of the berry is governed by the structural polysaccharides (dominant form of plant carbohydrates) consisting mainly of cellulose, hemicellulose and pectin. These polysaccharides can either be present in the cell wall or cell sap (Isherwood, 1970; Winkler *et al.*, 1974) and constitute most of the plant biomass. Polysaccharides contribute to the cell walls' highly dynamic structure that is important for growth and development, cell to cell communication and transport processes. It also provides a rigid support that allows the vine to stand upright and protect it against invading organisms (Warren, 1996).

Grape Pomace

The grape industry provides various processed products such as wines, juices, raisins and canned grapes (Larrauri *et al.*, 1996; Valiente *et al.*, 1995). The primary by-product of grape berry processing and wine production is by volume, grape pomace (Hang, 1988). The term is collectively used for the processed skins, seeds and stems (Igartuburu *et al.*, 1997; Larrauri *et al.*, 1996; Mazza, 1995) that are separated prior to fermentation in the production of white wines, or after partial fermentation of red wines (Boruff, 1953; Haas, 1976; Prescott *et al.*, 1993).

Grape pomace has been dried and sold as animal feed by several large wineries since the late 1930's (Famuyiwa and Ough, 1982). However, it has a low nutritional value and is consumable only by sheep and not cattle. Digestibility studies revealed that stem removal improved its nutritional value. Seeds extracted from grape pomace have an oil content between 13 and 18.4% with an average of 14.7% for all varieties (Hang, 1988), and provide grape-seed oil for health food and gourmet groceries (Amerine *et al.*, 1972; Heimoff, 1996; Valiente *et al.*, 1995). It is used as a natural source of nitrogen and phosphate when ploughed back into vineyards as a fertiliser and provides nutrients in a concentrated and stable form (Greene, 1998; Ingels, 1992; Mazza, 1995). Grape pomace is potentially beneficial in many ways as a variety of other products including tartrates, citric acid, anthocyanins, ethanol and food fibre can be obtained from this by-product (Figure 8) (Hang, 1988; Larrauri *et al.*, 1996). It is a suitable substrate for the production of citric acid used in the pharmaceutical, food and beverage industry, but the yield depends greatly on the optimum fermentation conditions. Anthocyanins extracted

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from pomace is probably the most valuable component and can be used in high acid foods such as soft drinks, jams and jellies (Mazza, 1995; Valiente *et al*., 1995).

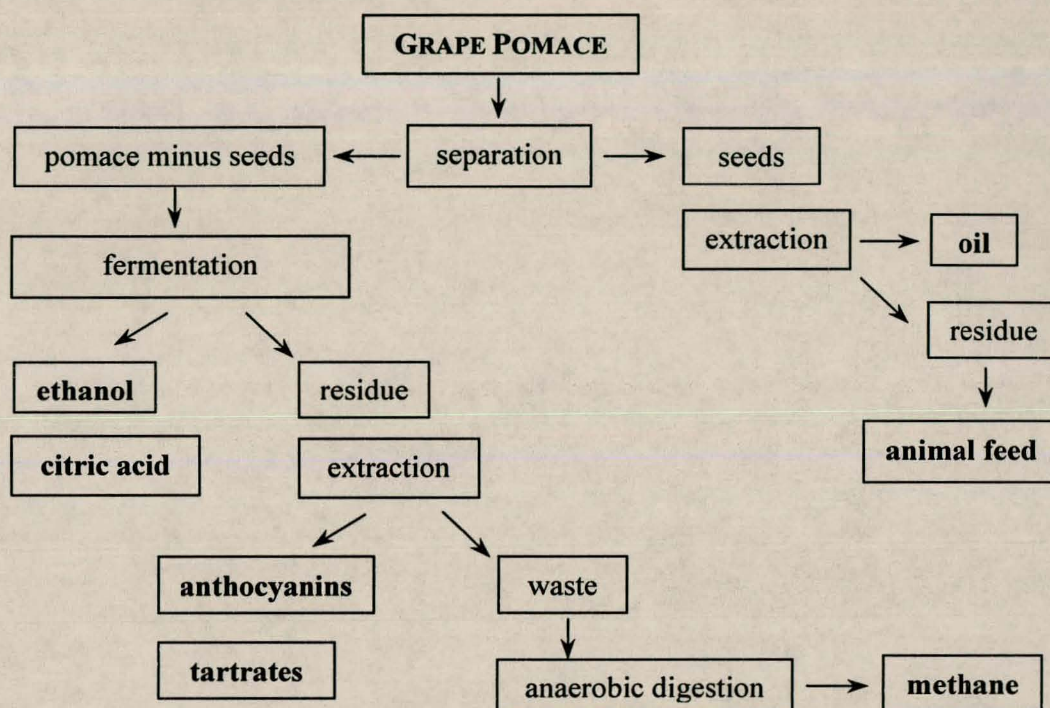

Figure 8. Flow diagram for the resource recovery from grape pomace (Hang, 1988; Mazza, 1995). The valuable end products are indicated in bold.

The high moisture content of freshly pressed grape pomace renders it susceptible to rapid microbial spoilage (Hang, 1988) whilst the huge quantities of space it occupies, contribute to environmental pollution (Mazza, 1995). Agricultural solid wastes have become an increasingly serious problem as disposal sites have become more difficult to obtain and more expensive to operate (Greene, 1998; Igartuburu *et al*., 1997; Rice, 1976). Several researchers have therefore investigated the potential for by-product recovery from these solids (Hang, 1988; Mazza, 1995; Rice, 1976). Grape pomace have been analysed by Valiente *et al.* (1995) for neutral sugar, uronic acid, amino acid and Klason lignin content, as it is considered as a potential fibre-rich food ingredient. It have been reviewed by Hang (1988) for its potential as a raw material for the production of a variety of saleable food ingredients. Antioxidant activity of both white and red grape pomaces have been evaluated by Larrauri *et al*. (1996) to develop products with high natural antioxidant capacities.

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Primarily, however, it is important to consider the possibility to re-use the grape waste in industrial processes, to change the character and reduce the quantity of waste. The reduction in volume of wastes, however, does not necessarily reduce the level of pollution, but may allow it to be treated at lower removal and operating costs (Rudolfs, 1953). To target these problem areas, new ways of recycling have to be found that are economically viable to the wine industry. To establish a novel solution to this problem, a proper knowledge of the chemical and microbial contents of grape pomace is required.

The pressed grape (pomace) contains about 60% moisture and represents 10 to 20% of the weight of the original fresh berry (Boruff, 1953; Famuyiwa and Ough, 1982; Larrauri *et al.*, 1996; Mazza, 1995). The stems, on which the berries are borne, consist of 78-80% water, 2-3.5% tannin and 2-2.5% other mineral substances. The crushed berry (skin, seeds and pulp) is therefore a source of tannin, water, sugars, acids, lignin and pectic matter (Table 3). The relative quantities vary with the specific grape used, the particular wine being processed, winemaking practices, the fermentation process and the availability of distillation facilities (Famuyiwa and Ough, 1982; Hang, 1988; Larrauri *et al.*, 1996). All these factors could be either beneficial or detrimental to the pomace end product and therefore contribute to the quantity and chemical composition of grape pomace and its potential for conversion of by-products.

Table 3. The chemical composition of grape pomace (Valiente *et al.*, 1995).

Components	% (dry matter)
Total neutral sugars including fibre	± 23
Uronic acids	± 5
Klason lignin	± 54
Protein	± 10
Ash	± 8

The polysaccharides present in the grape pomace can be defined as biomass in the context of biotechnology, generally meaning "the organic matter that grows by the photosynthetic conversion of solar energy" (Coughlan, 1985; Glazer and Nikaido, 1995). These polysaccharides are the most abundant form of organic material in nature (Wong and Saddler, 1993). The high market value of ethanol makes it a major product of interest that could potentially be recovered from grape pomace. The conversion of pomace to ethanol can be accomplished in three stages: (1) the hydrolysis of

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polysaccharides to fermentable sugars, (2) conversion of sugars to ethanol by means of fermentation, and (3) the recovery of the ethanol (Figure 9). All three stages must be simple and inexpensive, produce low levels of by-products and high concentrations of alcohol (Glazer and Nikaido, 1995).

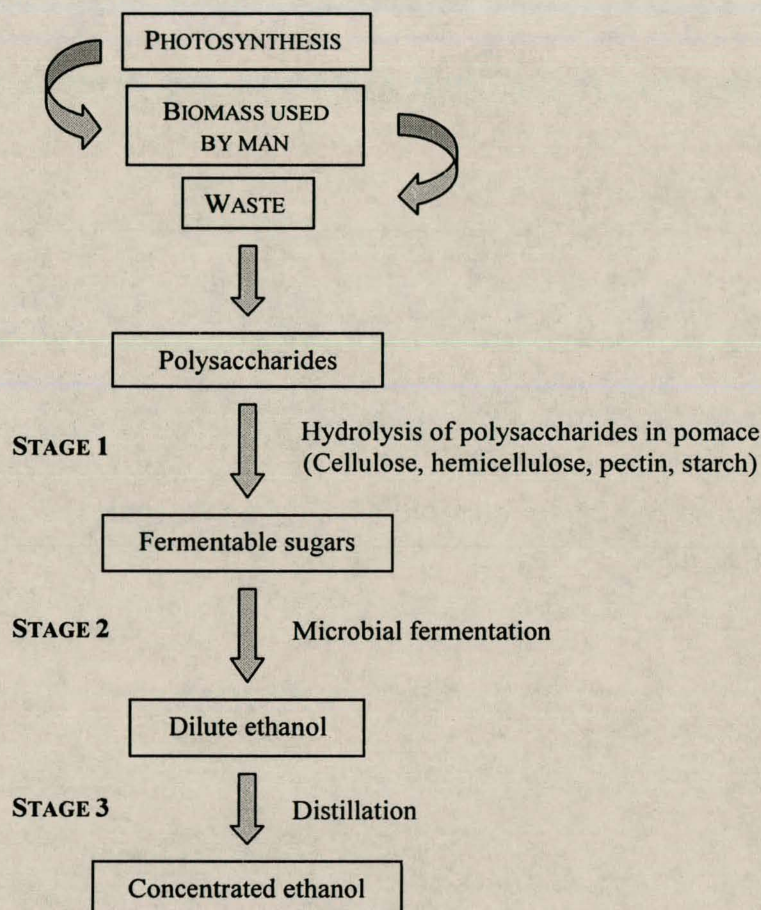

Figure 9. The bio-conversion of grape pomace to ethanol as an alternative for waste treatment (Glazer and Nikaido, 1995).

THE CONVERSION OF BY-PRODUCTS TO FERMENTABLE SUGARS

The cell walls of plants contribute to the fundamental aspects of plant biology that include the morphology, growth and development of the plant cells (Katõ, 1981). The walls that surround the growing plant cells consist of structurally complex polymers that could be either polysaccharides or glycoproteins. These primary cell walls contribute to

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a 0.1-0.2 μm thick layer composed of approximately 90% polysaccharide and 10% protein (Kretovich, 1966; McNeil *et al.*, 1984). The secondary cell wall, just outside the primary cell wall, consists of three (an outer, middle and inner) layers (Figure 10) that are also made up of various amounts of polymers that interact and change depending on their function (Bacic *et al.*, 1988). The middle lamellae (0.2-1.0 μm) fills the space between the secondary and primary cell walls and serves to bind them together (Glazer and Nikaido, 1995). The occurrence, abundance, distribution and structure of a polymer, as well as their relationship with other cell wall components, are dependent on the species, tissue and growth conditions (age) (Wong and Saddler, 1993).

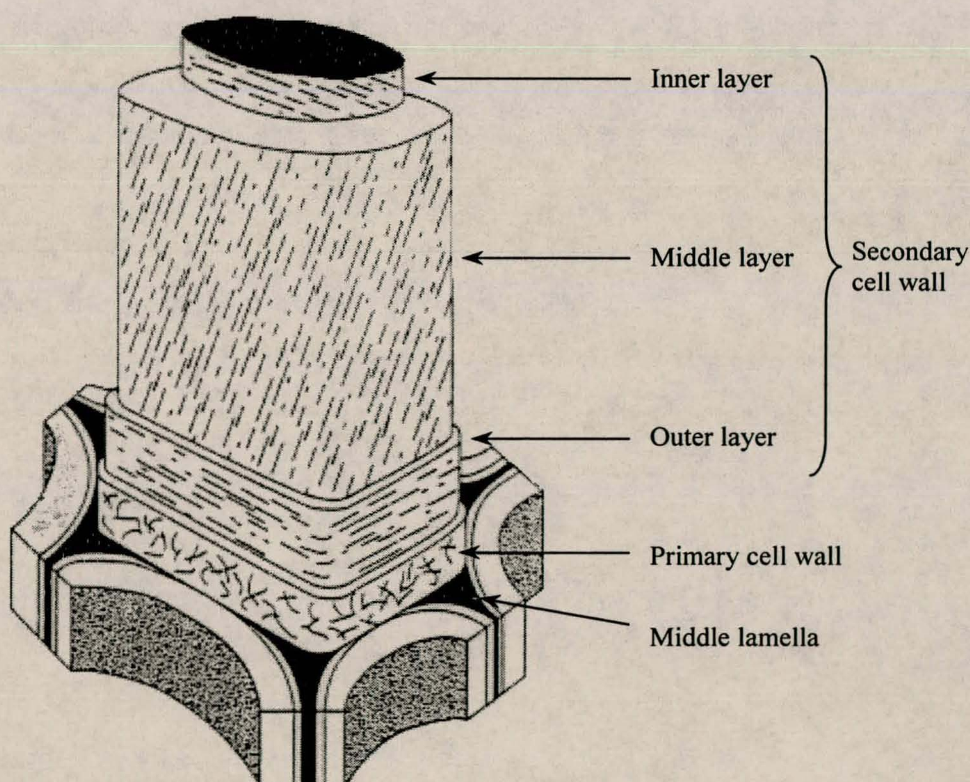

Figure 10. A simplified representation of a plant cell wall (Glazer and Nikaido, 1995).

The polysaccharides of the cell wall are chemically divided into cellulose, hemicellulose, starch and pectin (Kretovich, 1966). These polymers are abundant in nature and constitute the major part of living matter. Polysaccharides are composed of simple or derived sugars connected by glycosidic bonds between the hydroxyl group (OH) on the anomeric carbon of the one monosaccharide and any hydroxyl group of the

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other monosaccharide (Stanley and Zubay, 1993b). These polysaccharides have a remarkable diversity due to the variety of monosaccharides present in nature.

Polysaccharides can be classified as hetero- or homopolymers according to their structure (Stanley and Zubay, 1993b). Polymers composed of one type of sugar or building block are called homopolymers and those composed of more than one type are termed heteropolymers. These two types can be further divided into branched or linear polymers (Davidson, 1967). The size of the polymer is given in terms of the degree of polymerisation (DP), which is the number of monosaccharides present in a single complex macromolecule. Polysaccharides can also be classified according to their function or the distinct role they fulfil: the storage of chemical energy or a structural function (MacGregor and Greenwood, 1980; Zoecklein *et al.*, 1995).

Microorganisms produce polymers of varying composition, structure and properties. The microbial polysaccharides are used to produce emulsions, to stabilise suspensions, to encapsulate materials, to flocculate particles and to modify the flow characteristics of fluids (Glazer and Nikaido, 1995). These microorganisms may produce a multiplicity of enzymes for the efficient degradation of the polysaccharides required for cell growth (Warren, 1996). The microbial degradation of the polysaccharides has a diverse group of glycoside hydrolases with different specificity and modes of action. Each enzyme system that is produced by different microorganisms for the hydrolysis of a particular polysaccharide, contains similar enzymes from the same families (Warren, 1996).

The ability to decompose and obtain carbon and energy from cellulose, hemicellulose, starch and pectin is widespread among fungi and bacteria. There can, however, be physical and structural barriers on the surfaces of the epidermal walls of plants that protect it against infections. These barriers or compounds are also polymers and restrict the microbes and their enzymes from penetrating the plant tissues. The compounds are species specific and vary with age, location and environment (Bacic *et al.*, 1988; Kolattukudy *et al.*, 1981; McNeil *et al.*, 1984).

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BIOSYNTHESIS OF POLYSACCHARIDES

The synthesis of polysaccharides occurs generally during photosynthesis where sugars are produced and is then converted to the different end products. Hexoses are the primary building blocks of polysaccharides and come in a large variety. All hexoses are derivatives of glucose through a serious of conversions that occurs either at the level of the monophosphorylated sugar (glucose-1-phosphate) or the nucleoside diphosphate sugar (uridine diphosphate-glucose) (MacGregor and Greenwood, 1980). The nucleoside sugar is the activated substrate for the formation of polysaccharides and is produced by the reaction of a monosaccharide phosphate with a nucleoside triphosphate (Figure 11) (Atkinson and Zubay, 1993). Each monosaccharide is transferred from a nucleotide sugar to the non-reducing end of the growing polysaccharide chain.

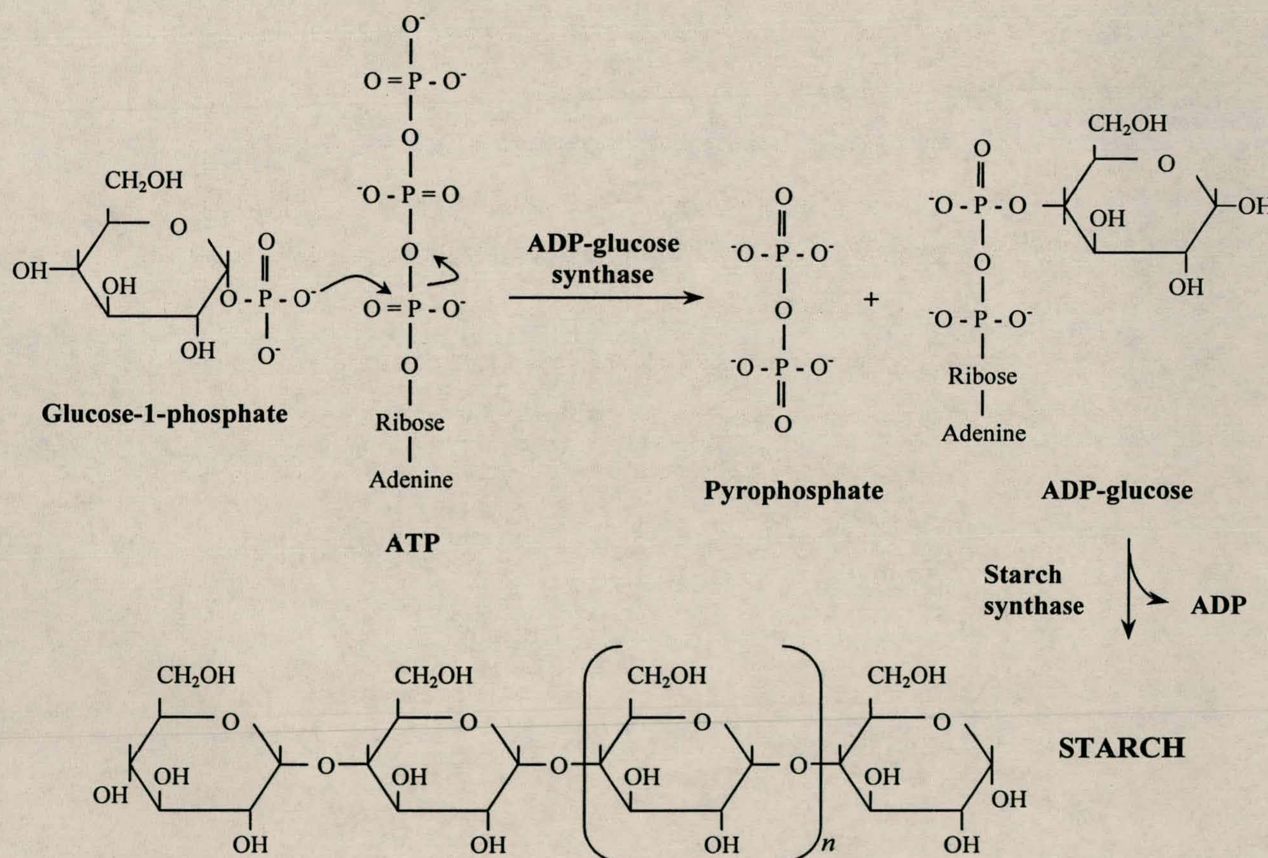

Figure 11. Polysaccharide biosynthesis using starch as an example (Atkinson and Zubay, 1993; Stanley and Zubay, 1993a).

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The general pathway for polysaccharide synthesis is indicated in Figure 12 (Fincher and Stone, 1981). Each stage is catalysed by a specific enzyme and polysaccharides are therefore products of polysaccharide synthetases and glycosyl transferases (Bacic *et al.*, 1988). The amounts of cell wall polysaccharides formed are controlled by the supply of monosaccharides as precursors, their activation to the nucleotide form and interconversion, and polymerisation or polysaccharide assembly (Fincher and Stone, 1981). Post-polymerisation modifications (e.g. esterification and etherification) occur when branching sugars are further synthesised by the respective branching enzymes. These enzymes catalyse the breakage of an oligosaccharide from the non-reducing end of a polymer chain, and reattach it by a α-1,6 bond (MacGregor and Greenwood, 1980). The sugars are translocated to the sites of cell wall synthesis and converted to UDP-GLc [uridine 5'-(α-D-glucopyranosyl pyrophosphate)] where deposition occurs. Adenosine (A), cytidine (C), guanosine (G), or thymidine (T) may replace the nucleoside portions of uridine (U) (Atkinson and Zubay, 1993).

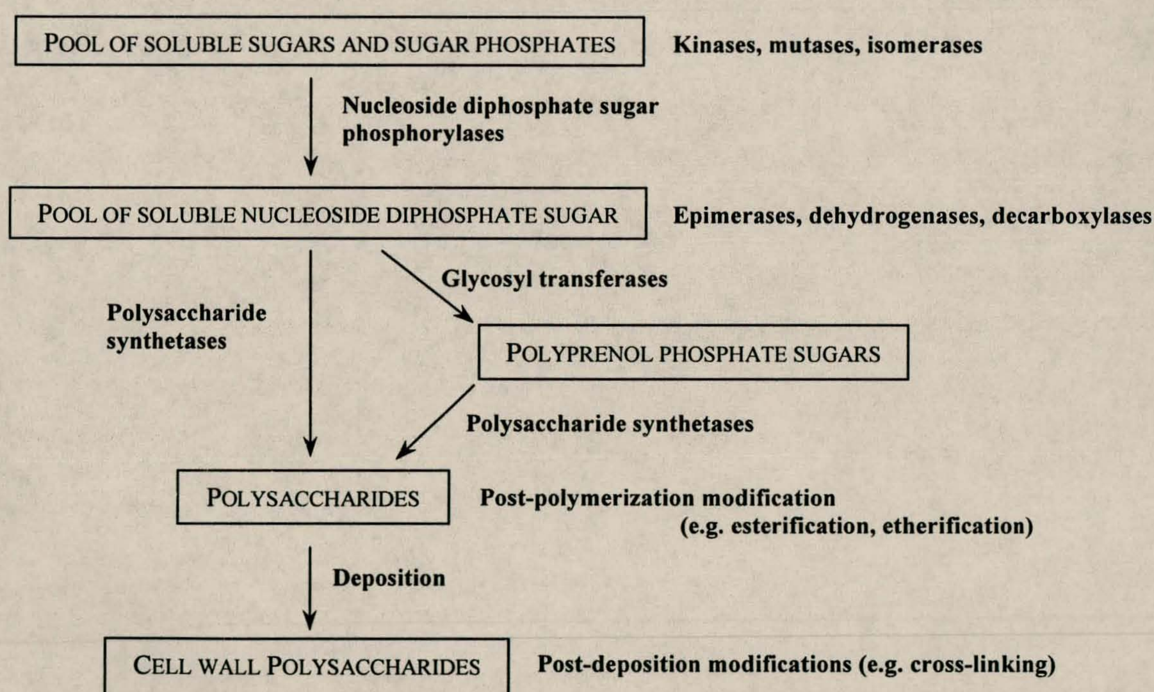

Figure 12. Different stages of the general synthesis and interconversion of monosaccharides for cell wall polysaccharide formation (Fincher and Stone, 1981). Involvement of different types of enzymes are indicated in bold.

Polysaccharides can also be produced via the gluconeogenesis pathway (Peynaud and Ribéreau-Gayon, 1971) where sugars are synthesised from less reduced metabolites

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such as the intermediates from the tricarboxylic acid (TCA) cycle. Malic acid, for example, can be converted via several enzymatic reactions to sugars in the grape and therefore to polysaccharide synthesis (Figure 13). During the growth and development of the grapevine and especially during the ripening of the grape berry, it was observed that the sugar content increases and the acidity level decreases (Lavee and Nir, 1986). Gluconeogenesis and different polysaccharides being synthesised may explain this sugar-acid phenomenon.

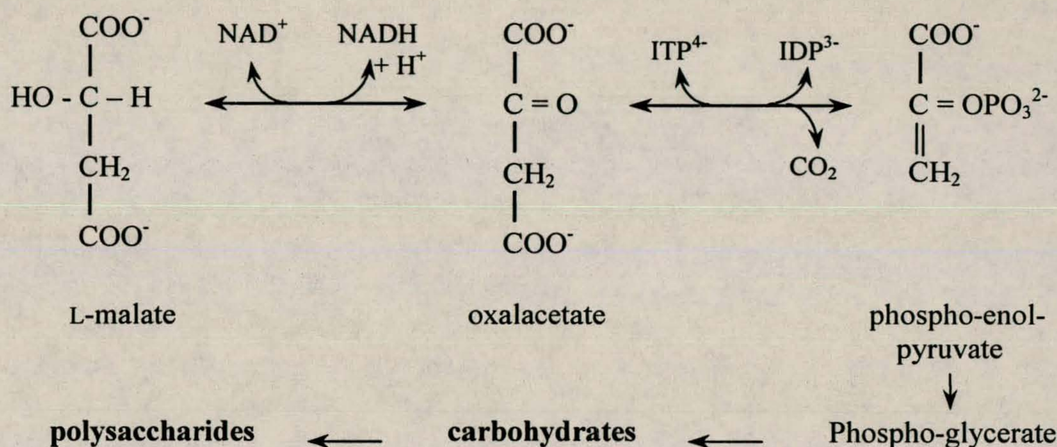

Figure 13. Carbohydrate synthesis from L-malic acid (Peynaud and Ribéreau-Gayon, 1971).

DEGRADATION OF POLYSACCHARIDES

Polysaccharide degradation is termed the reduction in the molecular weight of a polymer. Four different mechanisms that include chemical (acid or alkali), physical (thermal), microbial and enzymatic degradation can degrade polysaccharides (Detroy and Julian, 1982; Gowariker *et al.*, 1986). Each of these mechanisms has technological and ecological advantages and disadvantages. The degradation of the polymers by acid or alkaline hydrolysis results in toxic by-products that are costly to treat and therefore have serious economic implications. Hydrolysis by purified enzymes is expensive and the source and stability of enzymes influences their application. In contrast, hydrolysis by natural occurring microbial populations have several advantages as it is inexpensive, extremely stable, the most important natural process in the environment and does not result in pollution problems (Kubicek *et al.*, 1993; Pretorius, 1994). It is therefore not surprising that microorganisms are mainly responsible for the degradation of polysaccharides in nature.

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Microorganisms are characterised by their ability to carry out certain metabolic reactions and therefore their capacity to synthesise specific enzymes for degradation. These enzymes can be specific and attack a certain substrate, or non-specific and attack various substrates (Uhlig, 1998). Microorganisms also have the ability to carry out single-step or multi-step transformations of organic compounds that are not easily accomplished by conventional chemical methods. These microbes are the most versatile and adaptable forms of life and the capability of these living organisms to produce useful products from wastes can therefore be quite beneficial to mankind.

Studies have been done on the individual polysaccharide hydrolases, including their three-dimensional structures, mechanisms of hydrolysis of glycosidic bonds, catalytic important residues, specificity and relative efficiencies (Warren, 1996). The polysaccharides with their long extended molecules closely packed together in structures providing form, support and some protection, contribute to their resistance to enzymatic hydrolysis. Insoluble polysaccharides restrict the overall efficiency of the enzymes (Warren, 1996). High molecular weight oligomers are unable to enter microbial cells and corresponding hydrolytic enzymes have to be secreted or expressed on the cell surface (Prescott *et al.*, 1993). In eukaryotes, the enzymes' secretary pathway starts from the endoplasmic reticulum and moves through the Golgi bodies and vesicles to the membrane (Kubicek *et al.*, 1993). The enzymes are secreted into the growth media (plant) or stay confined to the microbes' cell surface in eukaryotes and prokaryotes (Biely, 1993).

In some fungi, attachment to the substrate, encysting and developing of a rhizoid system enable them to penetrate the plant cell wall and release polysaccharide hydrolases (polysaccharases). Many fungi therefore survive in the form of their conidia that contain a whole set of enzymes that are capable of hydrolysing polysaccharides and related compounds (Kubicek *et al.*, 1993). The conidial surface can contain enzymes under various nutrient conditions that are produced whenever the organism sporulates. The fragments liberated from the polysaccharides enter the cell and provide the signal for accelerated synthesis of the respective enzymes (Kubicek *et al.*, 1993).

Microorganisms that secrete extracellular enzymes differ in their specificity and properties (Fogarty and Kelly, 1990). Differences between the extent of microbial

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degradation and between the types of degradation products are dependent on the substrate and most importantly on the enzyme involved (Puls and Schuseil, 1993). The complete hydrolysis of chemically diverse plant polysaccharides therefore requires several different microbial systems that work together as organisms differ in their degradation capabilities (Glazer and Nikaido, 1995). The potential for a particular organic substrate to be decomposed by microorganisms should be based on elemental composition, structure of and linkage between repeating units, nutrients present in the environment, the microbial community present and abiotic conditions (pH, O_2, osmotic pressure) (Prescott *et al.*, 1993).

CELLULOSE

Cellulose, named by Payen in 1838, is the best known of all the plant polysaccharides (Nevell and Zeronian, 1985). It is the major homopolymer found in the cell walls of plants and contributes to about 20-30% of the primary cell walls (McNeil *et al.*, 1984). It is the most abundant of organic compounds (Béguin, 1990) consisting of 50% of all the carbon found in plants (Stanley and Zubay, 1993b). Its role is exclusively structural and the high tensile strength of cellulose enables the plant to withstand osmotic pressure and to resist mechanical stress (Leschine, 1995). In the secondary walls of plant cells, cellulose forms several sheets in which microfibrils are embedded in a matrix of hemicellulose and lignin (Coughlan, 1985; Knowles *et al.*, 1987; Ljungdahl and Eriksson, 1985). Together with its water-insoluble character, these characteristics make cellulose resistant to degradation (Bayer *et al.*, 1994; Béguin and Aubert, 1994).

STRUCTURE

The cellulose polymer is a linear chain of thousands of glucose monomers that are linked by β-1,4-glycosidic bonds (Goksøyr and Eriksen, 1980; McNeil *et al.*, 1984; Uhlig, 1998). The straight chain is due to each glucose unit flipping 180° relative to the previous one (Coughlan, 1990; Pretorius, 1997). Thus, the basic repeating unit consists of two glucose units termed cellobiose, a disaccharide (Leschine, 1995; Nevell and Zeronian, 1985). The cellulose polymer is stabilised by internal hydrogen bonds and the degree of polymerisation (DP) is high, ranging from 30 to 15000 units (Pretorius, 1997; Van Rensburg *et al.*, 1998). Microfibrils are formed when hydrogen bonds

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between different chains of cellulose interact strongly to form long and large aggregates (McNeil *et al.*, 1984). These cellulose fibrils combine to form larger fibrils that provide the framework of layers of the plant cell wall (Glazer and Nikaido, 1995). The cellulose fibrils consist of regions of high order to form the crystalline insoluble fibres, whereas the regions of lesser order intersperse the crystalline domains to form amorphous regions (Béguin and Aubert, 1994; Ghosh and Ghosh, 1992; Leschine, 1995). Crystalline cellulose is therefore resistant to microbial and enzymatic degradation, whereas the amorphous polymer can be hydrolysed much faster (Warren, 1996).

MICROBIAL DEGRADATION OF CELLULOSE

Cellulose degradation in nature occurs aerobically, resulting in carbon dioxide and water, or anaerobically, resulting in methane and carbon dioxide (Béguin and Aubert, 1994; Coughlan, 1990). Cellulolytic microorganisms therefore play an important role in the biosphere by recycling carbon (Finch, 1985). They are both widespread and abundant in the natural environment and can be found in all biota where cellulose accumulates (Béguin and Aubert, 1994; Coughlan, 1985). These organisms exist in mixed populations with many other non-cellulolytic strains of fungi and bacteria, but are the predominant polymer-degrading species. The hundreds of species of fungi and bacteria able to degrade cellulose include aerobes, anaerobes, mesophiles and thermophiles (Coughlan, 1990; Finch, 1985; Glazer and Nikaido, 1995). All these organisms produce a number of enzymes with different specificities that act together in a combined fashion and lead to the complete degradation of amorphous cellulose. A large number of microorganisms grow on cellulose, but relatively few are able to produce the complete enzyme system necessary to hydrolyse crystalline cellulose (Coughlan, 1990). The cellulolytic enzymes are synthesised in intracellular locations and are then transported to the cell surface (bacterial or fungal) or excreted to the extracellular medium to act extracellularly on the cellulose substrate (Béguin and Aubert, 1994; Ghosh and Ghosh, 1992).

Several enzymes sequentially or simultaneously act on the cellulose polymer to produce the glucose monomer (Figure 14). The cellulase system is divided into different groups and consists of a complete set of three general classes of enzymes (Knowles *et al.*, 1987):

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(1) cellobiohydrolases (CBH), which attack cellulose molecules stepwise from the non-reducing ends, liberating cellobiose subunits;

(2) endoglucanases (EG), which cleave β-glucosidic bonds at random in the middle of cellulose molecules; and

(3) β-glucosidases, which hydrolyse cellobiose and low molecular weight cellodextrins into glucose (Leschine, 1995; Ljungdahl and Eriksson, 1985; Pretorius, 1994; Van Rensburg *et al.*, 1998).

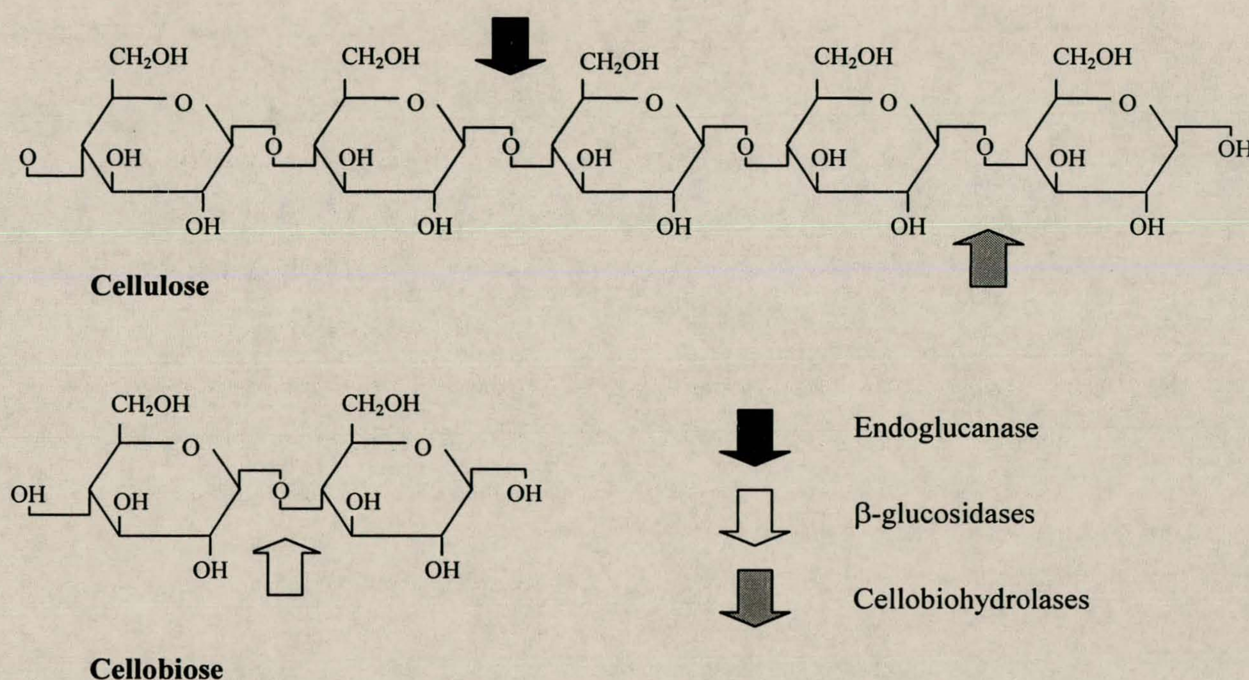

Figure 14. Structure and enzymatic degradation of cellulose (Pretorius, 1994; 1997). Endoglucanases cleave β-glucosidic bonds, β-glucosidases hydrolyse cellobiose and cellodextrins, and cellobiohydrolases attack cellulose from the non-reducing ends.

Although these enzymes differ in their substrate specificity and mode of action, they hydrolyse β-1,4-linkages. All three hydrolytic enzymes must be present at the same time for the maximum rate and extent of cellulose hydrolysis (Béguin, 1990; Ljungdahl and Eriksson, 1985).

CELLULOLYTIC FUNGI AND BACTERIA

Aerobes with cellulolytic enzymes, especially fungal cellulases, are non-complexed and have been investigated extensively due to the potential for the industrial degradation of cellulose to produce fermentable carbohydrates (Finch, 1985; Ljungdahl and Eriksson, 1985). Fungi are also the main degraders of plant material and their cellulase systems

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do not tend to form aggregates (Bayer *et al.*, 1994). With the help of cellulase, fungi degrade the polymer to glucose that not only supports the growth of the fungus, but also the growth of other microbial populations (Ghosh and Ghosh, 1992). The mechanism of enzyme degradation consists of the same three general classes of enzymes, but differ in the relative quantities. Cellulolytic enzymes have been studied particularly in the soft-rot fungi *Trichoderma reesei* and white-rot *Phanerochaete chrysosporium* (Knowles *et al.*, 1987; Ljungdahl and Eriksson, 1985).

Cellulolytic anaerobic organisms, especially the bacteria *Clostridium thermocellum,* have particularly been studied for its mode of action (Béguin and Aubert, 1994). Biomass is converted by a complexed nutritional chain to methane and carbon dioxide by anaerobic bacteria in the natural environment such as soil and compost (Ljungdahl and Eriksson, 1985). Bacteria differ from the fungal system due to the secretion of cellulases either as soluble extracellular enzymes or tightly assembled into large complexes called cellulosomes (Béguin, 1990; Béguin and Aubert, 1994). These cellulosomes have important physiological significance as they promote the adherence of the bacteria to cellulose (Bayer *et al.*, 1994; Leschine, 1995) and is more active against crystalline cellulose and therefore degrade cellulose completely (Warren, 1996). The cellulosome is primarily found attached to the cell surface during bacterial growth and is released into the medium when the cells enter the stationary phase (Stutzenberger, 1990). The cellulosome contains several endoglucanases and exoglucanases and function similar to the fungal system when cellulose is degraded.

HEMICELLULOSE

Hemicellulose is in close association with cellulose in the plant cell wall where it is considered to have a structural function (Eriksson *et al.*, 1990). Hemicellulose is a low-molecular-weight heteropolymer composed of both linear and branched sugar residues. These residues include pentoses (D-xylose, L-arabinose), hexoses (D- and L-galactose, D-mannose, L-rhamnose, L-fucose) and uronic acids (D-galacturonic acid and glucuronic acid) (Puls and Schuseil, 1993). The degree of polymerization is, however, less than cellulose and average between 150 to 200 residues (Eriksson *et al.*, 1990; Nevell and Zeronian, 1985). The most common hemicelluloses are characteristically different in

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their composition and structure and can be grouped as xylans, galactans or mannans depending on the sugar type that forms the backbone of the polymer (Henrissat, 1992). Xylan is the most extensively studied hemicellulose as it is the second most abundant renewable polysaccharide in nature, next to cellulose (Crous *et al.*, 1995). It is also an important component of the hemicelluloses of the secondary cell wall that occur in hardwood and herbaceous plants (MacGregor and Greenwood, 1980).

XYLAN STRUCTURE

The xylan structure contributes 30% to the cell wall material of the plant and seems to be involved in seed germination, fruit softening and plant defence mechanisms (Biely, 1993). The structure is complex and variable involving both linear and branched chains. The linear chains consists of 90% β-1,4-linked xylose residues and the branched chains of small different heteropolysaccharides, especially L-arabinose and D-glucuronic acid (Biely, 1993; Davidson, 1967). The various xylans arise from the absence or presence of branches, their difference in molecular size and the amounts and linking of the sugars to the xylan backbone (Coughlan and Hazlewood, 1993).

MICROBIAL DEGRADATION OF XYLANS

Microorganisms that utilise xylan as a carbon source and have the necessary enzymes for its breakdown, are beneficial in economical and ecological processes since they decrease the viscosity and increase the diffusion of the substrates. Microbial xylanases are biologically active in plant cells as xylan is exposed on the outer surface and is completely soluble (Jeffries, 1994). Fungi and bacteria contain various xylanases that degrade the hemicellulose backbone, as well as several accessory enzymes that are required to hydrolyse the side chains. The complete breakdown of xylan requires endo-1,4-β-xylanases (1,4-β-xylan xylanohydrolases) which attack the polysaccharide backbone and result in xylo-oligosaccharides (Figure 15). These xylo-oligosaccharides are hydrolysed by β-xylosidases to form D-xylose from the non-reducing end (Biely, 1985; 1993; Eriksson *et al.*, 1990). The accessory (debranching) enzymes are usually glycosidases and esterases, depending on the main chain substituents (Jeffries, 1994; Pretorius, 1997).

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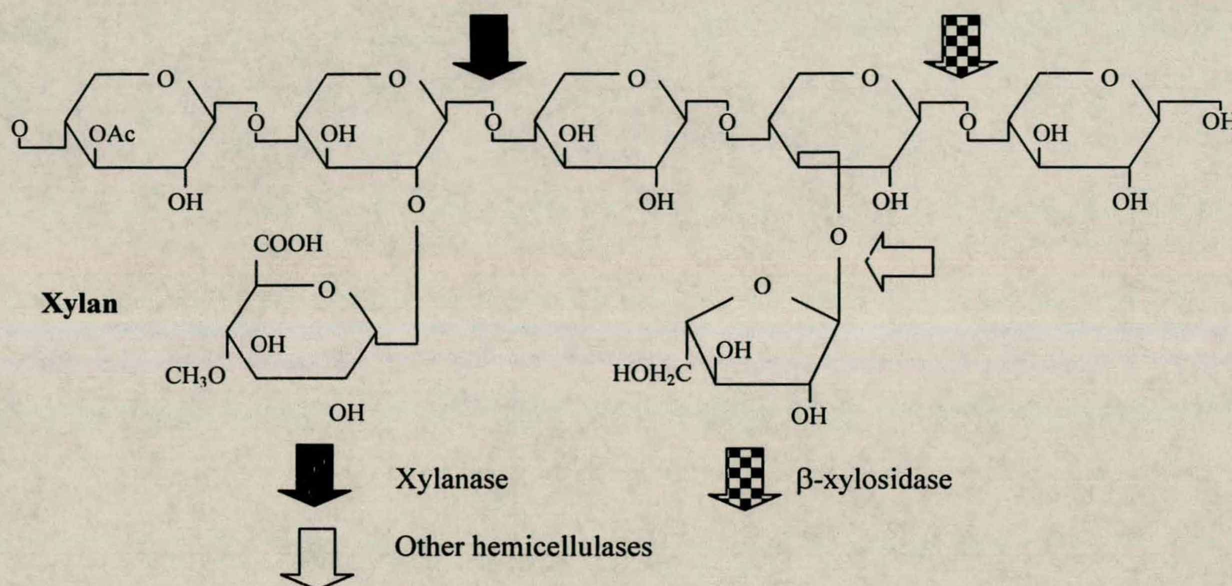

Figure 15. Structure and enzymatic degradation of xylan (Pretorius, 1994; 1997). Xylanase attack the xylan backbone, β-xylosidase hydrolyse oligosaccharides to xylose, and the other hemicellulases are accessory enzymes that attack the main chain substituents.

FUNGAL AND BACTERIAL XYLANASES

The enzyme systems of fungi and bacteria are similar, but differ in their size, optimum temperature and pH depending on the various side chains. Many of these organisms also produce cellulases due to the close association of cellulose and xylan in plant cell walls. A wide variety of fungal xylanases have been studied as they first attack the soluble hemicellulose in the cell wall (Jeffries, 1994). Most of the xylanases are small extracellular enzymes of around 20 kDa. The fungi that produce xylanases, can tolerate temperatures below 50°C, have an optimum pH around 5 and are normally stable at pH 2 to 9 (Viikari *et al.*, 1993). Xylanases from filamentous fungi such as *Aspergillus* and *Trichoderma*, are well known and have been purified and characterised to determine their potential applications (Crous *et al.*, 1995; Viikari *et al.*, 1993).

Some bacteria have the ability to grow at high temperatures (90°C) and produce thermostable xylanases that can hydrolyse xylans completely or partially. Bacterial xylanases have a pH optima slightly higher than fungal xylanases. Two types of xylanases can be detected in bacteria; those with a low molecular weight (< 30 kDa) and alkaline pI that are mainly used for bleaching kraft pulps, and those with a high molecular weight (40-72 kDa) and acidic or neutral pI (Jeffries, 1996; Viikari *et al.*,

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1993). A number of bacterial systems have been characterised in detail, e.g. *Clostridium* and *Pseudomonas*.

PECTIN

All plants contain pectins (Gainvors *et al.*, 1994) that are largely responsible for the integrity and coherence of plant tissues (Rombouts and Pilnik, 1980). Pectin is an acid polysaccharide that is water-soluble, and is another high molecular weight structural heteropolysaccharide abundantly present in plant extracts. The molecular weight of pectin varies, however, with its origin (Kretovich, 1966; Sakai, 1992). It is necessary for the biochemical and physical (texture) modifications of the cell wall that cause fruit softness and is therefore present during fruit growth, ripening and storage (Gainvors *et al.*, 1994; Uhlig, 1998; Voragen *et al.*, 1995; Whitaker, 1990). In higher plants, pectic substances occur mainly in the middle lamellae and primary cell walls, but in fruits they are found dissolved between cells (Laing and Pretorius, 1993a; MacGregor and Greenwood, 1980; Rombouts and Pilnik, 1980; Sakai, 1992). The amount of pectin decreases in the order of the middle lamellae, primary and secondary cell walls (Voragen *et al.*, 1995).

STRUCTURE

Pectic substances are carbohydrate polymers consisting mainly of polygalacturonic acid (pectate) or its methyl ester (pectin) (Pretorius, 1997). This pectic substance is esterified at the carboxyl groups with methanol and more or less neutralised at the free groups. The pectin molecule has a backbone structure of D-galacturonic acid pyranose ring units that are glycosidically linked with α-1,4 bonds (Gainvors *et al.*, 1994; Voragen *et al.*, 1995). Pectin contains other neutral sugar units attached to the galacturonic acid, such as galactose, arabinose and rhamnose (Zoecklein *et al.*, 1995). Protopectin is the pectic substance normally found fixed in plant tissues and that occur in all higher plants (Sakai, 1992).

MICROBIAL DEGRADATION OF PECTIN

Pectin degradation is very important to fermentation industries, including winemaking. Pectin can be attacked by various enzymes that occur in many higher plants and are

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produced by various organisms (Voragen *et al.*, 1995). Three large groups of microbes, namely fungi, actinomycetes and other bacteria, have the ability to degrade pectin. The enzymatic breakdown caused by microbes is classified into two groups based on their mode of action (Figure 16):

1. De-esterifying enzymes:
   (1) Pectin-esterases, which release methyl ester groups (methanol)
2. De-polymerising enzymes:
   (1) Polygalacturonases, which hydrolyse α-1,4 glycosidic bonds between two non-methylated galacturonic acid residues.
   (2) Lyases, which catalyse β-elimination between two esterified galacturonic acid residues. They are distinguished on the basis of substrate preference being either pectin lyase or pectate lyase (Gainvors et al., 1994; Laing and Pretorius, 1993b; Pretorius, 1997; Sakai, 1992; Whitaker, 1990; Zoecklein et al., 1995).

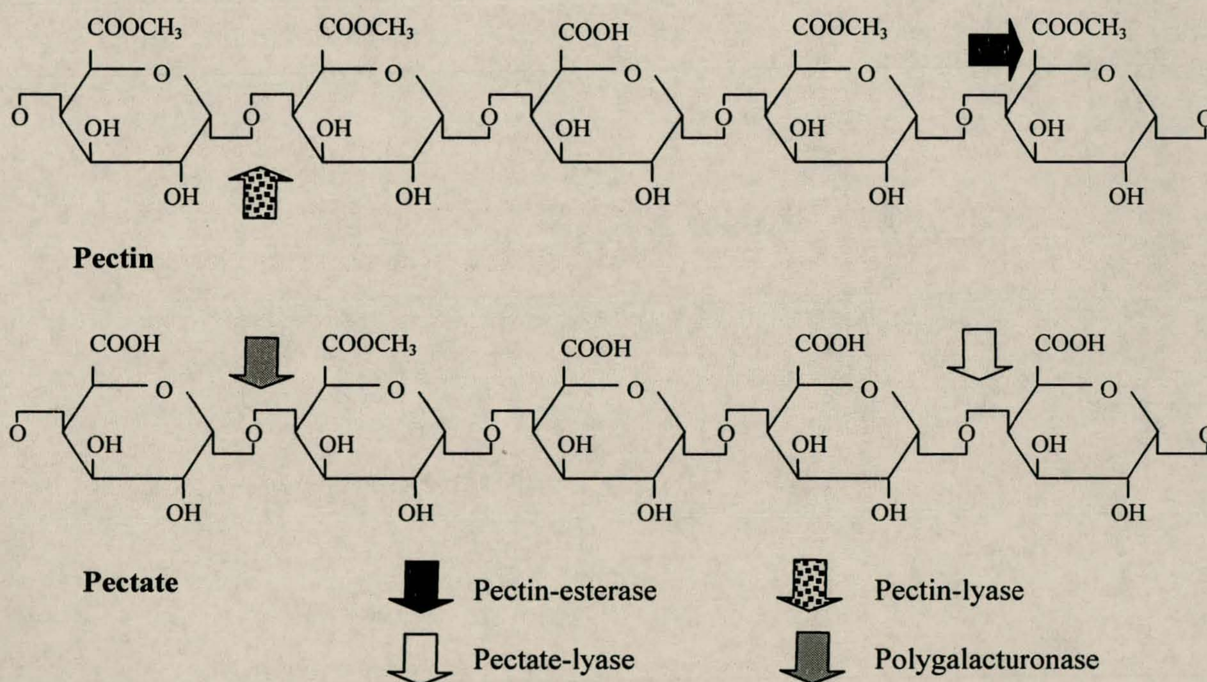

Figure 16. Structure and enzymatic degradation of pectin (Pretorius, 1994; 1997). Pectin esterase releases methyl ester groups, polygalacturonase hydrolyse α-1,4 glycosidic bonds, and the lyases catalyse β-elimination.

FUNGAL AND BACTERIAL PECTINASES

Microorganisms that degrade pectin can be either saprophytic or pathogenic and cause the disintegration of plants and plant material (Sakai, 1992). These enzymes play an essential role in the rotting of fruits. Enzymatic attack of pectic substances results in the

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accumulation of a variety of products, making pectin the major natural source of methanol (Rombouts and Pilnik, 1980). Pectin-esterases are produced by numerous fungi, some bacteria and a limited range of yeast species. Pectin lyases are produced by only a few molds, whereas bacteria produce mainly pectate lyases. Yeasts are generally not known as being powerful pectolytic organisms (Laing and Pretorius, 1993a; b). The soil is a large reservoir of pectolytic microorganisms that contribute to the decomposition and mineralization of plant deposits (Rombouts and Pilnik, 1980).

STARCH

Starch is the major glucose-containing carbohydrate polysaccharide next to cellulose and serves as the major reserve carbohydrate in all higher plants (MacGregor and Greenwood, 1980; Mullins *et al.*, 1992; Weaver, 1976; Zobel and Stephen, 1995). It is composed exclusively of glucose units bound by α-glycosidic linkages, whereas cellulose is composed of β-D-glucose units (Antranikian, 1992). Due to the difference in conformation, starch requires its own enzymatic systems for its degradation. It can also be utilised as a carbon and energy source by a large variety of animals, plants and microorganisms (Ramesh *et al.*, 1995).

STRUCTURE

This macromolecule is heterogeneous and consists of two high molecular-weight compounds, namely amylose (15-25%) and amylopectin (75-85%) (Antranikian, 1992; Pretorius, 1997). These two compounds show significant differences in their physical properties such as structure, molecular size, solubility in water and susceptibility to enzymatic hydrolysis (Fogarty and Kelly, 1980; Ramesh *et al.*, 1995). Amylose has a linear chain where the glucose residues are linked between C_1 and C_4 in the alpha orientation. The length of the chain varies from a few hundred to 6000 residues. In contrast, amylopectin is a branched polymer composed of α-1,4-linked glucan with α-1,6-linked branched points at every 17-26 glucose residues (Antranikian, 1992; Warren, 1996; Zobel and Stephen, 1995). This molecule is one of the largest in nature and may have a molecular weight of more than one million dalton.

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MICROBIAL DEGRADATION OF STARCH

Plant starch is primarily a water-insoluble granule and degradation occurs in three phases: (1) reduction of starch to soluble maltodextrins, (2) debranching and degradation of maltodextrins to glucose, and (3) further metabolism of glucose and export of the products from the site of storage.

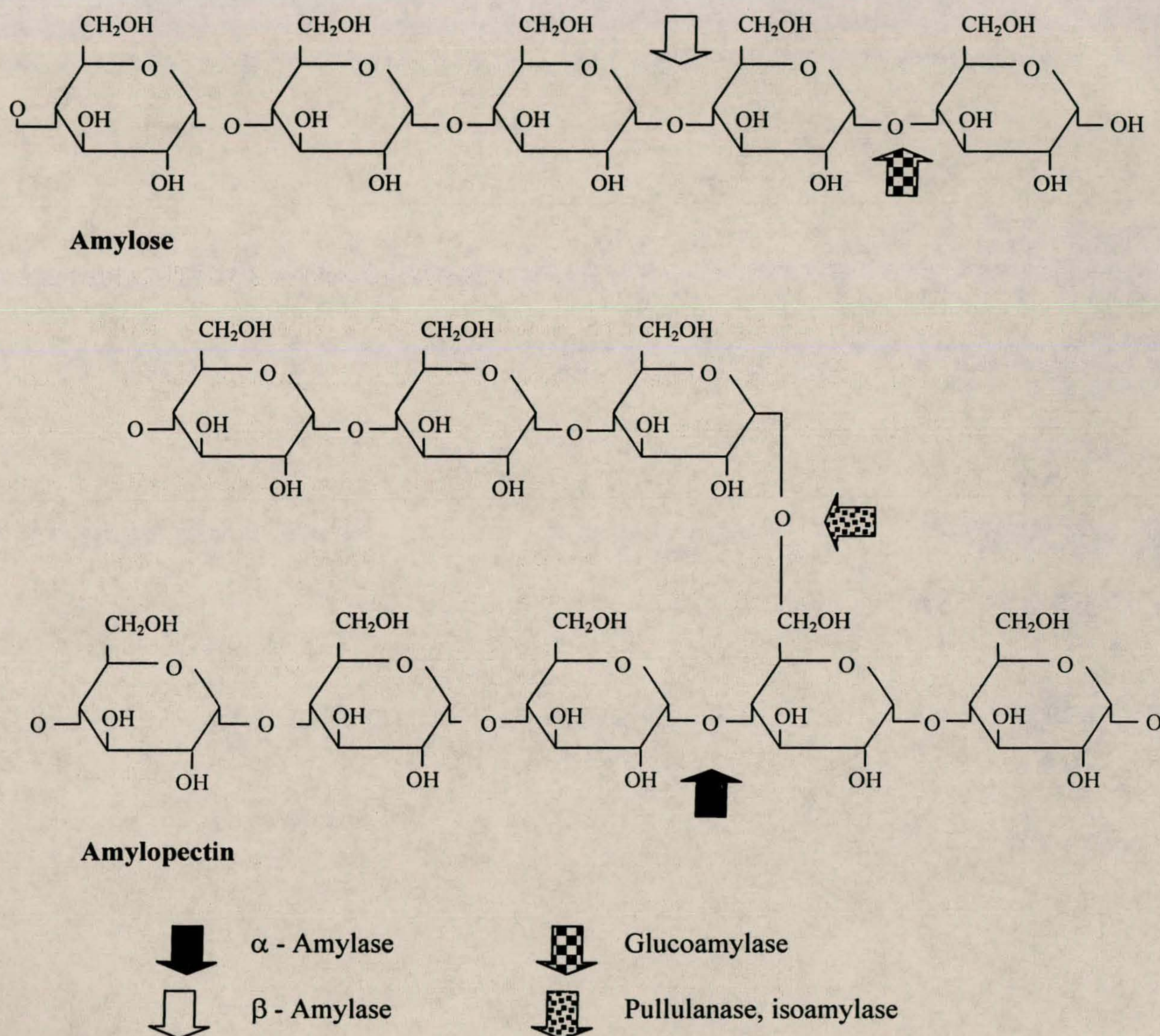

Figure 17. Structure and enzymatic degradation of starch (Pretorius, 1994; 1997). α-Amylase are endo-acting enzymes, β-amylases are exo-acting enzymes, pullulanases and iso-amylases are debranching enzymes hydrolysing α-1,6 linkages, and glucoamylases cleave the starch molecule and release glucose sub-units.

A variety of microorganisms produce starch-degrading enzymes that may be divided into the following classes (Figure 17):

(1) Exo-acting enzymes, e.g. β-amylases, hydrolyse α-1,4 bonds and cannot by-pass α-1,6 linkages;

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(2) Endo-acting enzymes, e.g. α-amylases, hydrolyse α-1,4 bonds and by-pass α-1,6 linkages;

(3) Debranching enzymes, hydrolyse only α-1,6 linkages;

(4) Cyclodextrin-producing enzymes, hydrolyse starch to non-reducing cyclic D-glucosyl polymers called cyclodextrins, which have been detected exclusively in bacteria;

(5) Saccharification enzymes, e.g. glucoamylases, hydrolyse both α-1,4 and α-1,6 bonds from the non-reducing end (Antranikian, 1992; Fogarty and Kelly, 1980; Pretorius, 1997; Warren, 1996).

FUNGAL AND BACTERIAL AMYLASES

β-Amylase is an extracellular enzyme in microorganisms that degrades amylose, amylopectin and glycogen from the non-reducing chain ends (Antranikian, 1992; Fogarty and Kelly, 1980; 1990). The degradation is, however, incomplete and results in the disaccharide maltose or malto-oligosaccharides. β-Amylase is, however, rare in microbes, and more commonly present in plants (Antranikian, 1992), but microorganisms such *as Bacillus, Pseudomonas* and *Streptomyces* that have β-amylases have been isolated and characterised to be used commercially for the production of maltose syrups. This enzyme is unstable at temperatures above 60°C and pH optima is displayed in the neutral region (Fogarty and Kelly, 1980; 1990).

The α-amylases from different microbial sources have been studied, including *Bacillus*, *Candida*, *Pseudomonas*, *Penicillium* and *Aspergillus*. The enzyme has a maximum activity at high temperatures with an optimum at 75°C. It has an optimum pH between 4.8 and 6.5, but is usually stable between pH 5.5 and 8.0 (Fogarty and Kelly, 1980). Some of the microorganisms that produce α-amylases display different specificities and interesting properties that have generated more opportunities for industrial applications.

Debranching enzymes can be divided into direct (degrade unmodified glycogen and/or amylopectin) or indirect enzymes (requires substrate modifications prior to debranching) (Fogarty and Kelly, 1990; Steup, 1988). Direct debranching enzymes can be further divided into two types, namely pullulanases and iso-amylases. Pullulanases require that each of the two chains linked by a α-1,6 glucosidic bond contains at least

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two α-1,4 linked glucose units. Pullulanases are thermostable and mostly acidophilic and are produced in large quantities for industrial processes by a number of organisms, such as *Bacillus* (Antranikian, 1992). The pH optimum of pullulanases is between 5.0 and 7.0 with a temperature optimum of 45-50°C (Fogarty and Kelly, 1980). Iso-amylases cleave α-1,6 linkages of amylopectin and glycogen and have a specificity for large branched polysaccharides. The pH optima range from 5.0 to 6.4 and temperature optima generally lies between 4.0 and 52°C (Antranikian, 1992; Fogarty and Kelly, 1980).

INDUSTRIAL APPLICATIONS OF MICROBIAL POLYSACCHARASES

Microorganisms play various important roles in the environment and the global recycling of organic matter (Figure 18) (Prescott *et al.*, 1993). They have the ability to use natural or synthetic organic substances as a source of nutrients and energy. Microorganisms therefore have a remarkable range of degradation abilities as they can grow on a vast diversity of potential substrates. They vary greatly in their tolerance to pH, temperature, water availability, pressure, salinity and other environmental factors for survival. Degradation of polysaccharides by microorganisms (biodegradation) is, however, not always desirable and may convert compounds to toxic products or generate products that could even be detrimental to other organisms (Prescott *et al.*, 1993).

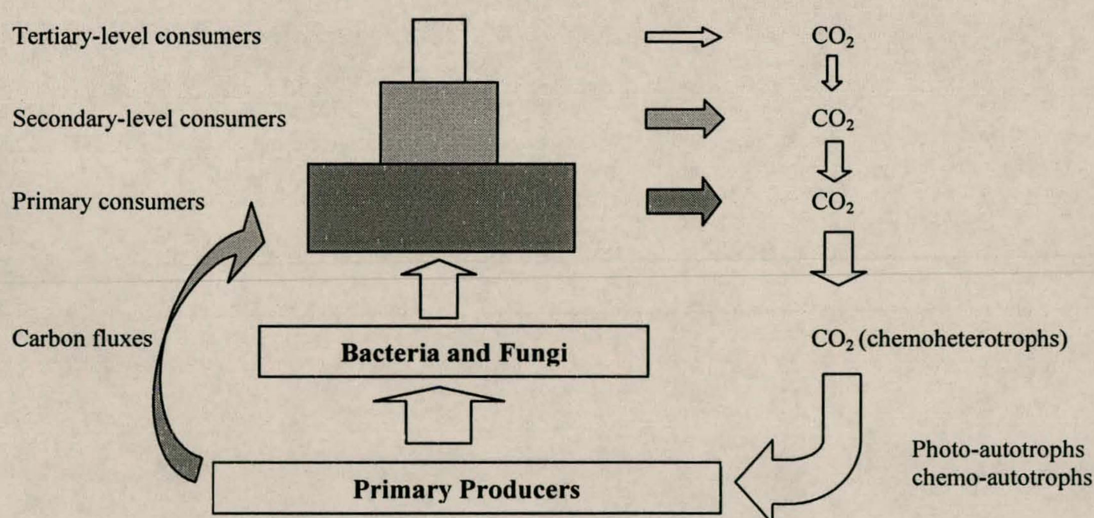

Figure 18. Ecological role of microorganisms. Carbon is fixed by the primary producers, which use light or chemically bound energy. The main decomposers of organic matter are bacteria and fungi, whereas some microorganisms serve as consumers (Prescott *et al.*, 1993).

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The application of microorganisms and/or their enzymes in the industries should be able to solve some industrial problems due to their specificity, non-toxicity, absence of side products and mild reaction conditions, and therefore reduce environmental pollution (Aunstrup *et al.*, 1979; Gacesa and Hubble, 1998). The microbial carbohydrases (carbohydrate cleaving enzymes) are the most important industrial enzymes produced by volume and value (Uhlig, 1998), and will be discussed in the following section.

MICROBIAL SOURCES OF ENZYMES

All living cells (plant and animal tissues, microorganisms) have the ability to produce enzymes that may provide possible useful enzyme products (Underkofler, 1976). The number of enzymes produced on an industrial scale (approximately 30) is only a fraction of the total number of enzymes that have been discovered, i.e. 2500 different enzyme-catalysed reactions listed in the International Union Handbook of Enzyme Nomenclature (Gacesa and Hubble, 1998). Most of the industrial enzymes are produced from approximately 20 microbial sources (bacterial or fungal), as well as several non-microbial sources (plants and animals). Plant and animal enzymes are involved in specialised functions, but are not readily available as definite factors limit their supply. Plant enzymes depend on the available land and labour, and are subjected to climatic, weather and seasonal variations, whereas animal enzymes are by-products of the meatpacking industry. Microorganisms, on the other hand, have an unlimited supply of enzymes that can be economically and easily obtained in large amounts (Gacesa and Hubble, 1998; Underkofler, 1976). Microbial enzymes may sometimes have a disadvantage due to the multiplicity of enzymes a single organism can produce. These enzymes may be non-specific for a biochemical conversion or be the cause of undesirable reactions. However, microbial enzymes are favoured for industrial enzymes because of their technical and economic advantages (Underkofler, 1976).

A variety of microbial enzymes and related compounds are manufactured on commercial scale. The microorganisms producing the enzymes must be harmless, grow rapidly and be highly variable in terms of their activity. In environmental microbiology the majority of enzymes used industrially are derived from the two genera *Bacillus* and *Aspergillus* as the bacteria and fungi display spectacular versatility (Underkofler, 1976). The genus *Bacillus* is known to produce α-amylases, β-amylases and β-glucanases, whereas *Aspergillus* produces α-amylase, β-glucanase, cellulase, glucoamylase and

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pectinase. Only a few other microbial species are used for commercial enzyme production and are listed in Table 4 (Aunstrup *et al.*, 1979). Microbial enzymes have increasingly been used for applications that previously used plant and animal enzymes. These include the partial replacement of amylases from malted barley and wheat with amylases from *Bacillus* spp. and *Aspergillus* spp. in the beer, baking and textile industries.

Table 4. Microbial enzymes for industrial use (Aunstrup *et al.*, 1979; Underkofler, 1976).

Enzyme	Microbial Source
α-Amylase	*Aspergillus orizae*
	Bacillus amyloliquefaciens
	Bacillus licheniformis
β-Amylase	*Bacillus cereus*
	Bacillus megaterium
	Bacillus polymyxa
β-Glucanase	*Aspergillus niger*
	Bacillus amyloliquefaciens
Cellulase	*Trichoderma reesei*
	Aspergillus niger
Glucoamylase	*Aspergillus niger*
	Rhizopus sp.
Glucose isomerase	*Bacillus* sp.
	Streptomyces sp.
Glucose oxidase	*Aspergillus niger*
Invertase	*Saccharomyces* sp.
Pectinase	*Aspergillus niger*
	Aspergillus sp.

APPLICATIONS IN WASTE TREATMENT

In the past, waste was generally discarded untreated, allowing nature to dispose of those that were biodegradable and therefore simply returned to the environment the resources that were previously extracted from it. This situation is, however, no longer sustainable as the increasing volumes of waste create pollution-associated problems. These problems are due to the scarcity of places to dispose of them, the increasing population densities, the rising standards of living and the exploitation of new materials (Martin, 1998). These problems led to research into the biotechnology and bioconversion of waste to accelerate the natural recycling process and to produce usable products.

Bioconversion processes may be subjected to several advantages and disadvantages, but recently the use of enzymes for waste processing has increased both due to economic and environmental demand. The most abundant waste materials available to be

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recycled into useful products are probably the polysaccharides resulting from agricultural waste. The application of enzyme technology has been most useful for the production of a wide variety of by-products from polysaccharides like cellulose and starch.

AMYLASES

The major application of microbial enzymes has been the use of glucose isomerase in conjunction with α-amylase and glucoamylase to convert starch to mixtures of glucose and fructose that are used in the manufacturing of food products and beverages (Antranikian, 1992; Uhlig, 1998). Starch is first gelatinised by heat treatment above 100°C, followed directly by partial hydrolysis for the thinning of starch. The enzymes are therefore required to function at high temperatures and should be heat stable, such as the amylases of *Bacillus* that function at temperatures up to 110°C. Due to the process' dependence on the enzyme activity, the degree of hydrolysis can be controlled. The enzymatic degradation results in fewer by-products, and provides more specificity and higher yields of the desired end products (Aunstrup *et al*., 1979; Fogarty and Kelly, 1980).

PECTINASES

The most commercial pectinase preparations are of fungal origin, especially derived of *Aspergillus*, and contain varying ratios of pectin esterase (PE), pectin lyase (PL) and polygalacturonase (PG). Pectinases work on pectic substances that occur as structural polysaccharides in the middle lamella and primary plant cell wall. The use of pectic enzymes in the wine and juice industry improves the yield and quality of the products (Aunstrup *et al*., 1979; Sakai, 1992; Uhlig, 1998). The pectinases are used to extract and clarify fruit-juices, lower the viscosity of the products and to isolate essential oils from citrus peels (Laing and Pretorius, 1993b; Whitaker, 1990). Pectinases can also be used to produce finely dispersed constituents from carrots and other vegetables for baby foods (Rombouts and Pilnik, 1980).

CELLULASES

Cellulases and hemicellulases can be applied to cellulose and related fractions of hemicellulose in agricultural wastes and residues (Coughlan, 1985). Cellulases are mainly from *Aspergillus*, *Trichoderma* and *Penicillium* species. Cellulases can be used

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as a feed supplement for cattle, but the use of the enzyme has been limited as components of digestive aids in the pharmaceutical industry. The enzymes are useful for the extraction of green-tea components, the production of unicellular vegetables, modifying food tissues, removing soybean seed coats and improving the tensile strength of paper (Bisaria, 1998; Goskøyr and Eriksen, 1980). It is also added to laundry powder to shave off the microfibrils that tend to stick out of cotton fibres after several washing cycles (Béguin and Aubert, 1994).

HEMICELLULASES

Xylanases (hemicellulases) are readily obtained from *Trichoderma* and *Aspergillus* species. Xylanolytic enzymes can be applied in conjunction with or in the absence of cellulolytic enzymes. Fuel crops, agricultural and forestry residues and waste contain a large amount of xylans that frequently pollute streams and rivers (Biely, 1985; Glazer and Nikaido, 1995) and are enzymatically degraded by xylanases and cellulases. Degradation of wastes that are generated in the paper and pulp industry involves the use of xylanolytic enzymes to reduce and dilute the waste quantity (Biely, 1993; Coughlan and Hazlewood, 1993). Xylanases are also used to modify cereal flours to improve the quality (volume and texture) and to improve bread leavening (Eriksson *et al.*, 1990; Jeffries, 1996; Viikari *et al.*, 1993). These enzymes can also be applied to extract juices, flavours, spices, oils and pigments and to convert xylans into sweeteners like xylitol (Béguin and Aubert, 1994; Bisaria, 1998; Wong and Saddler, 1993).

LIMITING FACTORS

Since microorganisms are predominantly responsible for the degradation of polysaccharides in the biosphere, there are a number of factors that influence their enzyme action (Bacon, 1979). The microbial source of the enzyme and its cost and stability when used in a continuous operation, are the main factors that influence the microbial enzyme application. Other contributing factors include reaction time, pH, temperature, concentration of the enzyme and substrate, and the presence or absence of inhibiting or activating factors (Uhlig, 1998; Underkofler, 1976).

Some enzymes are affected by the configuration of the ring structure, the glycosidic bonds, it's non-reducing ends and chain-end modifications. Parameters such as the chemical structure and molecular weight of the polymer should therefore also be

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considered as limiting factors. We must also consider that one polysaccharide may prevent another from being digested when they are incorporated together in the plant cell wall (Bacon, 1979). Furthermore, the physical and structural barriers of the plants and the processing operations in the industry limit the microbes and their enzymes (Kolattukudy *et al.*, 1981).

Many enzymes of current or potential importance in commercial practice are subject to repression by a carbon source present in the medium as a mixture or released from the polysaccharides prior to hydrolysis (Table 5) (Demain, 1990; Eriksson, 1979). Advances in enzyme technology, however, hold promise for the efficient utilisation of polysaccharides as abundant sources of sugar substrates for fermentation processes, such as the production of ethanol.

Table 5. Carbon source repression of enzymes of commercial interest (Demain, 1990).

Enzyme	Microorganism	Repressing carbon source
α-Amylase	*Bacillus licheniformis*	glucose, fructose, maltose, glycerol, acetate, succinate
β-Amylase	*Bacillus megaterium*	glucose, maltose
Cellulase	*Trichoderma reesei*	glucose, glycerol, starch
Glucoamylase	*Aspergillus niger*	glycerol, sorbitol, fructose, xylose, α-ketoglutarate, pyruvate
β-1,3 Glucanase	*Streptomyces* sp.	glucose, glycerol
β-Glucosidase	*Trichoderma reesei*	glucose
α-Galactosidase	*Monascus* sp.	glucose, sucrose, glycerol
β-Galactosidase	*Escherichia coli*	glucose
Invertase	*Saccharomyces cerevisiae*	glucose, fructose, mannose
Pectinase	*Acrocylindrium* sp.	glucose, fructose, mannose, xylose, glycerol, malate, pyruvate, succinate, fumarate, acetate, α-ketoglutarate
Glucose isomerase	*Streptomyces phaeochromogenes*	glucose

THE CONVERSION OF FERMENTABLE SUGARS TO ALCOHOL

SUGAR AS A SUBSTRATE

The name "sugar" is applied to everything having a sweet taste. The most important of the natural sugars include malt, milk, grape, fruit and cane that are respectively known as maltose, lactose, glucose, fructose and sucrose (Mackenzie, 1913). These sugars are

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all composed of carbon (C), hydrogen (H) and oxygen (O) elements, therefore belonging to the group of substances known as carbohydrates (Kretovich, 1966; Mackenzie, 1913). Sugars also have many other common properties that include their sweet taste, easy solubility in water, very slight solubility in alcohol and insolublility in ether. The sugars (mono- and disaccharides) also share the function of being generally fermented by fungi and bacteria, i.e. converted to ethanol and CO_2 (Mackenzie, 1913).

Plant matter represents an abundant inexpensive source of organic matter that can be converted to feedstock chemicals (basic building blocks that serve as raw materials to synthesise other chemicals) by microbial fermentation and chemical processes. Fungi (mainly yeast) and a few bacteria can utilise and ferment the simple sugars hydrolysed from polysaccharides (cellulose, hemicellulose, pectin and starch), but the range of substrates are, however, limited (Glazer and Nikaido, 1995). Substrates that can be utilised include disaccharides such as sucrose and maltose, as well as monosaccharides such as glucose and fructose.

THE DISACCHARIDES

Disaccharides are carbohydrates ($C_{12}H_{22}O_{11}$) composed of two simple sugars bonded by a glycosydic linkage that can be cleaved to form monosaccharides (Figure 19) (Kretovich, 1966; Mackenzie, 1913; Stanley and Zubay, 1993b). The disaccharides differ as they can contain two hexoses, two pentoses or a hexose and a pentose, and the disaccharide sub-units can be linked together in different ways (Kretovich, 1966). Disaccharides that exist freely are found in plants as products of their own metabolic activities and only occur in very small amounts (e.g. sucrose). Most disaccharides in plant tissues are usually found as products of polysaccharide degradation and therefore many of the disaccharides appear in the polysaccharide fraction (maltose, galactose and cellobiose) (Avigad, 1982). It is also rare to find significant accumulation of disaccharides as rapid hydrolysis to monosaccharide constituents usually occurs. This catabolic reaction ensures that substrates for the metabolic requirements of the growing tissues are provided. The presence of higher levels of disaccharides can, however, be noticed during the ripening of fruits and in mechanically damaged or infected tissue (Avigad, 1982).

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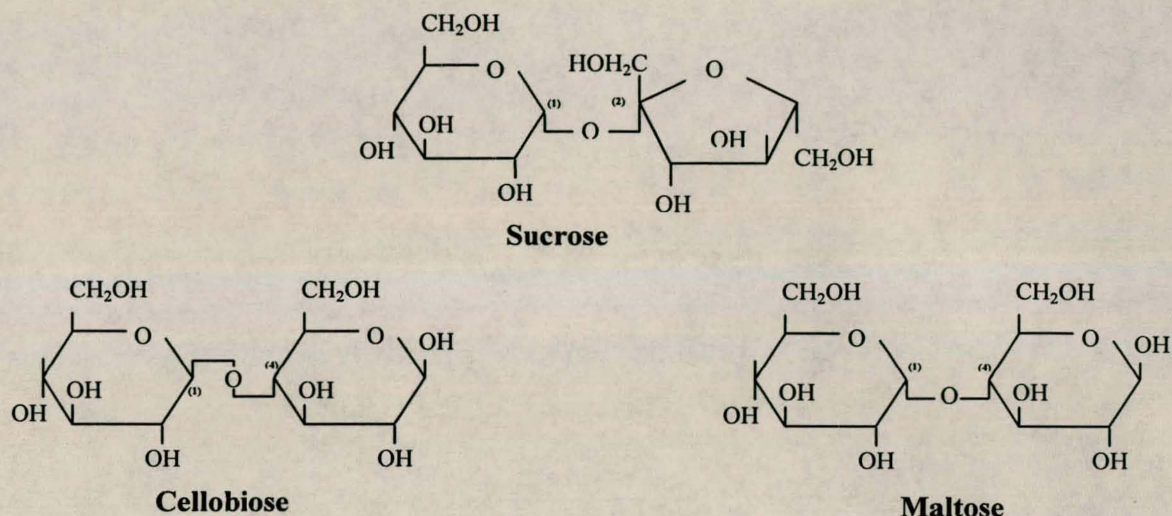

Figure 19. Examples of disaccharides present in plant cells (Kretovich, 1966; Stanley and Zubay, 1993b).

SUCROSE

Sucrose, or cane sugar, plays a central and vital role since it is the major transportable metabolite in the plant (Avigad, 1982; Mackenzie, 1913). It is synthesised and channelled during photosynthesis. Sucrose is then delivered to all plant organs where it serves as a source of organic carbon to build permanent structural elements, and as a metabolic fuel to produce energy (Zoecklein *et al.*, 1995). This disaccharide is set aside for future use in growth periods and consists of glucose and fructose units that are α-1,2-β glycosidically linked (Stanley and Zubay, 1993b). It is found in leaves, stems, seeds, fruits, berries and roots and dissolves readily in water (Kretovich, 1966). Sucrose is synthesised and hydrolysed by the same enzyme, sucrose synthase, and these processes are easily reversible (Avigad, 1982). It is itself unfermentable, but the products of its hydrolysis are readily utilised.

MALTOSE

The disaccharide maltose, or malt sugar, is a product of degradation of the storage polysaccharide starch (Avigad, 1982; Mackenzie, 1913; Stanley and Zubay, 1993b). It is a major intermediate as maltose (comprising of two glucose residues linked with a α-1,4 glycosidic bond) is rapidly utilised in the plant tissues to provide glucose for metabolism. Maltose can be found where starch has accumulated in the storage tissues (seeds) and is relatively noticeable in the fruit of the grape (Avigad, 1982). Maltose

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production can also be found as a major product of photosynthesis, suggesting that glucose molecules produced by photosynthetic assimilation are exported and about half are converted into maltose. Maltose is utilised by a predominant pathway where the action of the α-glucosidase (maltase) cleavage enzyme splits maltose into two free glucose molecules (Kretovich, 1966).

CELLOBIOSE

This disaccharide is a product of polysaccharide degradation and stands in the same relationship to cellulose that maltose does to starch (Mackenzie, 1913). It is one of the most common disaccharides detected as a product of cellulose hydrolysis and is the major end product of exoglucanase activity on cellulose, which is then hydrolysed to glucose. This disaccharide consists of two glucose subunits that are β-1,4 glycosidically linked (Stanley and Zubay, 1993b). Cellobiose does not occur free in nature and has a faintly sweet taste (Mackenzie, 1913).

THE MONOSACCHARIDES

Monosaccharides are the simplest (least complex) and smallest carbohydrates. They usually occupy key roles in energy metabolism and supply carbon skeletons for the synthesis of other more complex compounds (Stanley and Zubay, 1993b). Monosaccharides are usually structurally related and can be inter-converted. They have different conformations that result in dioses, trioses, tetroses, pentoses and hexoses; all represented by the general formula $C_nH_{2n}O_n$ containing only carbon, hydrogen and oxygen (Kretovich, 1966; Mackenzie, 1913). The most common sugars have five or six carbons; these pentoses (five) and hexoses (six) are present in abundance in plant cells (Figure 20).

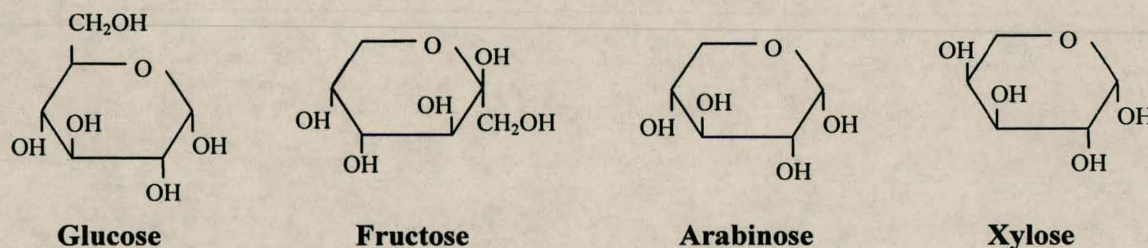

Figure 20. Examples of monosaccharides in their pyranose conformation present in plant cells (Kretovich, 1966). Glucose and fructose represent the hexoses and arabinose and xylose represent the pentoses.

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Glucose

Glucose is commonly called "grape sugar" as it is found in large quantities in grapes (Mackenzie, 1913). It occurs in nearly all parts of plants and is synthesised mainly through photosynthesis in the leaves and to some extent in the green, unripe berry. Glucose is also produced from the hydrolysis of numerous polysaccharides such as cellulose and starch, as well as disaccharides such as sucrose, maltose and cellobiose (Kretovich, 1966). Since glucose has many chemical and physiological properties, it can be utilised by a number of organisms as a source of carbon and energy. It is also the most common sugar used as a substrate for fermentation studies (Mackenzie, 1913) and is readily available for experiments as it is commercially produced.

Fructose

Fructose is much sweeter than sucrose and glucose and provides the sweet taste to fruits and vegetables (Mackenzie, 1913; Winkler *et al.*, 1974). It is found in the green parts of plants, in the nectar of flowers and in fruits (Kretovich, 1966). It is commonly known as fruit sugar and can be produced from a number of substances upon hydrolysis, including inulin (a variety of starch), levulin and levan. The hydrolysis of sucrose produce fructose in equal amounts as glucose (Mackenzie, 1913). Fructose can also be utilised by a number of organisms and is produced commercially, as it is readily inter-converted to glucose by means of isomerisation (Glazer and Nikaido, 1995).

Xylose and Arabinose

Xylose and arabinose are the only two pentoses of importance on account of their wide occurrence and easy obtainability. Arabinose and xylose (or wood sugar) can be derived from the polysaccharide hemicellulose, the most common being xylan (Kretovich, 1966; Mackenzie, 1913). It can either be hydrolysed from the backbone of the structure or as part of the side chains. Pentoses are, however, difficult to assimilate and ferment by microorganisms. Naturally xylose-fermenting microorganisms have been identified but are, however, less suitable for industrial ethanol production. Xylose is, therefore, not always easily fermentable by various organisms and is converted via xylitol to its isomer xylulose in the presence of the xylose isomerase enzyme (Glazer and Nikaido, 1995; Hahn-Hägerdal *et al.*, 1994). Recombinant microorganisms have, therefore, been constructed that have the ability to utilise xylose as it is the most abundant sugar available in nature after glucose (Hahn-Hägerdal *et al.*, 1999).

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FERMENTABLE SUBSTRATES

Organisms that have the ability to utilise the major carbohydrates as substrates to produce significant quantities of ethanol by means of fermentation are usually limited. Substrates can follow two pathways of utilisation that are common for most organisms. The complex carbohydrates can either be hydrolysed by extracellular enzymes and the simpler sugars may then be transported into the cell, or it can be transported into the cell and hydrolysed by intracellular enzymes (Glazer and Nikaido, 1995). The uptake of a substrate, as well as its metabolism, is determined by regulatory mechanisms, as certain substrates may be preferred above the others when mixtures occur. The fermentation of the various substrates therefore does not occur in a simultaneous fashion and the identity and concentration of substrates will have a significant effect on the end product.

There are many organisms capable of utilising sugars as substrates. Some of the organisms have the ability to utilise the complex polysaccharides, but most of them prefer the mono- and disaccharides as they are readily available and easy to utilise. These substrates restrict the organisms to a limited range of sugars to provide carbon for various biosynthesis of constituents and provide energy for the cell. Furthermore, microorganisms do not necessarily produce ethanol from the sugar that is utilised, but a substantial amount of other products may be produced (Jay, 1996; Prescott *et al.*, 1993). Some of the selected organisms that have the ability to utilise different substrates are indicated in Table 6 (Glazer and Nikaido, 1995).

Table 6. Examples of organisms with the ability to produce ethanol and the substrates they ferment (Glazer and Nikaido, 1995).

Yeasts	**Substrates**
Saccharomyces cerevisiae	glucose, fructose, galactose, maltose, maltotriose and xylulose
Saccharomyces rouxii (osmophilic)	glucose, fructose, maltose and sucrose
Kluyveromyces fragilis	glucose, galactose and lactose
Kluyveromyces lactis	glucose, galactose and lactose
Candida pseudotropicalis	glucose, galactose and lactose
Candida tropicalis	glucose, xylose and xylulose
Bacteria	
Zymomonas mobilis	glucose, fructose and sucrose
Clostridium thermocellum	glucose, cellobiose and cellulose
Clostridium thermohydrosulfuricum	glucose, xylose, sucrose, cellobiose and starch
Thermoanaerobium brockii	glucose, sucrose and cellobiose
Thermobacteriodes acetoethylicus	glucose, sucrose and cellobiose

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PRETREATMENT OF BIOMASS

A number of factors in terms of biomass characteristics limit the rate and extent of substrate utilisation. Proper pretreatment of various forms of biomass are therefore a prerequisite for the efficient conversion into various products especially when large amounts of waste biomass are generated. Numerous pretreatment methods capable of enhancing the susceptibility of the biomass have been found to make the sugars they contain readily available to microorganisms for alcoholic fermentation. These methods include mechanical, physical, chemical, biological and enzymatic pretreatments, or a combination of these (Table 7) (Bisaria, 1998).

Table 7. Pretreatment methods and representative examples for enhancing the susceptibility of the microbial action (Bisaria, 1998).

Pretreatment method	Representative examples
1. Mechanical	Ball milling, hammer milling, extrusion
2. Physical	Steaming, wetting, pulping, freezing, irradiation
3. Chemical	Swelling agents (NaOH, NH_3), dilute and concentrated mineral acids (HCl, H_2SO_4), gases (SO_2, NO_2), amonia-based solvents (NH_3)
4. Biological	white-rot and soft-rot fungi, bacteria
5. Enzymatic	xylanases
6. Combined pretreatments	amonia freeze-explosion; high-temperature milling; alkali plus ball milling, steam explosion

Most pretreatment processes have been used extensively and can be further divided into representative examples causing an underlying result in different forms of biomass (Detroy and Julian, 1982). The advantages and disadvantages of the methods are, however, largely dependent on the type of biomass, the pretreatment conditions (time and temperature) used, the cost involved and the requirement of the specific industry for pretreatment. The methods do, however, have specific requirements, such as energy for the mechanical pretreatment that is dependent on the severity of the process. This pretreatment gives fine substrates that result in higher concentrations of higher bulk density. Swelling agents is the most common chemical pretreatment used. The chemical pretreatment are however not used for bioconversion purposes because of their recycling and environmental costs. Biological pretreatments are mainly concerned with the use of bacteria and fungi, but due to the lengthy pretreatment time, it is very detrimental to the pretreatment industries. The steam-explosion process seems to be the

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most promising of the combined pretreatment processes as lower energy requirements and no recycling are needed (Bisaria, 1998).

MICROBIAL FERMENTATION

The microbial fermentation of sugars originated more than 5000 years ago and led to the manufacturing of alcoholic beverages (Glazer and Nikaido, 1995). Fermentation is known to be the "life without air", but in the broad sense the term is commonly used as "a process in which chemical changes in an organic substrate are caused by the action of enzymes elaborated by microorganisms" (Jay, 1996). Biochemically, it is said to be an energy-yielding process where organic molecules serve as both electron donors and acceptors (Glazer and Nikaido, 1995; Jackson, 1994; Jay, 1996; Prescott *et al.*, 1993). The type of fermentation is named according to the main products of fermentation and is often characteristic of the particular microbial group (Prescott *et al.*, 1993). Figure 21 shows a few common fermentation pathways where microorganisms ferment sugars to lactate, ethanol, glycerol, succinate, formate, acetate, butanediol and additional products. An important process is alcoholic fermentation, where an organism (fungi, bacteria or algae) ferment sugars to ethanol and CO_2; pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol (pathway 2 in Figure 21).

The term fermentation does, however, has several different meanings where industrial microbiology and biotechnology are concerned (Prescott *et al.*, 1993):

(1) Aerobic or anaerobic process involving the mass culture of microorganisms;
(2) any biological process that occurs in the absence of O_2;
(3) food spoilage;
(4) the production of alcoholic beverages;
(5) an organic substrate used as the electron donor and acceptor;
(6) an organic substrate used as a reductant, and the use of the same partially degraded organic substrate as an oxidant (electron acceptor); or
(7) growth dependent upon substrate-level phosphorylation.

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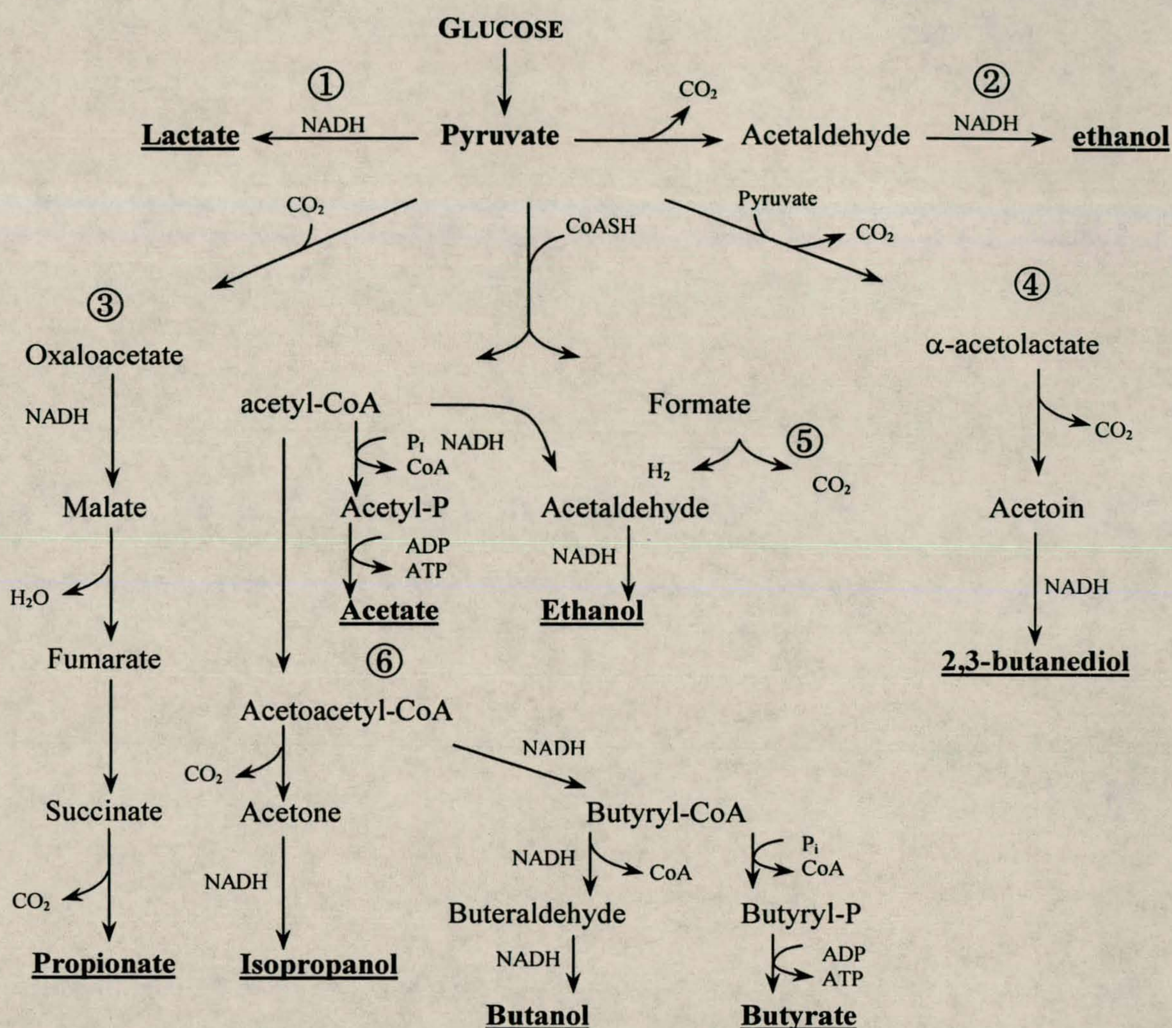

Figure 21. Simplified pathways of the type of fermentation characteristic of a particular microbial group (Prescott *et al.*, 1993). The major end products are bold and underlined.
① Lactic acid bacteria (*Bacillus, Streptococcus, Lactobacillus*) [Lactic acid fermentation]
② Yeast, *Zymomonas* [Alcoholic fermentation]
③ Propinionic acid bacteria (*Propionibacterium*)
④ *Enterobacter*, *Serratia*, *Bacillus* [Butanediol fermentation]
⑤ Enteric bacteria (*Escherichia*, *Enterobacter*, *Salmonella*, *Proteus*)
⑥ *Clostridium*

The industrial application of fermentation processes by microbes and the recent advances in biotechnology already produced several products of interest to the consumer. These products include wine, beer, ethanol, cheese and other fermented food products from fruits, vegetables, beans and related substrates (Jay, 1996; Prescott *et al.*, 1993). The fermentation processes that currently exist are primarily based on easily fermentable substrates such as sugar and starch. Biotechnological improvements in the

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fermentation industry are therefore necessary to enhance the rate of substrate utilisation, increase product yield, increase the energy efficiency of the recovery process (distillation) and to develop new sugar feedstocks (Detroy and Julian, 1982).

ORGANISMS USED IN FERMENTATION INDUSTRIES

Microorganisms have been used in many fermentation industries that include the dairy, meat and fish, wine, beer and other alcoholic beverages, as well as bread and other plant product industries (Table 8) (Jay, 1996). Fermentation has been an important way of preserving food as their substrates contain readily utilisable carbohydrates (Prescott *et al.*, 1993). These microorganisms cause chemical and or textural changes in the product and can be used to create new food flavours and aromas. They can either be present as the natural microbial growth or as an inoculated population.

Table 8. Fermented products produced in certain fermentation industries (Prescott *et al.*, 1993).

Industry	Microorganisms	Fermented product
Dairy	*Streptococcus thermophilus*	Yogurt
	Lactococcus lactis	Butter
	Lactobacillus bulgaricus	Mozzarella Cheese
Meat	*Lactobacillus plantarum*	Sausage
Plant products	*Leuconostoc mesenteroides*	Olives
	Pediococcus cerevisiae	Pickles

The fermenting microorganisms in the wine industry (Table 9) are predominantly yeast that can be native inhabitants of the grape, vineyards or winery equipment, spoilage organisms, or selected alcoholic fermenters (Boulton *et al.*, 1996). The yeasts that are important to the winemaker, are the native yeasts and those used as starter cultures. The native yeasts that naturally inhabit the grapes can be classified into two groups, the selected wine yeast and the "wild" yeast (Jackson, 1994). The wine yeasts include the different strains of *Saccharomyces* and some *Schizosaccharomyces* and *Zygosaccharomyces* genera, whereas the "wild" yeasts are the non-*Saccharomyces* species that perform natural or spontaneous alcoholic fermentation. The most important advantage of the natural fermentative organisms is their unique capability to provide distinctive characteristics (flavours) to wine. One disadvantage is that there are a variety of yeasts that can produce off-flavours. Using pure yeast cultures for fermentation result in rapidly active fermentation, predictable sugar-to-alcohol ratio, complete utilisation of fermentable sugars and improved alcohol tolerance.

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Table 9. Selected species of wine-related yeasts (Amerine *et al.*, 1972; Jackson, 1994).

Genus	Species in wine
Wine yeast	
Saccharomyces	*cerevisiae, bayanus*
Schizosaccharomyces	*japonicus* var. *japonicus*
Zygosaccharomyces	*balii, bisporus, cidri, florentinus, rouxii*
"Wild yeast"	
Candida	*stellata, apicola, sake, utilis, krusei, kefyer, mesenterica, incummunis, intermedia, valida, diversa, lambica, vini,*
Kloeckera	*apiculata, japonica, corticis*
Hansenula	*anomala, fabrianii, jadinii, subpelliculosa*
Pichia	*delftensis, fermentans, kluyveri,*
Brettanomyces	*anomallus, lambicus, intermedius, custersii*
Dekkera	*intermedia, bruxellensis*
Hanseniaspora	*osmopohila, uvarum, valbyensis*
Debaromyces	*hansenii*
Torulaspora	*delbruecikii*
Kluyveromyces	*marxianus, var. bulgaricus*
Saccharomycodes	*ludwigii*

MICROBIAL PRODUCTION OF ETHANOL

Ethanol can be used as a solvent, in beverages, in hydrocarbon synthesis and for biological energy (ATP) (Detroy and Julian, 1982). It is also used as a gasoline dilutant and the possibility to replace or extend gasoline, led to the establishment of fermentation plants for the production of fuel ethanol. Fuel ethanol proved to have significant advantages with respect to urban air quality and global climate change, and is substantially less toxic than gasoline and methanol (Lynd *et al.*, 1991).

The production of ethanol includes three basic steps: (1) conversion of starch or cellulose to fermentable sugars; (2) fermentation of the sugars to alcohol; and (3) alcohol recovery by distillation or separation into water-free (anhydrous) ethanol (Figure 9) (Glazer and Nikaido, 1995). The sources of this fermentation fuel include fruits and cellulose-containing waste materials. As was shown in Table 8, ethanol production is widely distributed among different yeast and bacteria that differ in their ethanol yield. These variations are attributable to the operation of four different metabolic routes for the production of ethanol (Detroy and Julian, 1982):

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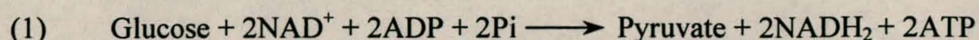
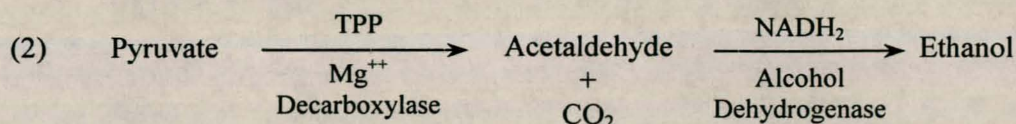
Route 1. Glycolysis

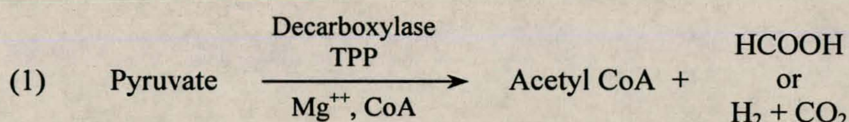
Glucose is quantitatively converted to ethanol and carbon dioxide via pyruvate, the route best known to yeast.

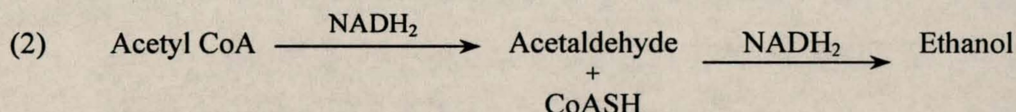




Route 2. Thioclastic reaction

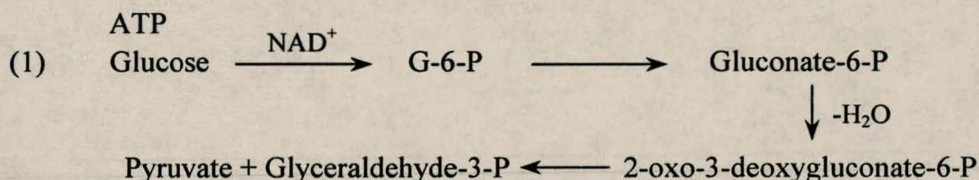
Clostridia and *Enterobacteriaceae* cleave pyruvate to yield acetyl coenzyme A (CoA), which is followed by a reduction to acetaldehyde and ethanol.

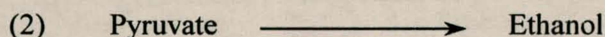




Route 3. Entner-Doudoroff pathway

Bacteria of the genus *Zymomonas* take up glucose and produce ethanol 3 to 4 times more rapidly than yeast by utilising the Entner-Doudoroff route. The fermentation balance is, however, similar to yeast.





Route 4. Heterolactic fermentations

Glucose is fermented by heterolactic microorganisms to produce lactate and ethanol via the Pentose-phosphate pathway with xylulose-5-PO_4 as intermediate.

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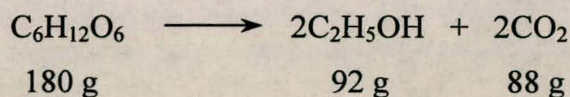
The formation of ethanol and carbon dioxide from a fermentative sugar (glucose) is, however, a more complex scheme as known by the more detailed glycolysis pathway. Although organisms have four different routes of producing ethanol, the best known conversion results in the equation that was established by Gay-Lussac in 1810, calculating the theoretical yield of alcohol to be 51.1% by weight (Glazer and Nikaido, 1995):



$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

$$180\text{ g} \qquad 92\text{ g} \qquad 88\text{ g}$$

ORGANISMS THAT PRODUCE ETHANOL

Ethanol is the primary product of fermentation in the brewery, winery, distillery and sake producing industries. The primary importance for these industries to consider is the organisms ability to utilise the substrate, the maximum level of ethanol produced and the organisms' ability to tolerate ethanol. Since different fermentation industries employ different fermentation conditions, the strains employed can tolerate differing levels of ethanol (Casey and Ingledew, 1986). Other factors that also influence ethanol yield are the temperature, carbohydrate level, substrate concentration and water activity.

Organisms that have the ability to produce ethanol are widely distributed (Table 6). *Saccharomyces cerevisiae* or *Zymomonas* are, however, the safest and most effective microorganisms to ferment sugars to ethanol (Glazer and Nikaido, 1995). An ideal alcohol fermenter must have certain characteristics (Glazer and Nikaido, 1995):

(1) the ability to rapidly ferment a broad range of carbohydrate substrates;
(2) produce low levels of by-products;
(3) the ability to withstand the high osmotic pressures due to high concentrations of sugar substrates (osmotolerance);
(4) temperature tolerance;
(5) ethanol tolerance;
(6) the ability to produce high concentrations of ethanol;
(7) a high cell viability to ensure repeated recycling; and
(8) flocculation and sedimentation characteristics should be appropriate to facilitate cell recycle.

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SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

DEFINITION

Separate hydrolysis and fermentation is where a polysaccharide is chemically or enzymatically hydrolysed to a mono- or disaccharide that can subsequently be fermented to ethanol. This is the conventional two-step process where both steps can be carried out at their respective optimum conditions (Glazer and Nikaido, 1995). The major disadvantage is that a lower polysaccharide concentration and higher enzyme load are required to obtain reasonable ethanol yields. Recent advances in biotechnology led to the single-step process for the conversion of a polysaccharide to ethanol known as simultaneous saccharification and fermentation (SSF). Hydrolysis and fermentation are carried out or take place in a single vessel and has several advantages (Bisaria, 1998):

(1) the removal of sugars enhanced the rate of polysaccharide hydrolysis and inhibit polysaccharase activity;
(2) the enzyme loading is lower;
(3) the product yield is higher;
(4) the requirement for aseptic conditions decreased;
(5) limiting the initial polysaccharide concentration may also limit the ethanol concentration that is not inhibitory to the organism or the polysaccharase;
(6) the process time considerably reduced; and
(7) the use of a single reactor.

The most important requirement for the system is the optimum conditions with respect to temperature, pH and substrate concentration. The SSF process must, however, still fulfil certain other requirements to produce ethanol (Bisaria, 1998):

(1) saccharification and fermentation conditions must be compatible;
(2) a high ethanol yield and production rate;
(3) the culture must have a high ethanol tolerance;
(4) the enzyme must be minimally inhibited by ethanol;
(5) the level of β-glucosidase must be optimal in certain polysaccharides to minimize accumulation of disaccharides;
(6) ethanologen must be resistant to the lytic effects of polysaccharase; and
(7) polysaccharase must be resistant to proteolytic enzymes of the ethanologen.

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ORGANISMS THAT CAN PERFORM SSF

Only a few organisms that have the ability to perform SSF occur naturally and no modification has been brought to them. As there are only a few of these organisms, genetically engineered organisms were constructed to produce the useful product in a single vessel. Most organisms, especially yeasts, are unable to hydrolyse complex polysaccharides, but they are excellent fermenters. Yeast has complete GRAS (generally regarded as safe) status and has several properties making it an attractive host for the expression and production of enzymes that are of biotechnological interest. If the genes of polysaccharide degrading enzymes are expressed in yeast, it would expand its substrate range and make it more favourable to use in industries. The genes of organisms that has the ability to saccharify complex polysaccharides have been purified and characterised and expressed in yeast (Pretorius, 1997). This resulted in genetically engineered organisms that have the ability to simultaneously saccharify and ferment purified complex polysaccharides (Table 10).

Table 10. The hydrolase genes of donor organisms that have been expressed in *S. cerevisiae*.

Activities	Donor	References
Endoglucanases	*Trichoderma reesei,*	Bailey *et al.*, 1993,
	Clostridium thermocellum	Sacco *et al.*, 1984
Cellobiohydrolases	*Phanerocheate chrysosporium*	Van Rensburg *et al.*, 1996
β-Glucosidase	*Apergillus niger*	Penttilä *et al.*, 1984
Cellobiase	*Endomyces fibuliger*	Van Rensburg *et al.*, 1998
Xylanases	*Aspergillus niger*	Luttig *et al.*, 1996
β-xylosidase	*Bacillus pumilis,*	La Grange *et al.*, 1997
	Trichoderma reesei	Margolles-Clark *et al.*, 1996
Accessory xylan	*Apergillus niger,*	Crous *et al.*, 1996
enzymes	*Trichoderma reesei*	Margolles-Clark *et al.*, 1996
Pectate lyase	*Erwinia chrysanthemi*	Laing and Pretorius, 1992
Polygalacturonase	*Erwinia carotovora*	Laing and Pretorius, 1993a
Pectin lyase	*Glomerella cingulata*	Templeton *et al.*, 1994
α-amylase	*Bacillus amyloliquefaciens*	Kovaleva *et al.*, 1989
	Lipomyces kononenkoae	Steyn and Pretorius, 1995
β-amylase	*Aspergillus oryzae*	Hata *et al.*, 1992
Pullulanases	*Klebsiella pneumoniae*	Janse and Pretorius, 1993
Combination:		
Amylopullulanase	*Lipomyces kononenkoae*	
Pectate lyase	*Erwinia chrysanthemi*	
Polygalacturonase	*Erwinia carotovora*	
endo-β-1,4-glucanase	*Butyrivibrio fibriosolvens*	
cellobiohydrolase	*Phanerocheate chrysosporium*	Petersen *et al.*, 1998
cellobiase	*Endomyces fibuliger*	
endo-β-D-xylanase	*Aspergilluse niger*	
exo-β-1,3-D-glucanase	*Saccharomyces cerevisiae*	

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Since grapes are the world's largest fruit crop that is used in wine making, hydrolysis (saccharification) and fermentation of waste materials such as grape pomace can yield ethanol for the fortification of dessert wines, for fuel alcohol or other applications (Mazza, 1995). Variations in ethanol yield can be expected due to the chemical composition of grapes from different varieties and the type of processing conditions, such as the method of hydrolysis and the fermentation system used (Hang, 1988). A solution for the problems associated with waste treatment, was to investigate the organisms present in pomace and exploit it to perform SSF.

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BIOPROCESSING OF GRAPE POMACE

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BIOPROCESSING OF GRAPE POMACE

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ABSTRACT

Grape pomace is, by volume, the primary by-product that is generated during grape berry processing and wine production. Grape pomace is considered to be an agricultural waste product and its processing is of considerable economic and environmental interest. The feasibility of bioprocessing was preceded by chemical analysis of representative samples of grape pomace. Enzymatic hydrolysis of grape pomace with different purified enzymes confirmed that enzymatic biodegradation could occur. Natural occurring microorganisms associated with grape pomace were isolated and their ability to degrade complex substrates was evaluated. These organisms were compared to two recombinant strains of *Saccharomyces cerevisiae* encoding genes for various hydrolytic enzymes. The extent of, and various hydrolysis products produced by the various organisms, as well as their ability to ferment the resulting monosaccharides, was evaluated. The results indicate that the yeast *Pichia rhodanensis* and the two recombinant *S. cerevisiae* strains are capable of hydrolysing polysaccharides from grape pomace (cellulose, hemicellulose, starch and pectin) thereby releasing fermentable sugars that could be used for the subsequent production of ethanol.

INTRODUCTION

Grape pomace consists of the fibrous material that remains after the juice has been extracted from grape berries. It consists of the processed skins, seeds and stems (Hang, 1988; Mazza, 1995), and is separated from the grape juice prior to fermentation of white wines, or after partial fermentation of red wines (Prescott *et al.*, 1993). Grape pomace is potentially useful in many ways; it can be used as animal feed and the seed extracts provide grape seed oil for health food and gourmet groceries (Amerine *et al.*, 1972; Greene, 1998; Heimoff, 1996; Valiente *et al.*, 1995). For many years it has been ploughed back into the soils of vineyards as a natural source of nitrogen and phosphate

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and provide nutrients in a concentrated and stable form (Greene, 1998). The high moisture content of grape pomace renders it susceptible to rapid microbial spoilage (Hang, 1988). This contributes to environmental pollution as copious amounts are produced by an ever increasing number of wineries (Greene, 1998; Mazza, 1995). Factors contributing to the quantity and quality of pomace, are harvest and processing conditions, the microorganisms present, and the changes in the biochemical content.

Grape pomace consists of the four major polysaccharides in nature, i.e. cellulose (consisting of glucose subunits), hemicellulose (glucose, mannane, xylan and arabinose subunits), starch (glucose subunits) and pectin (D-galacturonic acid subunits) (Hulme, 1970). Starch serves mainly as an energy reserve in plants, while cellulose, hemicellulose and pectin are closely associated in an integrated structure that plays an important role in the cell wall (Glazer and Nikaido, 1995). These polysaccharides should, therefore, at least be partially degraded to make the other substrates more accessible for microbial degradation to release the mono- and disaccharides. Residual levels of sucrose, glucose, fructose and traces of other reducing sugars are also present in grape pomace. Together with the polysaccharides, the available carbohydrate fractions of grape pomace are of considerable interest to the wine industry as they may contribute to the production of fuel alcohol.

Although the wine yeast *Saccharomyces cerevisiae* is well known for its ability to produce ethanol from various fermentable sugars, it is not able to hydrolyse complex polysaccharides (Glazer and Nikaido, 1995). The genes encoding some of the enzymes required for the hydrolysis of polysaccharides have been cloned and expressed in *S. cerevisiae* to degrade these polysaccharides to mono- and disaccharides (Petersen *et al.*, 1998; Van Rensburg *et al.*, 1998).

The ability of two cellulolytic recombinant strains to hydrolyse the polysaccharides present in grape pomace was evaluated. Furthermore, natural-occurring microorganisms were isolated from grape pomace and evaluated for their ability to hydrolyse cellulose, hemicellulose, pectin and starch to fermentable sugars. Effective hydrolysis of the poly- and disaccharides, followed by fermentation of the sugars to ethanol, could result in a simple and cost-effective process that produce few by-products and reasonable concentrations of alcohol.

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MATERIALS AND METHODS

Strains and plasmids

The various yeast strains and plasmids used in this study were the industrial wine yeast strain VIN7, *Saccharomyces cerevisiae* Y294 (MATα *leu2-3, 112 ura3-52, his3, trp1-289*) obtained from W. H. van Zyl, and *S. cerevisiae* with either plasmid pECCB (Ap^R *URA3*, *END1*, *CBH1*, *CEL1*, *BGL1*) or pBEE (Ap^R *URA3, END1, BEG1, EXG1*) obtained from P. van Rensburg (1997; 1998).

Isolation of naturally occuring microorganisms

Grape pomace (freshly pressed or exposed to air for a few days) was diluted to 1 g in 10 ml sterile 0.85% NaCl. A dilution series from 10^{-1} to 10^{-8} was made and 100 µl of each dilution was spread onto agar plates. Malt extract agar plates (MEA; 3% malt extract, 0.5% soy peptone and 1.5% agar [Merck Biolab Diagnostics, Midrand, SA]), containing 20 µg/ml chloramphenicol (Boehringer Mannheim, Germany) were used to isolate yeast and other fungal species. Bacterial species were isolated with plate count agar (PCA; 0.5% tryptone, 0.25% yeast extract, 0.1% glucose and 1.5% agar) plates, containing 10 µg/ml filter sterilised cyclohexamide (Sigma, St. Louis, MO) (Ausubel *et al.*, 1989). The plates were incubated at 30°C (for mesophiles) or 40°C (for thermotolerant organisms) for two days. Sequential plating onto fresh plates enabled the isolation of pure colonies.

Screening procedures for isolates

Colony and cell identification. Pure colonies were obtained and identified by their characteristic growth on the agar media. This included the general form and shape of the colony margin or edge, the colony elevation or cross-section, the size, colour and appearance of the colony. Microscopy was used to study the shape and structure of the yeast and fungi, and standard physiological tests [Gram staining and catalase (H_20_2) activity] were used to identify the bacteria (Harley and Prescott, 1993).

Enzyme activity assays on polysaccharide plates. Microorganisms isolated from grape pomace were aerobically evaluated for their ability to hydrolyse cellulose, hemicellulose, amylose and pectin. Enzyme activities were determined as zones

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produced by the colonies after growth on selective MEA or PCA plates containing the respective polysaccharides.

The CMC plates contained 0.1% carboxymethylcellulose (Sigma P-C4888) and the xylan plates contained 0.1% Bichwood xylan (Sigma X-0502). Colonies on the xylan and CMC plates were incubated until a colony diameter of 5 mm was obtained (approximately 2 days). The plates were stained by flooding with 0.1% Congo red (Merck) for 15 minutes while shaking at 50 rpm, and destained by rinsing with 1 N NaCl for 15 minutes (Teather and Wood, 1982). A clear zone around the colony indicated enzyme activity and the varying levels thereof was determined by the diameter of the zone. The isolates were compared to *S. cerevisiae* strain Y294, industrial wine yeast strain, VIN7, and the recombinant *S. cerevisiae* strain for their ability to utilise cellulose.

Starch hydrolysis was evaluated by streaking the respective organism onto a MEA or PCA plate containing 0.1% starch (Merck). The plates were incubated for 24 hours at 30°C and 40°C respectively. Gram's iodine (Saarchem, Krugersdorp, SA) was used to indicate the presence of starch by placing several drops on each of the streaks (Harley and Prescott, 1993). When the iodine interacts with the starch, it forms a blue to brown complex. A clear zone around the line of growth indicates that the starch has been hydrolysed.

Pure cultures were also streaked onto pectin plates [6.7 g/l Yeast Nitrogen Base (Difco laboratories), 1% glucose, 1% apple pectin (Sigma), 0.5% agar and 50 mM pH 5.5 phosphate buffer ($K_2HPO_4 \cdot KH_2PO_4$)] and then incubated at 30°C for 10 days (Cruickshank and Wade, 1980; Gainvors *et al.*, 1994). The surface of the agar was flooded with 0.1% ruthenium red (Sigma) for 2 hours and then washed with several changes of sterile distilled water for 2 hours to remove the ruthenium red from the surface. The ruthenium red penetrates deeply into the medium and indicates degradation around the colony. Pectin esterases (PE) produce zones with a darker staining than the background; pectin lyases (PL) produce yellow zones or clear zones with yellow margins; and polygalacturonase (PG) activity is indicated as a colourless or pale zone surrounding the colony (Cruickshank and Wade, 1980).

Other identification procedures. The yeast species that were isolated were further identified using the ID 32C yeast API strips (bioMérieux sa 69280 Marcy-l'Etoile, France). The yeasts' ability to grow in a defined medium is measured with strips

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containing 22 carbohydrate assimilation tests, five organic acid assimilation tests, two amino acid assimilation tests, one colourometric test and one growth test. The probable identification of the yeast, as well as its ecology, is indicated by this system. The yeasts isolates were also sent to the Centraal Bureau voor Schimmelcultures (Netherlands) for a more detailed and precise identification.

HPLC analysis of pomace

Optimisation of pre-treatment for pomace. HPLC analysis (Aminex HPX087C carbohydrate analysis column) of the pomace was done to determine the relative concentrations of the different sugars that could be released by hydrolysis. Different pre-treatments were performed on the grape pomace:

(1) Wet pomace was homogenised with a household blender for 6 minutes and 1 g was suspended in 100 ml sterile double distilled water (ddH_2O).

(2) A suspension of wet pomace (1 g in 100 ml ddH_2O) was homogenised for 2 minutes as described above.

(3) Pomace was dried at 60°C for 48 hours, homogenised with a household coffee grinder (grounded) (Valiente *et al.*, 1995) and autoclaved for 15 minutes at 121°C, 2 kPa. One gram of dried pomace was added to 100 ml ddH_2O.

(4) A suspension of the dried and grounded pomace (1 g in 100 ml ddH_2O) was autoclaved.

(5) Fifteen grams of dried, grounded and autoclaved pomace was added to 100 ml ddH_2O and incubated at 60°C for 2 hours.

(6) Dried and grounded pomace was sterilised by radiation (11 hours with ^{60}Co), 15 g were added to 100 ml ddH_2O and incubated at 60°C for 2 hours.

These different pomace concentrations and pre-treatments were used to optimise the availability of sugars. All samples were centrifuged (12000 rpm, 5 minutes) and the supernatant filtered through a 0.22 μm membrane filter before HPLC analysis.

Optimisation of hydrolytic treatments. (1) Acid hydrolysis: Intact polysaccharides can not be detected by HPLC analysis, therefore suspensions of pomace in ddH_2O were hydrolysed with sulphuric acid (12 M H_2SO_4 for 2 hrs at 20°C, followed by 0.8 M H_2SO_4 for 3 hrs at 100°C) to release the mono- and disaccharides (Ausubel *et al.*, 1987; Valiente *et al.*, 1995). Different time intervals were also used to optimise the acid

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hydrolysis. After hydrolysis, the samples were exposed to a vacuum to vaporise all the excess acid and washed twice with 1 ml methanol. After removal of the methanol, the pomace was suspended in 1 ml ddH_2O and filtered with a 0.22 μm membrane prior to HPLC analysis.

(2) Enzymatic hydrolysis: A suspension of 15 g pomace per 100 ml ddH_2O was incubated for 24 hours at 30°C with purified commercial *Aspergillus niger* enzymes [450 units cellulases, 138 units hemicellulases or 1.4 units pectinases (Sigma)] to determine hydrolysis. The samples were centrifuged and the supernatant filtered for HPLC analysis.

Microbial Hydrolysis of Grape Pomace

The yeast isolates that demonstrated enzymatic activity on purified polysaccharides were evaluated for their ability to hydrolyse the polysaccharides present in the grape pomace using various assays.

Plate assays. Yeast isolates were streaked onto MEA plates containing 0.1% dried, grounded and autoclaved pomace as the only carbon source, and incubated for 24 hrs at 30°C. Hydrolysis was determined by the presence of a clear zone around the colony using 0.1% Congo red as indicator for cellulose and hemicellulose, Gram's iodine for starch, and 0.1% ruthenium red for pectin.

Liquid assays. Yeast cultures were pre-grown at 30°C in 5 ml YPD (1% yeast extract, 2% peptone and 2% glucose) medium containing 20 μg/ml chloramphenicol. Cells were inoculated in 1% dried and grounded pomace to an initial cell density of 1×10^6 per ml. Cultures were incubated at 30°C while shaking and growth was monitored at an absorbency of 600nm over a period of 5 days. Samples were taken at 24 hour intervals, centrifuged (12000 rpm) for 5 minutes and the supernatants assayed. The pH was measured with Panpeha multi-colour strips (Riedel-de Haën, Seelze) and the release of reducing sugars and oligosaccharides was determined by the dinitrosalicylic acid (DNS) method described by Miller *et al.* (1960). Ethanol concentrations were determined enzymatically by the alcohol dehydrogenase/NADH absorbance-method (Boehringer Mannheim, Germany).

Secreted enzymes. To avoid the immediate utilisation of the released sugars, the isolates were inoculated in 1% pomace to an initial cell density of 1×10^6 per ml and

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incubated for 24 hours at 30°C. Subsequently, the suspension was centrifuged (10000 rpm, 10 minutes) and filtered through Wattman paper. The supernatant (8 ml) was added to fresh pomace (0.1 g in 2 ml ddH_2O) and incubated either aerobically or anaerobically for 24 hours. The concentration of reducing sugars and oligosaccharides and levels of ethanol were determined in the fresh pomace suspensions as described previously.

Congo Red absorbance assays. Congo Red was used as an indicator to determine hydrolysis spectrophotometrically (Van Rensburg *et al.*, 1997). A grape pomace suspension (15% pretreated for 2 hrs at 60°C) was inoculated with the respective isolates and incubated aerobically at 30°C for 5 days. The pre-treated pomace was also incubated with purified commercial enzymes (Table 1) to determine hydrolysis. Aliquots of 1 ml were removed at intervals, heated at 100°C for 15 minutes and cooled to room temperature. Subsequently, 200 μl Congo red (100 μg/ml) was added and diluted with 800 μl 0.05 mM citrate-phosphate buffer (pH 6.2) to 2 ml. The absorbance of the Congo red/pomace complex was measured at 540nm (Wood *et al.*, 1988); a decrease in colour intensity would indicate hydrolytic activity. Parallel to the absorbance assays, the growth, pH, DNS values, ethanol production, as well as HPLC analysis, were performed.

Table 1. Different concentrations of purified hydrolytic enzymes (Sigma) used in this study.

Enzyme	**Units used (minimum units required)**
Cellulase from *Aspergillus niger*	1 (0.3 units/mg solid)
Hemicellulase from *A. niger*	5 (5 units/mg solid)
Xylanase from *Aureobasidium pullulans*	30 (30-60 units/mg solid)
Pectinase from *A. niger*	0.28 (0.28 units/ml)
Pectin esterase from orange peel	10 (3.8-26.9 units/ml)
Pectin lyase from *A. niger*	1 (0.4-1.2 units/ml)
α-glucosidase from *S. cerevisiae*	125 (125 units/mg protein)
Amyloglucosidase from *A. niger*	30-60 (30-60 units/mg protein)
Cellulase and hemicellulase from *A. niger*	450 and 138 units

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RESULTS AND DISCUSSION

Isolation and screening of naturally occurring microorganisms

Naturally occurring microorganisms were isolated from two batches of wet pomace (freshly pressed) and dry pomace (exposed to air for a few days) obtained from Stellenbosch Farmer's Winery, Stellenbosch, South Africa. Preliminary identification of the isolated organisms using solid medium incubated aerobically revealed 13 yeast, 14 mycelial fungal, one actinomycete and 46 bacterial species based on colony morphology. Further analyses for the hydrolysis of pure solutions of cellulose, hemicellulose and starch at 30 and 40°C (Table 2), revealed yeast and bacteria with varying enzymatic activities (Table 3). Although significant results were obtained at 40°C, subsequent experiments were performed at 30°C as it was the optimum conditions used in industries.

Table 2. Percentage of organisms isolated from either wet or dry pomace that have the ability to hydrolyse cellulose (CMC), xylan or starch at 30 and 40°C, respectively.

		% of isolated yeasts capable of hydrolysis		**% of isolated bacteria capable of hydrolysis**	
Polysaccharide	**Temperature**	**Wet**	**Dry**	**Wet**	**Dry**
Cellulose	30°C	9	56	27	27
	40°C	83	100	50	33
Xylan	30°C	18	42	9	0
	40°C	83	75	33	39
Starch	30°C	64	42	82	82
	40°C	33	88	100	94

Only nine yeast, eight bacteria, one actinomycete and three fungal species displayed activities on all these polysaccharides. These respective organisms were also evaluated for pectin esterases, polygalacturonases and pectin lyases activity (Table 3). The nine yeast isolates that were able to hydrolyse purified polysaccharides were further identified using the ID 32C yeast API system. Table 4 indicates the percentage homology with a known species. Preliminary identification of the yeast species was done with the API system, but further identification performed by the CBS yeast division revealed different results for some of the species (Table 4).

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Table 3. Organisms isolated from grape pomace and their ability to hydrolyse purified cellulose (CMC), starch, xylan and pectin: **C,** Cellulase; **X,** Xylanase; **A,** Amylases; **PG,** Polygalacturonase; **PE,** Pectin esterase; **PL,** Pectin lyase.

Bacteria	**Description**	**C**	**X**	**A**	**PE**	**PG**	**PL**
B1	Yellow, Gram + cocci, H_2O_2+	++	++	+++	-	+++	-
B2	Off-white, Gram + cocci, H_2O_2+	++	++	++	++	+++	-
B3	Clear, Gram + cocci, H_2O_2+	+++	++	++	-	+++	-
B4	Clear, Gram + bacilli, H_2O_2+	++++	++	++++	-	-	-
B5	Off-white, Gram + bacilli, H_2O_2+	+++	++	++	-	-	-
B6	White, Gram + cocci, H_2O_2+	+	+	++	-	+++	-
B7	White, Gram + cocci, H_2O_2+	+	+	++	-	-	-
B8	White, Gram + bacilli, H_2O_2+	++	+++	++++	-	-	-
Yeast							
Y1	Shiny white colony	+++	+++	+	-	-	+++
Y2	Off-grey colony	+++	+++	++	+++	-	-
Y3	White colony	+++	+++	+	-	-	++++
Y4	Shiny off-white colony	+++	+++	+	-	-	+++
Y5	Off-grey colony	++	++	+	++++	-	++++
Y6	Shiny white colony	+++	+++	+	-	++	++
Y7	Shiny off-white colony	++	++	+	-	++	++
Y8	Off-white colony	++	++	-	++++	-	++++
Y9	White colony	+	+	++	-	++++	-
Fungi and Actinomycetes							
Mucor sp.	Grey, hairy	++	+++	++	++++	++++	-
Penicillium sp.	Light blue, flat powdery	+++	+++	+++	-	-	-
Aspergillus sp.	Black, cottony	++++	++++	++++	-	++++	++++
Streptomyces sp.	Grey, flat	+++	+++	+++	-	-	-

(+): varying levels of enzymatic activity; (-): no activity

Table 4. Identification of yeast isolates by the API-system and the CBS yeast division.

API-system		**CBS**
Preliminary Identification	**%**	**Conclusive Identification**
1. Candida lusitaniea	95.9	*Pichia rhodanensis*
2. Candida krusei	87.6	*Candida krusei*
3. Saccharomyces cerevisiae	99.9	*Saccharomyces cerevisiae*
4. Candida lusitaniea	95.9	*Pichia rhodanensis*
5. Candida krusei	87.6	*Candida krusei*
6. Candida lusitaniea	93.7	*Pichia rhodanensis*
7. Candida kefyr	99.9	*Kluyveromyces marxianus*
8. Candida krusei	87.6	*Candida krusei*
9. Trichosporon mucoides	85.6	*Zygoascus*

%, percentage probability based on ID 32C yeast API system.

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HPLC analysis of grape pomace

The relative amounts of different polysaccharides will greatly influence the effectiveness of the various organisms with respect to hydrolysing these substrates, as well as the type and concentration of fermentable sugars. As discussed in previous sections, the chemical composition of the pomace can differ greatly between the different varieties of grapes. HPLC analysis was therefore used to determine the levels of various sugars present in the grape pomace. The different pomace pretreatments (Table 5) revealed that the best option indicated by the glucose and fructose values, was to sterilise the dried and grounded pomace by autoclaving. This treatment gave highly reproducible results and lowered the degree of fungal contamination. HPLC analysis indicated high concentrations of residual glucose and fructose. An interesting observation was that the levels of glucose were much higher than fructose (Table 5), confirming that glucose is the predominant monosaccharide in grapes.

Table 5. HPLC analysis of different physio-chemical treatments of grape pomace as indicated by the percentage of dry weight of sugars.

Pre-treatment	Maltose (%)	Glucose (%)	Fructose (%)	Cellobiose (%)	Xylose (%)	Arabinose (%)
0.1% Wet pomace	0.068	0.068	0.002	0.029	0	0.010
Acid hydrolysed	**0.182**	**0.185**	**0.391**	**0.138**	**0**	**0**
0.1% Dried, ground	0.069	0.077	0.012	0	0	0.017
Acid hydrolysed	**0.168**	**0.200**	**0.295**	**0.120**	**0**	**0.309**
0.1% Autoclaved	0	0.075	0.014	0	0	0.018
Acid hydrolysed	**0.031**	**0.075**	**0.024**	**0.009**	**0**	**0.030**
0.1% Dried, ground 50°C for 2hr	0.009	0	0	0	0	0.019
1.5% Dried, ground 60°C for 2hr	0	0.360	0.040	0	0	0.004

Different acid hydrolytic treatments on pomace were evaluated for the release of monosaccharides. A significant increase in the concentrations of the mono- and disaccharides maltose, glucose, fructose and arabinose was observed after acid hydrolysis, suggesting the presence of polysaccharides that can be hydrolysed to fermentable sugars (Table 5). Treatment of grape pomace with hydrolysing organisms should therefore release sufficient mono- and disaccharides for further fermentation. The fructose values were higher than glucose after hydrolysis, which could imply that a substantial amount of polysaccharides with fructose as a subunit were hydrolysed. The

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presence of the disaccharides maltose and cellobiose also indicated that the hydrolytic treatments were incomplete. Suspensions of pomace using extended times of exposure to sulphuric acid were evaluated to optimise the release of only monosaccharides. The results were, however, inconclusive since the ring structures of the monosaccharides were also hydrolysed.

Enzymatic hydrolysis with different purified enzymes revealed that high concentrations of monosaccharides, especially glucose and fructose, could be released from pomace (Table 6). Individual treatments with hemicellulase, cellulase or pectinase released comparable amounts of monosaccharides. However, when simultaneously used, higher amounts of glucose and fructose were released, indicating that combinations of the various hydrolytic enzymes are required for the optimal hydrolysis of the polysaccharides present in grape pomace. This suggests that microbial degradation of grape pomace could take place if the microorganism produce one or more of these enzymes.

Table 6. HPLC analysis of enzymatic hydrolysis of grape pomace with purified enzymes (15% pomace pre-treated at 30°C for 24 hrs).

Enzyme (units/15g dried pomace)	Glucose (%)	Fructose (%)	Mannose (%)	Arabinose (%)	Galactose (%)
Cellulase (450)	0.542	0.103	0.000	0.001	0.002
Hemicellulase (138)	0.576	0.092	0.000	0.001	0.000
Pectinase (1.4)	0.635	0.094	0.000	0.001	0.001
Cellulase (450), hemicellulase (138) and pectinase (1.4)	0.813	0.119	0.001	0.001	0.000

Microbial hydrolysis of grape pomace

Plate assays. The respective CMC, xylan, pectin and starch substrates were substituted by grape pomace that presumably contained various amounts of these polysaccharides. The assays were performed to determine whether the isolated microorganisms were capable of hydrolysing pomace in a solidified media. The pomace particles, however, made it difficult to identify and measure the zones around a colony. Small or no zones were obtained, probably due to the low polysaccharide concentration and complex structure of the pomace (Table 7).

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Table 7. Hydrolysis of various polysaccharides present in 0.1% grape pomace using plate assays for the yeast isolates.

CBS identification	Cellulose & Hemicellulose	Starch	Pectin
1. Pichia rhodanensis	+	+	-
2. Candida krusei	-	-	-
3. Saccharomyces cerevisiae	-	-	+
4. Pichia rhodanensis	-	+	-
5. Candida krusei	-	-	+
6. Pichia rhodanensis	+	+	+
7. Kluyveromyces marxianus	+	-	+
8. Candida krusei	-	-	+
9. Zygoascus	+	-	+
Control: Aspergillus sp.	+	+	+

(+): enzyme activity; (-): no activity

Liquid assays. The liquid assay was chosen as an alternative method due to the difficulty in screening polysaccharide degradation on plates. Although positive growth was observed (Table 8), no conclusion could be made since the pomace particles in the suspension made it difficult to evaluate. The levels of reducing sugars decreased, suggesting that the sugars were utilised for cell growth upon hydrolysis. The pH levels remain unchanged at pH 6 and no ethanol production was observed since aerobic conditions were used.

Table 8. Yeast isolates' ability to hydrolyse solutions of 1% pomace.

CBS identification	Growth A_{600}		Reducing sugars (A_{540})		Ethanol [g/l]	
T_{days}	T_0	T_5	T_0	T_5	T_0	T_5
1. Pichia rhodanensis	0.40	3.02	1.28	0.66	0.8	-0.0
2. Candida krusei	0.42	2.36	1.56	0.92	0.8	-0.0
3. Saccharomyces cerevisiae	0.34	1.56	1.28	0.86	0.8	-0.0
4. Pichia rhodanensis	0.41	2.38	1.47	0.74	0.7	-0.0
5. Candida krusei	0.46	1.42	1.47	0.66	0.7	-0.0
6. Pichia rhodanensis	0.34	2.41	1.83	0.60	0.8	-0.0
7. Kluyveromyces marxianus	0.34	2.93	1.77	0.85	0.8	-0.0
8. Candida krusei	0.42	2.21	1.34	0.83	0.7	-0.0
9. Zygoascus	0.41	3.02	2.02	0.93	0.3	-0.0
Negative control	0.30	1.04	0.65	1.17	0.0	-0.0

Secreted enzymes. The liquid assay was modified to nullify the utilisation of sugars for microbial cell growth. The supernatant containing the secreted enzymes from representative isolates of *Pichia rhodanensis*, *Candida krusei* and *Zygoascus* were used

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to evaluate the organisms' ability to hydrolyse the polysaccharides present in the pomace. The sharp increase in reducing sugars within 3 hrs (Table 9) was probably due to residual sugars that dissolved in the suspension. *Pichia rhodanensis* showed a further increase in reducing sugars after 6 hrs, suggesting hydrolysis of the polysaccharides by enzymes present in the supernatant.

For all three species, cultures containing only the supernatant showed a decrease in reducing sugars after 24 hrs, indicating possible contaminants that utilised the sugars upon release. However, inoculation with either the isolate itself or *S. cerevisiae* strain Y294 resulted in a stronger utilisation of the reducing sugars by the microbial cells. Re-inoculation with *S. cerevisiae* showed an increase in ethanol concentration after 6 hrs for all three species, but this was not sustained over the 24-hr period investigated. Anaerobic incubation of the grape pomace with the supernatant resulted in a significant increase in ethanol concentration for all three species tested. The most effective production of ethanol was observed for *P. rhodanensis* that also showed the most effective release of sugars. These results suggested that enzymes secreted by *P. rhodanensis* were able to hydrolyse some of the polysaccharides in grape pomace and ferment the released sugars to ethanol under anaerobic conditions.

Table 9. Levels of reducing sugars and ethanol concentrations after treatment with filtered growth media containing possible secreted enzymes.

CBS identification		**Reducing sugars(A_{540})**				**Ethanol Concentration [g/l]**			
	Time (hrs)	**T_0**	**T_3**	**T_6**	**T_{24}**	**T_0**	**T_3**	**T_6**	**T_{24}**
Pichia rhodanensis	*a	0.44	1.42	1.63	1.17	0.03	0.03	0.02	-0.02
Isolate 6	b		1.28	1.60	1.05		0.02	0.02	-0.03
	c		1.46	1.66	0.99		0.03	0.05	-0.07
	d		1.41	1.79	0.96		0.02	0.07	0.40
Candida krusei	a	0.41	1.58	1.47	1.29	0.00	0.03	0.01	-0.02
Isolate 8	b		1.41	1.35	1.34		0.01	0.01	-0.01
	c		1.51	1.47	1.16		0.07	0.08	-0.02
	d		1.61	1.78	1.09		0.01	0.05	0.16
Zygoascus	a	0.39	1.54	1.49	1.15	0.03	0.02	0.02	-0.06
Isolate 9	b		1.37	1.73	1.16		0.01	0.03	-0.05
	c		1.21	1.33	0.98		0.09	0.13	-0.00
	d		1.49	1.74	1.27		0.02	0.05	0.17

* a, only supernatant – aerobic growth
b, supernatant plus re-inoculation with isolate;
c, supernatant plus re-inoculation with *S. cerevisiae* Y294;
d, only supernatant – anaerobic growth

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Absorbance assays. A higher concentration of grape pomace was used (15% instead of 1%) to improve the quantification of microbial hydrolysis using absorbency of the Congo Red/polysaccharide complex at 540 nm. The negative control (no inoculum) in Figure 1 showed no breakdown of grape pomace polysaccharides over a period of five days. Commercial enzymes showed that a substantial amount of pomace was degraded within 3 hrs when high concentrations of cellulase (450 units) and hemicellulase (138 units) were added together (Figure 1).

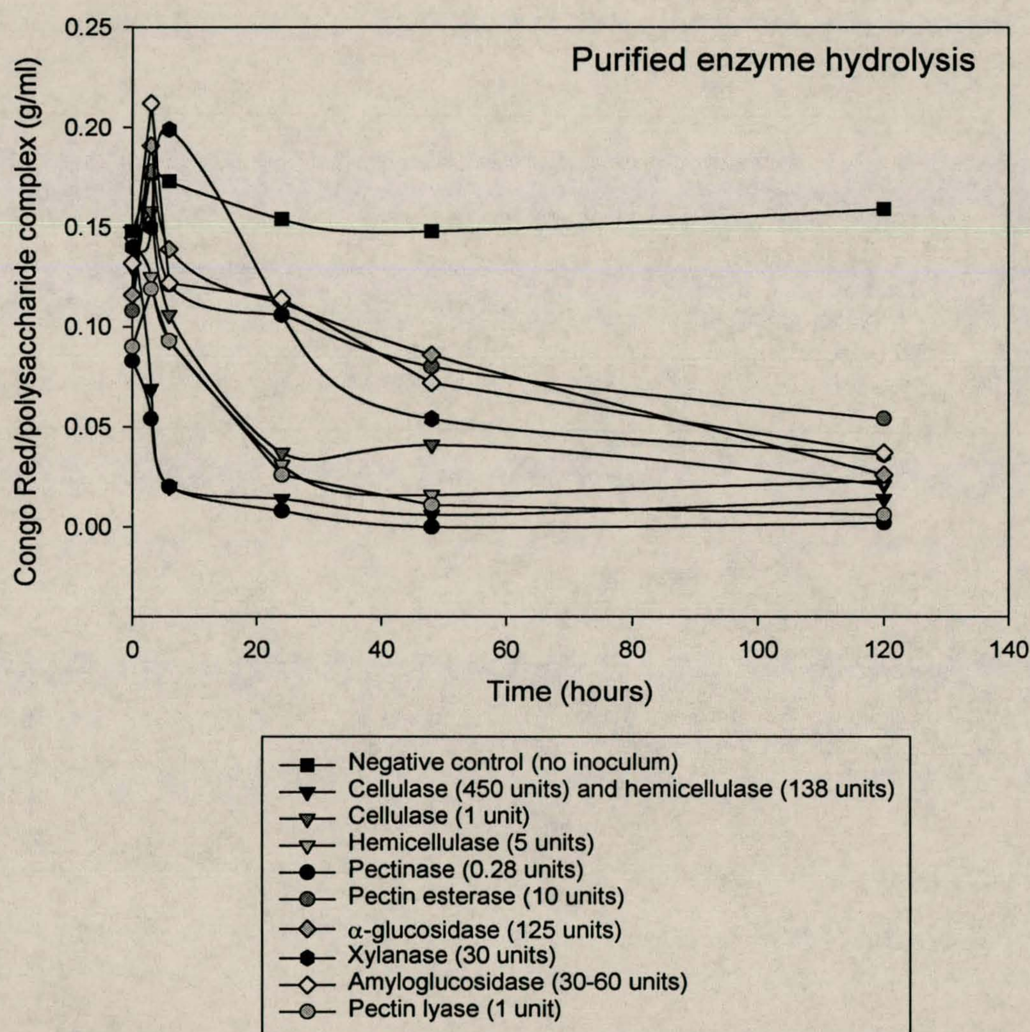

Figure 1. Purified commercial enzymes used for the enzymatic hydrolysis of grape pomace.

Using the minimal concentration of the respective enzymes shown in Table 1, hydrolysis was completed within 5 days. Cellulase and hemicellulase were equally effective when used separately at lower concentrations, but progressed at a slower rate than the combined enzymes. Pectinase and pectin lyase were the most effective

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enzymes, especially pectinase that performed similar to the combined cellulase and hemicellulase activity. Pectin esterase, α-glucosidase, xylanase and amyloglucosidase were not as effective, but 75% of the polysaccharides was nevertheless hydrolysed within 5 days. This confirmed that the absorbency assay could be used to evaluate hydrolysis of grape pomace, and that a combination of cellulases and hemicellulases were most effective to hydrolyse the polysaccharides in grape pomace.

Results discussed in previous sections indicated that *P. rhodanensis* was able to hydrolyse purified cellulose, hemicellulose, starch and pectin when incorporated into agar plates. The yeast isolates number 1 and 4 (*Pichia rhodanensis*) were compared to *S. cerevisiae* Y294, the industrial wine yeast VIN7 and the recombinant strains of *S. cerevisiae* containing either pECCB or pBEE for their ability to hydrolyse purified cellulose (Figure 2). Plasmid pECCB contains the four genes *END1, CBH1, CEL1, BGL1* that encode the enzymes required for the complete degradation of cellulose, whereas pBEE contains the three genes *END1, BEG1, EXG1* required for optimal glucanase degradation (Van Rensburg, 1997; 1998). The ability of the two *P. rhodanensis* isolates to degrade cellulose is similar to that of the recombinant *S. cerevisiae* strains containing the cellulolytic genes. The zones around each colony are, however, smaller than that of *S. cerevisiae* (pECCB) that has the ability to degrade complex crystalline cellulose.

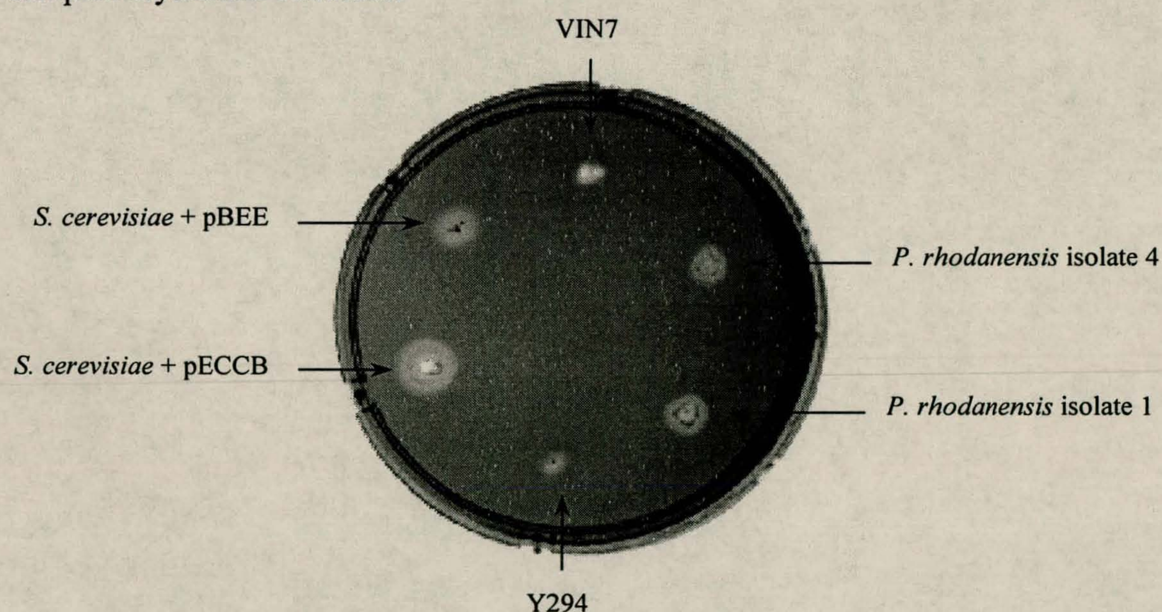

Figure 2. The ability of two *P. rhodanensis* isolates to hydrolyse CMC relative to *S. cerevisiae* strain Y294, recombinant *S. cerevisiae* strains with either plasmid pECCB or pBEE, and the industrial wine yeast strain VIN7.

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The *P. rhodanensis* isolates, *S. cerevisiae* Y294, the recombinant *S. cerevisiae* strains and the industrial wine yeast VIN7 were aerobically cultured in grape pomace and evaluated for hydrolysis using the Congo Red assay (Figure 3). All the strains indicated a small increase in the Congo Red/polysaccharide complex under aerobic conditions within the first 3 hours, similar to the negative control (no inoculum). The recombinant *S. cerevisiae* strain with plasmid pBEE confirmed its extracellular glucanase activity and was able to hydrolyse the polysaccharides almost completely after 5 days. The Congo red assay indicated that the recombinant *S. cerevisiae* strain with plasmid pECCB, which contains the four major genes required for the complete degradation of crystalline cellulose, *S. cerevisiae* Y294 and VIN7 did not degrade the pomace as effective as the *P. rhodanensis* isolates. Nevertheless, the results indicate that hydrolysis was almost completed after 120 hours for all the strains tested.

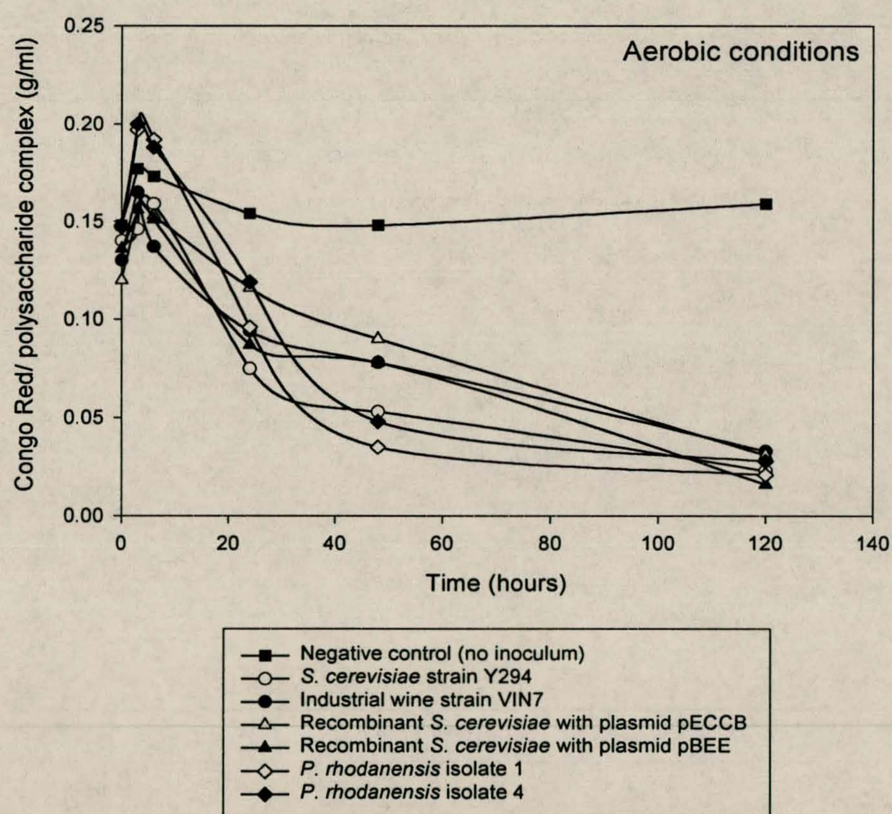

Figure 3. Aerobic enzymatic degradation of pomace polysaccharides by *P. rhodanensis, S. cerevisiae* Y294, VIN7 and two recombinant *S. cerevisiae* strains with either plasmid pECCB or pBEE.

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CONCLUDING REMARKS

Although several yeasts, bacterial and fungal species were isolated from grape pomace, only the yeast species were further evaluated as they are known to ferment sugars to alcohol. Various techniques were used to evaluate the ability of the yeast isolates to hydrolyse pomace. The yeast *P. rhodanensis* proved to be the most effective in its ability to hydrolyse the purified substrates (cellulose, hemicellulose, starch and pectin). Since grape pomace contains high concentrations of cellulose, hemicellulose, pectin and starch, *P. rhodanensis* should be able to hydrolyse the polysaccharides present in the grape pomace. Our results showed that *P. rhodanensis* was the most effective in the release of sugars and ethanol production from grape pomace (Table 9) and suggested that this yeast is able to hydrolyse some of the polysaccharides in grape pomace and ferment the released sugars to ethanol under anaerobic conditions.

Physiological evaluations done by the CBS yeast division concluded that *P. rhodanensis* has the ability to grow on xylose, maltose, cellobiose and sucrose as sole carbon sources. These sugars are all subunits of the polysaccharides' cellulose, hemicellulose, and starch and therefore indicate that the organism should have the ability to hydrolyse the polysaccharides present in pomace. Although promising results have been obtained, the efficacy of this isolate need to be further investigated to evaluate its applicability to the hydrolysis of pomace. The ability of the yeast to simultaneously saccharify grape pomace and ferment the sugars released in a single vessel could be of great commercial interest. The production of ethanol from sugars released from grape pomace and the industrial application thereof will have to be evaluated and optimised to determine the viability of bioprocessing of grape pomace.

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