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# Yeast β-Glucosidases

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## Introduction

For much of man's existence he has depended upon the conversion of cellulosic materials, in the gut of the herbivore, into animal foods such as meat and milk, and for power and transport by oxen and horses. For such reasons alone the degradation of cellulose in the rumen of the ox and the caecum of the horse requires understanding, if buildings and documents are to be protected against fungal and insect attack. More recently the vast quantities of cellulosic material produced as by-products from our forests and crops has been recognized as a potentially valuable resource which might yield food and fuel or chemicals but which is barely utilized at present. A detailed understanding of the mode of breakdown of cellulose in biological systems is obviously essential if a fermentation is to utilize or transform cellulosic materials economically. The first step in any such process is the disruption of the cellulose molecule.

Filamentous fungi, like most cellulolytic bacteria, hydrolyse cellulose with several enzymes: these include endoglucanases (1,4-β-D-glucan-glucanohydrolase, EC 3.2.1.4.), cellobiohydrolases (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), exoglucanases (1,4-β-D-glucan-glucohydrolase, EC 3.2.1.74), and β-glucosidases (1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.21). These enzymes together comprise the cellulasic complex (Mandels and Reese, 1964; Streamer, Eriksson and Petterson, 1975; Creuzet *et al.*, 1980; Stoppok, Rapp and Wagner, 1982).

The use of bacteria for the enzymatic breakdown of cellulose has two types of drawback: a number of cellulolytic bacteria (such as *Clostridium*

Abbreviations: CMC, carboxymethylcellulose; DCPIP, 2,6-dichlorophenolindophenol; IAA, indolylacetic acid; MBG, methyl-β-D-glucoside; PAGE, polyacrylamide gel electrophoresis; pCMB, *p*-chloromercuribenzoate; pNPG, *p*-nitrophenyl-β-D-glucopyranoside; SDS, sodium dodecyl sulphate; TEG, thioethyl-β-D-glucoside; TMG, thiomethyl-β-D-glucoside.

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*thermocellum*) are anaerobic, thus adding to the complexity of any industrial process; moreover, most of these bacteria have low exoglucanase activity. Their total cellulasic activity is thus lower than that of fungi such as *Trichoderma reesei*. However, only small amounts of β-glucosidase are produced by filamentous fungi (Sternberg, 1976; Yamanaka and Wilke, 1976; Sternberg, Vijayakumar and Reese, 1977). This leads to cellobiose accumulation, which in turn slows cellulose hydrolysis because cellobiose strongly inhibits endoglucanases and cellobiohydrolases (Halliwell and Griffin, 1973; Emert *et al.*, 1974). This drawback could be avoided in the following ways:

1. The isolation of β-glucosidase hyperproducing mutant strains;
2. The addition of β-glucosidase to the hydrolysis media;
3. The use of a Simultaneous Saccharification and Fermentation (SSF) process (Blotkamp *et al.*, 1978; Vause, 1983) involving a yeast strain capable of fermenting cellobiose and, ultimately, cellodextrins, i.e. β-1,4-oligopolymers of glucose.

Enzymatic systems capable of metabolizing cellobiose and soluble cellodextrins have thus been investigated in a large number of micro-organisms. Some of these enzymes are also able to hydrolyse heteropolymeric β-glucosides.

Most of the micro-organisms that have been investigated assimilate cellobiose by hydrolysis into glucose, using β-glucosidases. However, some fungi and bacteria can assimilate cellobiose by other means. The first enzyme involved may be a cellobiose phosphorylase (cellobiose:orthophosphate α-D-glucosyltransferase, EC 2.4.1.20), a cellobiose kinase (β-glucoside kinase) EC 2.7.1.85, (systematic name ATP : cellobiose 6-phosphotransferase), a cellobiose dehydrogenase (quinone) (cellobiose:quinone 1-oxidoreductase, EC 1.1.5.1; cellobiose:(acceptor) 1-oxidoreductase (EC 1.1.99.18), or a cellobiose oxidase (which can be included in the category EC 1.13.12). Before considering β-glucosidases—particularly yeast β-glucosidases—in detail we give a brief account of the above-mentioned enzymes.

## Enzymes which metabolize cellobiose and β-glucosides *(Figure 1)*

### CELLOBIOSE PHOSPHORYLASES

#### *Mode of action*

Some cellulolytic bacteria (*Cellvibrio gilvus*, *Clostridium thermocellum*, *Cellulomonas* sp.) grow faster on cellobiose than glucose as the sole carbon source. A similar feature has also been demonstrated in *Fomes annosus*, a basidiomycete which parasitizes conifer stems and roots (Hütterman and Volger, 1973). Furthermore, the anaerobic rumen bacterium *Ruminococcus flavefaciens* is completely unable to grow when the sole carbon source is glucose (Ayers, 1958). However, this organism possesses a glucokinase (EC 2.7.1.2) which phosphorylates glucose to glucose-6-phosphate: thus glucose-metabolizing enzymes are, in fact, present in cells of *R. flavefaciens*. The bacterium possesses a specific cellobiose transport system, whereas the lack

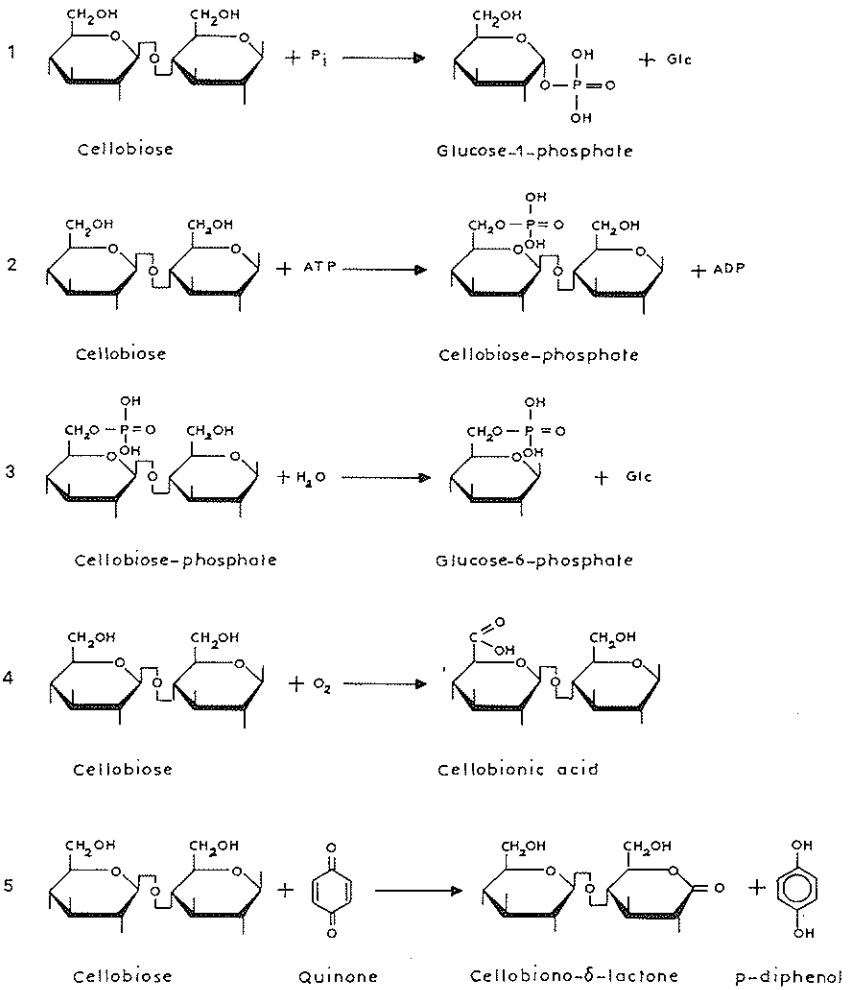


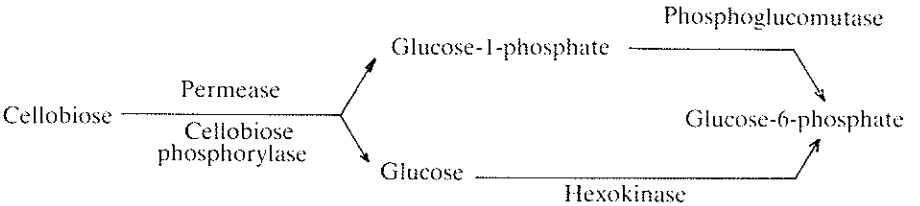

**Figure 1.** Mechanism of some enzymes that metabolize cellobiose. 1: cellobiose phosphorylase; 2: cellobiose kinase; 3: phospho-β-glucosidase; 4: cellobiose oxidase; 5: cellobiose : quinone oxidoreductase. Glc = glucose.

of such a system for glucose prevents that compound entering the cells. Study of the transformation of cellobiose in *Ruminococcus* cells showed that an intracellular cellobiose phosphorylase hydrolysed it into glucose and glucose-6-phosphate, in the presence of inorganic phosphate. This reaction is reversible: in the presence of glucose and glucose-1-phosphate, the cellobiose phosphorylase yields cellobiose and inorganic phosphate is released (Ayers, 1959).

The reasons why organisms possessing cellobiose phosphorylase grow better on cellobiose than on glucose are not clear. *Cellvibrio gilvus* appears to metabolize glucose and glucose-1-phosphate through two distinct pathways:

glucose-1-phosphate enters the Embden–Meyerhof glycolysis pathway, while glucose is metabolized by the pentose-phosphate pathway, to yield less energy than the glycolytic process. Thus, cellobiose metabolism by phosphorylative hydrolysis would yield more energy than would glucose (Hulcher and King, 1958; Swisher, Storvick and King, 1964).

*Clostridium thermocellum* consumes cellobiose preferentially in the presence of both glucose and cellobiose. In this strain, glucose metabolism is adaptive (the hexokinase is inducible), whereas the intracellular cellobiose phosphorylase is constitutive. A hexokinase of low activity and a phosphoglucomutase (EC 5.4.2.2) metabolize dextrose and glucose-1-phosphate:




*Clostridium thermocellum* also possesses an exocellular cellodextrin phosphorylase (1,4-β-D-oligoglucan : orthophosphate α-D-glucosyltransferase, EC 2.4.1.49 (Ng and Zeikus, 1982).

Cellobiose phosphorylase activities have also been identified in several *Cellulomonas* species, e.g. *C. fimi*, *C. uda*, *C. flavigena*, *C. cartae* and *C.* sp. (Sato and Takahashi, 1967; Schimz, 1979; Schimz, Broll and John, 1983). Micro-organisms that possess cellobiose phosphorylases may also produce β-glucosidases: for example *Clostridium thermocellum* and *Cellulomonas uda* produce constitutive β-glucosidases (Aït, Creuzet and Cattaneo, 1982; Stoppok, Rapp and Wagner, 1982).

### *Properties*

The properties of cellobiose phosphorylases have not been elucidated fully. Their optimum pH is about 6 or 7. They are induced by cellobiose (except in *Clostridium thermocellum*), or even by cellulose (*Cellulomonas* sp.). Their activity spectrum is narrow: they cleave cellobiose, but not *p*-nitrophenyl-β-D-glucopyranoside (pNPG).

## CELLOBIOSE KINASES AND PHOSPHO-β-GLUCOSIDASES

### *Mode of action*

Nelson and McBee (1957) demonstrated the existence of cellobiose kinase activity in *Clostridium thermocellum*, which also possesses a β-glucosidase and a cellobiose phosphorylase. This type of activity has also been found in the bacterium *Aerobacter aerogenes* (Palmer and Anderson, 1971), which metabolizes cellobiose in two stages: cellobiose is first phosphorylated by a β-glucoside kinase (EC 2.7.1.85); cellobiose monophosphate is then hydro-

lysed by 6-phospho-β-glucosidase (systematic name 6-phospho-β-D-glucosyl-(1,4) D-glucose glucohydrolase, EC 3.2.1.86):

$$\text{Cellobiose} + \text{ATP} \xrightarrow{\beta\text{-glucoside kinase}} \text{Cellobiose-6-phosphate} + \text{ADP}.$$

$$\text{Cellobiose-6-phosphate} + H_2O \xrightarrow{\text{Phospho-}\beta\text{-glucosidase}} \text{Glucose} + \text{Glucose 6 phosphate}.$$

This mechanism thus differs from that of cellobiose phosphorylase, which involves inorganic phosphate instead of ATP, is a one-stage process and yields glucose and glucose-1-phosphate. In *Aerobacter* a phosphoglucomutase is useless, because glucose-6-phosphate is the product of both reactions.

*Properties*

Palmer and Anderson (1972a, b) purified a β-glucoside kinase and a phospho-β-glucosidase from *Aerobacter aerogenes*. Both are induced by cellobiose. The β-glucoside kinase phosphorylates cellobiose, cellodextrins, some aryl-β-glucosides (but not *p*NPG), methyl-β-D-glucoside, and some diglucosides with configurations other than β-1,4. Similarly, the phospho-β-glucosidase has a wide activity spectrum, but it does not cleave non-phosphorylated β-glucosides. It is likely that ATP is the *in vivo* phosphorylating factor; ATP is more efficient than GTP, CTP or UTP.

## CELLOBIOSE DEHYDROGENASES AND CELLOBIOSE OXIDASES

*Mode of action and physiological role*

Unlike the enzymes described above, cellobiose dehydrogenases and cellobiose oxidases oxidize cellobiose rather than cleaving it. Cellobiose oxidases use molecular oxygen and directly yield cellobionic acid, whereas cellobiose dehydrogenases (cellobiose:quinone 1-oxidoreductase) utilize a quinone or a phenoxy radical as the electron acceptor, and yield cellobiono-δ-lactone, which is then transformed into cellobionic acid by a lactonase:

$$\text{Cellobiose} + O_2 \xrightarrow{\text{Cellobiose oxidase}} \text{Cellobionic acid} + H_2O$$

$$\text{Cellobiose} + \text{quinone} \xrightarrow{\text{Cellobiose dehydrogenase}} \text{Cellobiono-}\delta\text{-lactone} + \text{phenol}$$

$$\text{Cellobiono-}\delta\text{-lactone} + H_2O \xrightarrow{\text{Lactonase}} \text{Cellobionic acid}.$$

Cellobionic acid is then hydrolysed to glucose and glucono-δ-lactone by a β-glucosidase. Glucono-δ-lactone is transformed into gluconic acid, which is then phosphorylated, and metabolized by the pentose-phosphate pathway.

Cellobiose dehydrogenases have been found in lignolytic fungi such as *Chrysosporium lignorum*, *Polyporus versicolor*, *Sporotrichum pulverulentum* and *Fomes annosus* (Westermark and Eriksson, 1974; Eriksson, 1978; Hütterman and Noelle, 1982). *Sporotrichum pulverulentum* also possesses a cellobiose oxidase (Ayers, Ayers and Eriksson, 1978). In these organisms, the action of the cellobiose : quinone oxidoreductase is tightly linked to lignin metabolism, which supplies it with electron acceptors. *Figure 2* (Eriksson, 1978) illustrates cellulolytic metabolism in *Sporotrichum pulverulentum*, and shows the physiological role of cellobiose dehydrogenase in controlling cellulose breakdown. Glucose accumulates during cellulose hydrolysis and is partly transformed into glucono-δ-lactone which strongly inhibits β-glucosidases. Cellobiose concentration thus increases, inhibiting cellulases and thereby causing the formation of transglycosylation oligosaccharides. The action of cellobiose dehydrogenase and cellobiose oxidase thus minimize cellobiose accumulation.

Cellobiose dehydrogenases have also been found in the non-lignolytic organisms *Monilia* sp. (Dekker, 1980) and *Sporotrichum thermophile* (Coudray, Cavenascini and Meyer, 1982). In these fungi, this enzyme may alleviate cellulase inhibition by cellobiose, particularly in *Sporotrichum thermophile*, which does not produce β-glucosidase. These enzymes use, *in vitro*, artificial electron acceptors such as 2,6-dichlorophenol-indophenol (DCPIP) or benzoquinones. The *in vivo* acceptor is not known: it may be a quinone or a phenoxy radical synthesized by the organism itself. It is possible that these fungi are able to use quinones produced by lignolytic species with which they live in symbiosis.

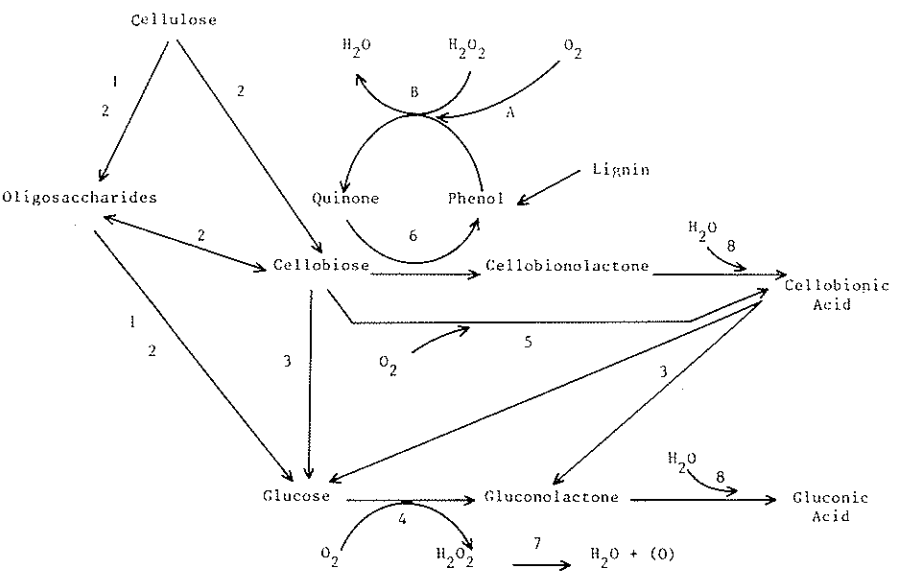



**Figure 2.** Cellulose metabolism in *Sporotrichum pulverulentum*. 1: endoglucanase; 2: exoglucanase; 3: glucosidase; 4: glucose oxidase; 5: cellobiose oxidase; 6: cellobiose dehydrogenase; 7: catalase; 8: lactonase; A: laccase (polyphenol oxidase); B: peroxidase.

*Properties*

Cellobiose:quinone oxidoreductases, and the cellobiose oxidase of *Sporotrichum pulverulentum*, are exocellular enzymes. They are induced by cellulose or cellulosic substrates which are not easily hydrolysed. Cellobiose, carboxymethylcellulose (CMC) and glucose are not good inducers in *Monilia* and *Polyporus*, but the cellobiose dehydrogenase of *Fomes annosus* is an exception in that it is induced only by a cellobiose and lignosulphonate mixture.

These enzymes have a narrow activity spectrum: they oxidize cellobiose, cellodextrins, and eventually diglucosides such as lactose or glucosyl-β-1,4-mannose. Those natural electron acceptors that are known are mainly benzoquinones (*Figure 3*). The optimum pH of the enzymes depends on the acceptor and ranges in value from 4 to 8; however, with DCPIP, which is the best acceptor for most cellobiose dehydrogenases, optimal pHs are 4–5. In the presence of DCPIP, the $K_m$, for cellobiose, of enzymes from *Monilia* sp. is 12·2 μM and from *Sporotrichum thermophile* is 6·9 μM (Dekker, 1980; Coudray, Cavenascini and Meyer, 1982).

## β-GLUCOSIDASES

Cellulolytic micro-organisms (particularly fungi) metabolize cellobiose by various different pathways. However, cellobiose assimilation by phosphorylative hydrolysis, dehydrogenation or oxidation is relatively unusual: most cellobiose-assimilating micro-organisms possess one or more β-glucosidases.

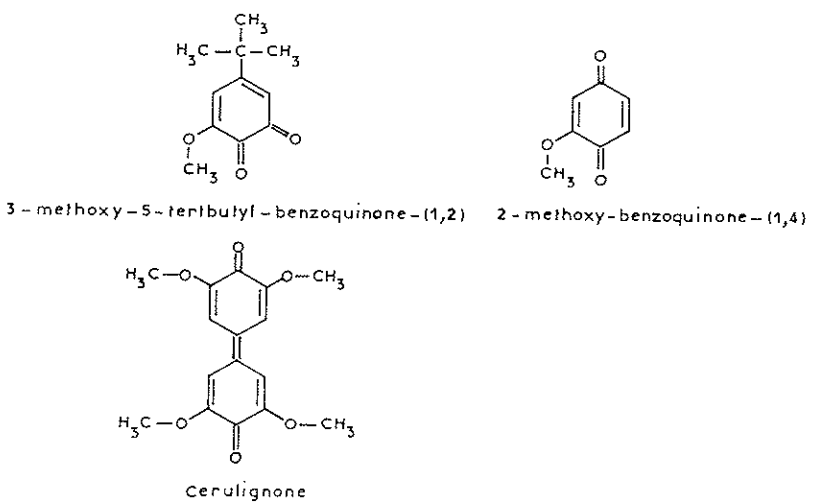



**Figure 3.** Benzoquinone natural electron acceptors for cellobiose : quinone oxidoreductases and the cellobiose oxidase of *Sporotrichum pulverulentum*.

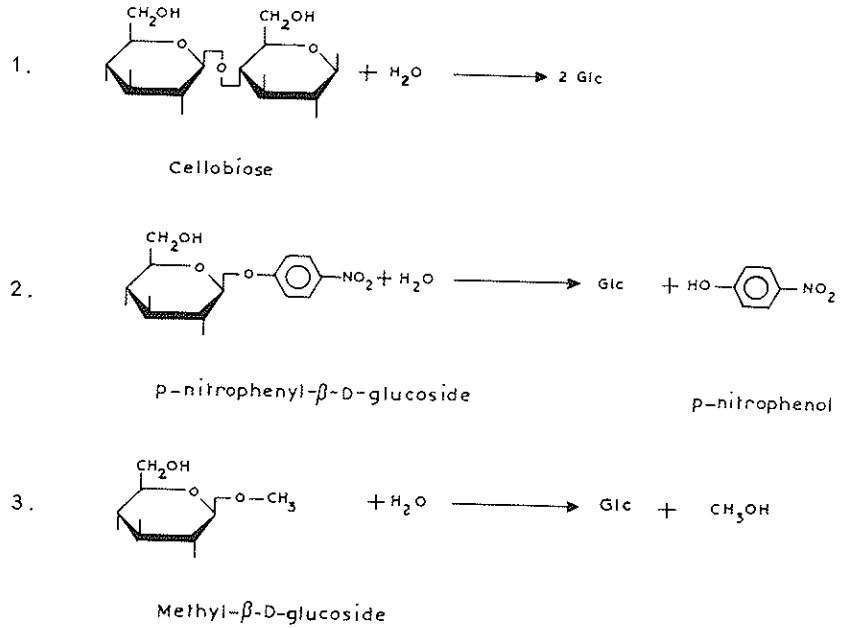



**Figure 4.** Action of β-glucosidases on: 1, cellobiose; 2, arylglucosides; 3, alkylglucosides. Glc = glucose.

### *Mode of action*

β-Glucosidases catalyse the hydrolysis of alkyl-β-D-glucosides (such as methyl-β-D-glucopyranoside), aryl-β-D-glucosides (such as pNPG), and glucosides containing only glucosidic residues (such as cellobiose) (*Figure 4*).

### *Distribution among organisms. Physiological roles*

β-Glucosidase activity has been found both in invertebrates and in vertebrates. Thus, the liver of *Helix pomatia* (the Burgundy snail) contains one or more enzymes which catalyse the hydrolysis of cellobiose, cellotriose and, to a certain extent, that of soluble cellodextrins of a higher degree of polymerization than 3 (Stevens, 1955). In addition, *H. pomatia* liver produces one or more cellulolytic enzymes. Two β-glucosidases have been isolated from Antarctic krill, *Euphausia superba* (Chen and Lian, 1986).

Human intestine contains several disaccharidases (four maltases, one trehalase and one lactase), which are involved in digestion; the lactase is also a cellobiase (Dahlqvist, 1966). Man also produces a β-glucosidase which can hydrolyse some heteroglucosides with a lipidic aglycone fraction, i.e. ceramyl glucosides. This enzyme is situated in the reticulo-endothelial cell lysomes and is essential to glucosyl ceramide catabolism; its absence causes Gaucher's disease, involving the accumulation of glucosyl ceramides in liver, spleen, and lymphatic ganglia (Frederickson and Sloan, 1972; Li and Li, 1983).

A number of β-glucosidases are found in higher plants. Their activity spectrum is often narrow, and directly linked to their physiological role and their location in plants. This role can be structural (action on cell walls), metabolic (accessory metabolic pathways), or related to defence against parasites.

During cell growth and differentiation in higher plants, hormones such as indolylacetic acid (IAA) induce biosynthesis of β-glucanase and β-glucosidase, thus increasing cell wall plasticity and facilitating cell multiplication, elongation and differentiation. Such a phenomenon has been found in oat (*Avena* sp.) coleoptiles. β-Glucosidases also play a part in fruit ripening (sometimes associated with β-glucanases) and encourage partial or total hydrolysis of the cell-wall middle lamella (Verma, Kumar and MacLaghlan, 1982).

Aryl-β-glucosidases are involved in pigment catabolism in plants. Pigments are often found in the form of heteroglucosides: their aglycone fraction, which is responsible for the colour, belongs to flavones or anthocyanins (White, Handler and Smith, 1968).

β-Glucosidases take part in cyanoglucoside metabolism in plants (*Figure 5*). Such activity has been found in *Trifolium*, *Vicia*, *Lotus* and *Triticum*, in tree leaves (*Sambucus*, *Populus*) or in fruits (bitter almond; *Prunus* sp.). Bitter almonds contain two β-glucosidases (A and B) which catalyse the two-stage hydrolysis of amygdalin (*Figure 6*). The role of cyanoglucosides and the biological significance of their hydrolysis are not clearly understood. Apparently, the β-glucosidases responsible for hydrolysis are not located in the same cells as the cyanoglucosides, so that hydrolysis occurs only when the cells are damaged. Reaction may then bring about cyanide release, thus defending the plant against its damaging pathogen (Knowles, 1976; Hösel, 1981). Apple (*Malus*) and pear (*Pyrus*) trees employ a similar system: their β-glucosidases hydrolyse arbutine (3-hydroxyphenyl-β-D-glucoside), in response to parasitic infections; this causes the release of hydroxyphenol, which is toxic to parasites (Garibaldi and Gibbins, 1975).

A number of rumen ciliate Protozoa are able to utilize oligosaccharides (Coleman, 1980); they produce intracellular α- and β-glucosidases (Delfosse-Debusscher *et al.*, 1979). Twenty-one ciliate species have been shown to synthesize relatively large amounts of β-glucosidase (Williams, Withers and Coleman, 1984), which is involved in the digestion of soluble polysaccharides ingested by the ruminant host. These Protozoa can also partly hydrolyse cellulose and hemicelluloses. β-Glucosidase activity has also been detected in protozoal parasites of the gut and vascular system (Avila *et al.*, 1979; Trissl, 1983).

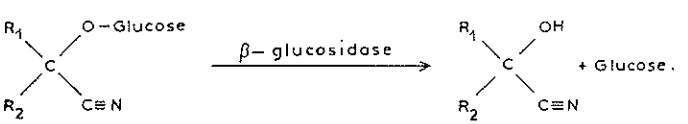



**Figure 5.** Action of β-glucosidases on cyanoglucosides.

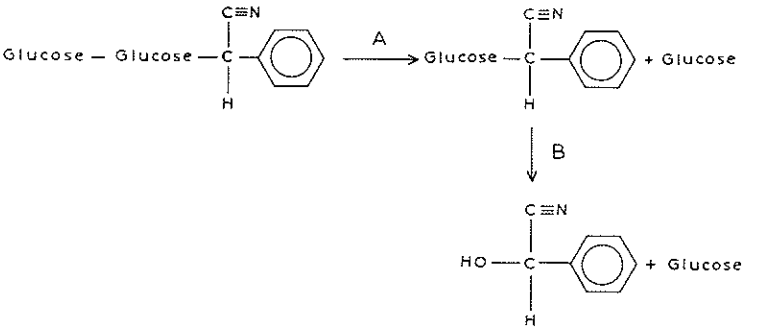



**Figure 6.** Two-stage hydrolysis of amygdalin by β-glucosidases A and B.

β-Glucosidases are relatively widespread in micro-organisms, particularly in the cellulolytic species, the best-known of which is the fungus *Trichoderma reesei*. The role of β-glucosidase in such organisms is the hydrolysis of cellobiose and soluble cellodextrins into glucose, i.e. the last stage of cellulose hydrolysis, in which the bulk of the glucose released is derived from β-glucosidase action (Sternberg, 1976). Although *Trichoderma* produces several endo- and exoglucanases, only one β-glucosidase has been shown to be synthesized by this fungus (Berghem and Pettersson, 1974; Wood and McCrae, 1982). Some other cellulolytic fungi also produce a single β-glucosidase, for example *Penicillium funiculosum* (Parr, 1983), *Aspergillus wentii* (Srivastava, Gopalkrishnan and Ramachandran, 1984), *Scytalidium lignicola* (Desai, Ray and Patel, 1983), *Fusarium solani* (Wood, 1971) and *Sporotrichum thermophile* (Canevascini and Meyer, 1979). However, there are some fungi which synthesize more than one β-glucosidase: for example *Monilia* sp. produces one endocellular and one exocellular enzyme (Dekker, 1981); *Neurospora crassa* and *Stereum sanguinolentum* produce an exocellular aryl-β-glucosidase and an endocellular cellobiase (Bucht and Eriksson, 1969; Eberhart and Beck, 1970). In these latter fungi, both enzymes are highly specific: the aryl-β-glucosidase does not cleave cellobiose, and the cellobiase does not hydrolyse aryl glucosides. Some cellulolytic fungi possess as many as three or four β-glucosidases; *Phanerochaete chrysosporium* produces two endocellular β-glucosidases (Smith and Gold, 1979); *Sporotrichum pulverulentum*, *Talaromyces emersonii* and *Sclerotium rolfsii* synthesize four enzymes (Deshpande, Eriksson and Petterson, 1978; McHale and Coughlan, 1981; Shewale and Sadana, 1981). The physiological role of this enzyme multiplicity has not been elucidated; nevertheless, it has been observed that in several cases the specificities and locations of the enzymes differ.

β-Glucosidases have been detected in bacteria. Some are cellulolytic aerobic genera, for example *Cellulomonas* (Rickard, Rajoka and Ide, 1981; Stoppok, Rapp and Wagner, 1982; Wakarchuk *et al.*, 1984), *Bacillus* (Sadler *et al.*, 1984), *Acetovibrio* (Mackenzie and Bilous, 1982) and *Rhizobium* (Abe and Higashi, 1982). Others are cellulolytic anaerobes: these include

*Clostridium* (Allcock and Woods, 1981; Park and Ryu, 1983), *Bacteroides* (Berg, Lindqvist and Nord, 1980; Forsberg and Groleau, 1982). Some thermophilic bacteria, for example *Thermoactinomyces* (Hägerdal, Harris and Kendall-Pye, 1979; Hägerdal *et al.*, 1979) and *Thermoanaerobacter* (Mitchell *et al.*, 1982), also produce β-glucosidases. Some authors (Schaefler, 1967; Schaefler and Maas, 1967; Defez and De Felice, 1981) have discussed the possibility of obtaining *Escherichia coli* mutants with the ability to metabolize β-glucosides with a β-glucosidase: the wild-type strain possesses the gene coding for β-glucosidase, but this gene is not active.

With the exception of some species belonging to the genus *Trichosporon* (Dennis, 1972; Stevens and Payne, 1977), yeasts are unable to hydrolyse cellulose. However, many yeast species possess β-glucosidase activity and assimilate cellobiose (Duerksen and Halvorson, 1958; Kaplan, 1965; Marchin and Duerksen, 1968a, b; Fiol, 1973; Gondé *et al.*, 1982; Kohchi, Hayashi and Nagai, 1985). This type of activity has been found in the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Candida* and *Brettanomyces*. All strains but two produce β-glucosidases that are endocellular or parietal (produced in the cell wall): the exceptions are *Candida molischiana* and *Candida wickerhamii*, which excrete β-glucosidases into the culture medium, thus enabling these yeast species to assimilate soluble cellodextrins, which usually do not cross the cell membrane and therefore cannot be hydrolysed (Gondé *et al.*, 1984). The presence of β-glucosidases in non-cellulolytic micro-organisms such as yeasts may initially appear hard to explain. However, the natural degradation of lignocellulosic material is usually performed by mixed flora, including yeasts, which probably consume some of the products of cellulose hydrolysis such as glucose, cellobiose and ultimately eventually cellodextrins. Nevertheless, some *Saccharomyces* and *Kluyveromyces* species produce β-glucosidases but cannot assimilate cellobiose: this is probably attributable to the lack of a membrane cellobiose-carrier system (Fiol, 1975, 1976; Barnett, 1976). The function of these enzymes is not clear, but some authors (Lehle, Cohen and Ballou, 1979; Kilker *et al.*, 1981) have related it to the glycosylation of proteins.

## Regulation of β-glucosidase biosynthesis in yeasts

### INDUCIBLE ENZYMES

Inducible β-glucosidases were found in the genus *Saccharomyces*. Induction of β-glucosidase was studied by Duerksen and Halvorson (1959) and Kaplan (1965) in *Saccharomyces cerevisiae*, by Herman and Halvorson (1963a) in *S. lactis*, McQuillan and Halvorson (1962a, b) in the hybrid yeast *S. fragilis* × *S. dobzhanskii*, and by Duerksen and Halvorson (1958). Many α-, β- and thio-β-glucosides were tested by Duerksen and Halvorson (1959) as potential inducers of β-glucosidase in *S. cerevisiae* (strain Yeast Foam), the most efficient being methyl-β-D-glucoside (MBG), thiomethyl-β-D-glucoside (TMG), and thio-ethyl-β-D-glucoside (TEG). TEG and TMG were gratuitous (non-substrate) inducers, i.e. they were not substrates of the enzyme, and

consequently could not induce enzyme biosynthesis via hydrolysis into glucose and thioethanol or thiomethanol. The optimal concentration for induction of β-glucosidase was about $10^{-2}$M. Other thiol derivatives of β-glucosides could lead to inhibition of the basal enzyme biosynthesis, while thio-phenyl-β-D-glucoside (TPG) was shown to be a competitive inhibitor of β-glucosidase induction when TMG or TEG were used as inducers. Natural β-glucosides, such as cellobiose, salicin, arbutin, amygdalin and aesculin were poor inducers.

Kaplan (1965) isolated from baker's yeast a strain capable of using cellobiose as an energy and carbon source for growth. This strain possesses overt cellobiase activity, localized in the cell membrane and discernible in whole cells, and latent activity, detectable following treatment of cells with *n*-butanol. This β-glucoside-hydrolysing system was found to be inducible: the best inducer was cellobiose, which enhanced biosynthesis of both overt and latent activity; MBG also induced this system, whereas other β-glucosides had little effect.

Herman and Halvorson (1963b) used the Y123 and Y14 strains in an attempt to elucidate the mechanism of β-glucosidase induction in *S. lactis*. MBG induced the formation of β-glucosidase in strain Y123 (the basal activity level was increased fourfold in the presence of $2 \times 10^{-2}$M MBG), but had no effect on enzyme biosynthesis in strain Y14; β-glucosidase was induced by glucose in both strains. Non-substrate inducers of β-glucosidases in other yeast strains (TMG and TEG, $10^{-2}$M) were not effective in *S. lactis*. Thus, inducer metabolism seems necessary in this species. Indeed, glucose and MGB did not act in the same way on β-glucosidase induction in *S. lactis*: crossing and sporulation experiments showed that induction by MBG was under the control of a single gene only (non-inducibility was dominant), whereas hybrid strains Y123 × Y14 exhibited various induction levels (ranging from 1 to 25); thus, more than one gene influenced the response to glucose induction. Another difference is that glucose induced other carbohydrases (at low concentrations: $10^{-3}$ to $10^{-6}$M), whereas MBG did not.

All strains mentioned above possess inducible β-glucosidases. These enzymes are produced at low basal levels, and their biosynthesis is increased by the addition of an inducer at low concentrations (from $10^{-2}$ to $10^{-6}$M). Although these strains are taxonomically close, neither the nature of the inducers nor the mechanism of the induction is constant.

## CONSTITUTIVE (NON-ADAPTATIVE) β-GLUCOSIDASES

Inducible β-glucosidases have been most commonly found in *Saccharomyces* species, whereas constitutive enzymes have been described in several yeast genera.

True constitutive producers of β-glucosidase have been described by Blondin *et al.* (1983), Leclerc *et al.* (1984), Gondé *et al.* (1985), and Kohchi, Hayashi and Nagai (1985) in *Dekkera* and *Candida* species. *Dekkera intermedia*, *Candida molischiana* and *Candida wickerhamii* produced equal amounts of β-glucosidase when grown on cellobiose, ethanol or glycerol minimal

media. Enzyme biosynthesis was not increased by MBG nor glucose at low concentration, unlike the case with *S. lactis*. Gondé *et al.* (1982, 1984) also described high β-glucosidase biosynthesis in *Brettanomyces* and other *Candida* species.

Other strains in the genus *Saccharomyces* have been described as constitutive β-glucosidase producers, although their basal level of enzyme biosynthesis can be increased further by the addition of inducers such as MBG or glucose at low concentrations (McQuillan, Winderman and Halvorson, 1960; McQuillan and Halvorson, 1962a, b; Herman and Halvorson, 1963b). These strains are sometimes designated 'semiconstitutive'.

### GLUCOSE REPRESSION OF β-GLUCOSIDASE BIOSYNTHESIS

Although low concentrations of glucose lead to β-glucosidase induction in certain *Saccharomyces* strains, higher levels prevent enzyme synthesis at normal rates. Herman and Halvorson (1963b) observed 60% inhibition of β-glucosidase biosynthesis in two *S. lactis* strains in the presence of $10^{-2}$–$10^{-1}$M glucose. This repression was of the competitive type, i.e. it could be reversed by the addition of inducers to the culture medium. Glucose repression in *S. lactis* was assumed to occur via glucose catabolites produced intracellularly. Although repression occurs with the hybrid yeast *S. fragilis* × *S. dobzhanskii* at glucose concentrations above $10^{-3}$M, inducers could not reverse this repression (McQuillan and Halvorson, 1962a). These authors related glucose repression of β-glucosidase biosynthesis to inhibition of several steps of the tricarboxylic acid cycle (McQuillan and Halvorson, 1962b). Glucose may also act by blocking the release of synthesized β-glucosidase from ribosomes in *S. fragilis* × *S. dobzhanskii*, as proposed by Hauge *et al.* (1961).

Glucose also represses the formation of β-glucosidase in constitutive strains such as *Dekkera intermedia*, *Candida molischiana* and *Candida wickerhamii*, in which enzyme biosynthesis is reduced by 90% in the presence of $5 \times 10^{-2}$M glucose. This repression is not reversed by cellobiose or other β-glucosides (Blondin *et al.*, 1983; Leclerc *et al.*, 1984; Gondé *et al.*, 1985).

## Purification and properties of yeast β-glucosidases

### LOCATION OF β-GLUCOSIDASES IN YEAST CELLS

In contrast to most filamentous fungi, yeasts seldom excrete β-glucosidases into the culture medium: the enzymes are intracellular in *Saccharomyces*, *Candida*, *Rhodotorula*, *Dekkera* and *Brettanomyces* species (Duerksen and Halvorson, 1958; Marchin and Duerksen, 1968a; Gondé *et al.*, 1984; Kohchi, Hayashi and Nagai, 1985). Intracellular yeast β-glucosidases are generally localized in the soluble cytoplasmic fraction (Blondin *et al.*, 1983; Leclerc *et al.*, 1984). Only small quantities of bound carbohydrate are found in the active enzyme fraction, which suggests that the enzyme proteins are not tightly bound to cellular organelles (Marchin and Duerksen, 1968a).

**Table 1.** Properties of some purified yeast β-glucosidases

| Organism | Optimal pH | Optimal temp. (° C) | Max. temp. for stability* (° C) | $\Delta H_1^{\neq}$ (Kcal/mol) | $K_m$ pNPG (mM) | $K_m$ cellobiose (mM) | $K_i$ glucose (mM) | pI | Mol. weight | Subunit mol. weight | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Hybrid *Saccharomyces* | | | | 16·0 | 0·086–1·25 | | | 6·0 | 325 000 | 110 000 | Hu *et al.*, 1960; Fleming and Duerksen, 1967a |
| *Saccharomyces lactis* | | | | | | | | | | | |
| Y 123 | 6·2 | | | 11·25 | 2·01 | | 3·15 | | | | Marchin and Duerksen, 1969; Fiol, 1973 |
| Y 14 | 6·8 | | | 16·23 | 1·57 | | 6·71 | | 80 000 | | |
| Y 1057A | 6·8 | | | 17·0 | 1·05 | | 23·0 | | | | |
| *Pichia vini* | 5·6 | | | | | 0·17 | | | | | |
| *Pichia vini* var. *melibiosi* | 6·4 | | | | | 0·7 | | | | | Fiol, 1973 |
| *Saccharomyces cerevisiae* | 6·8 | | | 13 | 0·095 | | 6·7 | | 313 000 | | Inamdar and Kaplan, 1966 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* | 6·4–6·8 | 45 | 45 | 16·6 | 0·08 | | 8·5 | | 300 000 | | Duerksen and Halvorson, 1958 |
| *Saccharomyces fragilis* | 5·8 | | | 15·8 | 0·110 | No activity | | | | | Fleming and Duerksen, 1967a |
| *Saccharomyces dobzhanskii* | 6·1 | | | 16·8 | 0·069 | No activity | | | | | |
| *Dekkera intermedia* | 5·0 | 55 | 50 | 16·0 | 0·20 | 55 | 3 | | 310 000 | | Blondin *et al.*, 1983 |
| *Candida guilliermondii* | 6·8 | 37 | 45 | 0·125 | 0·125 | | | | 48 000 | | Roth and Srinivasan, 1978 |
| *Candida pelliculosa* | 6·5 | 50 | 50 | | 0·5 | 37 | 6·5 | 4·9 | 360 000 | 90 000 | Kohchi, Hayashi and Nagai, 1985 |
| *Candida molischiana* | 4·5 | 55 | 60 | 12·0 | 0·10 | 130 | 7 | 3·5 | 120 000 | 64 000 | Gondé *et al.*, 1985 |
| *Candida wickerhamii* | | | | | | | | | | | |
| Extracellular | 4·5 | 50 | 50 | 10·4 | 4 | 225 | 230 | 3·3 | 130 000 | 98 000+38 000 | Leclerc *et al.*, 1984 |
| Intracellular | 6·0 | 50 | 50 | 9·8 | 0·14 | 3 | 9 | 3·5 | 130 000 | 48 000 | |

*Temperature at which half-life of the enzyme does not exceed 0·5 h.
†Thermal activation energy of the reaction.

Nevertheless, certain yeast β-glucosidases are not intracellular: *Candida molischiana* possesses an extracellular enzyme, 50% of which is bound to the cell membrane, the remainder being excreted into the culture medium (Gondé *et al.*, 1985). *Candida wickerhamii* strain CBS 2928 has been described as producing one endocellular and one exocellular β-glucosidase, 70% of which is bound to the cell membrane (Leclerc *et al.*, 1984). Similarly, one *S. cerevisiae* strain possesses an inducible system for the cleavage and transport of β-glucosides, which is inhibited by $Hg^{2+}$ ions at concentrations preventing these ions from entering the cells: this system is therefore located in the cell membrane (Kaplan, 1965; Kaplan and Tacreiter, 1966).

## MODES OF PURIFICATION

Most current modes of purification are applicable to yeast β-glucosidases. The endocellular location of these enzymes necessitates a preliminary extraction from cells, which can be achieved by autolysis, sonication, mechanical rupture in a French pressure cell or in a Braun homogenizer, or by protoplast preparation followed by cell rupture. The suspensions thus obtained are ultracentrifuged, and the last supernatant can be submitted to streptomycin sulphate precipitation in order to eliminate nucleic acids (Inamdar and Kaplan, 1966; Hu *et al.*, 1960). The resulting extracts may be precipitated by ethanol or ammonium sulphate, or directly submitted to chromatography. Most authors used ion-exchange chromatography, followed by a gel-filtration step, but some (Fleming and Duerksen, 1967a) additionally employed adsorption–desorption on calcium phosphate gel. Roth and Srinivasan (1978) used a different approach for purifying *Candida guilliermondii* enzymes, including an affinity chromatography step.

Extracellular enzymes may be recovered from the culture medium supernatant by acetone precipitation, then submitted to normal purification processes (Leclerc *et al.*, 1984; Gondé *et al.*, 1985).

Polyacrylamide gel electrophoresis (PAGE) or starch gel electrophoresis are used for verifying enzyme purity at the end of the purification procedure. Immersion of the gels in a buffered solution of *p*-nitrophenyl-β-D-glucopyranoside causes a yellow spot to appear where the protein band is situated.

## PHYSICAL PROPERTIES OF YEAST β-GLUCOSIDASES

Most yeast β-glucosidases are high molecular weight proteins: those from *Saccharomyces*, *Rhodotorula minuta*, *Candida pelliculosa*, and *Dekkera intermedia* have molecular weights ranging from 300 000 to 360 000. *S. lactis* strain Y14 β-glucosidase is pH-labile and its molecular weight varies from 80 000 to 320 000 according to pH conditions (Marchin and Duerksen, 1968b). There appear to be exceptions among *Candida* species: for example, Roth and Srinivasan (1978) have suggested a molecular weight of 48 000 for the enzyme from *C. guilliermondii*, and the molecular weights of β-glucosidases from *C. molischiana* and *C. wickerhamii* are 120 000 and 130 000 respectively (Leclerc *et al.*, 1984; Gondé *et al.*, 1985). In contrast, the

molecular weights of the β-glucosidases from filamentous fungi range more widely from 37 500 to 440 000 (Dekker, 1981; Meyer and Canevascini, 1981).

High molecular weights of yeast β-glucosidases are often related to oligomeric structure: this has been demonstrated in several instances in which the subunit molecular weight is approximately 80 000–100 000. The methods used for such determinations have mainly been SDS-PAGE, or urea denaturation followed by gel-filtration. *Candida* species are again exceptions, in that their subunit molecular weights are considerably lower, from 38 000 to 64 000 (Leclerc *et al.*, 1984; Gondé *et al.*, 1985). These enzymes comprise two rather than four subunits.

The hybrid yeast *Saccharomyces fragilis* × *Saccharomyces dobzhanskii* strain Y42 produces a variety of hybrid molecules of β-glucosidase, which result from combinations of two distinct polypeptides. These are similar to those synthesized by the parental strains, and probably are the subunits of the β-glucosidases of these strains (Fleming and Duerksen, 1967b). Few isoelectric points were determined in yeast β-glucosidases: data available for *Candida* enzymes are pH 4·9 in *C. pelliculosa* (Kohchi, Hayashi and Nagai, 1985), pHs of 3·3 and 3·5 in the extracellular and intracellular enzymes, respectively, of *C. wickerhamii* (Leclerc *et al.*, 1984), and pH 3·7 in the extracellular enzyme of *C. molischiana* (Gondé *et al.*, 1985). In this respect, therefore, the yeast enzymes resemble those of the filamentous fungi, in which the isoelectric points lie between pH 3 and 4, as shown by Wood (1971) and McHale and Coughlan (1981).

Many fungal β-glucosidases are glycoproteins (often extracellular), some containing up to 90% carbohydrates (Coughlan, 1985). This is seldom true for yeast enzymes, which generally contain less than 5% carbohydrate (Marchin and Duerksen, 1968a; Leclerc *et al.*, 1984; Gondé *et al.*, 1985; Kohchi, Hayashi and Nagai, 1985), as determined by periodate–fuschin staining following electrophoresis, or hydrolysis of the purified enzyme and carbohydrate assay by the Somogyi–Nelson method.

Yeast β-glucosidases are not very heat-stable, the half-lives of purified enzymes at temperatures higher than 45° C generally not exceeding 30 minutes (Duerksen and Halvorson, 1958; Roth and Srinivasan, 1978; Blondin *et al.*, 1983). This contrasts with enzymes of filamentous fungi, which may be unaffected by 30–60 minutes at 70° C (Shewale and Sadana, 1981; McHale and Coughlan, 1981, 1982), or even 90° C (Yoshioka and Hayashida, 1980a, b).

## KINETIC PROPERTIES OF YEAST β-GLUCOSIDASES

Optimal conditions for the activity of yeast β-glucosidases are usually in the neutral–acidic pH range and at 45–55° C. Reported optimal pHs for such intracellular enzymes vary from 5·5 in *Pichia vini* (Fiol, 1973) to 6·8 in various strains of *Saccharomyces* and *Candida*. However, the optimum pH for exocellular β-glucosidases of *Candida molischiana* and *Candida wickerhamii* is lower (about pH 4·5), and similar to that of exocellular fungal β-glucosidases (Lusis and Becker, 1973; Dekker, 1981; Dholakia and Modi,

1982). Rapid thermal denaturation occurs at the optimal temperatures, so that these enzymes cannot be used for more than a short period at this temperature. The enzyme of *Candida guilliermondii* is an exception in that its optimal temperature is only 37° C (Roth and Srinivasan, 1978). Thermal activation energies vary from 10 to 17 kcal/mol.

Yeast β-glucosidases generally metabolize cellobiose (cellobiase activity), soluble cellodextrins (up to cellohexaose), aryl-β-D-glucosides (such as *p*-nitrophenyl-β-D-glucopyranoside, or pNPG), and alkyl-β-D-glucosides (such as methyl-β-D-glucopyranoside). Exceptions, reported by Fleming and Duerksen (1967a), are *Saccharomyces fragilis* and *Saccharomyces dobzhanskii*, which synthesize β-glucosidases inactive against cellobiose, i.e. without cellobiase activity. Aryl-β-glucosides are more easily hydrolysed by yeast enzymes than are cellobiose or other glucosides such as gentiobiose: $K_m$s are lower and reaction rates higher. $K_m$s also decrease when the chain length of the substrate increases: the exocellular β-glucosidases of *Candida wickerhamii* and *C. molischiana* have more affinity for cellodextrins than for cellobiose, but the $V_{max}$ is lower, so the effectiveness of the enzymes is roughly the same (Leclerc *et al.*, 1984; Gondé *et al.*, 1985). As β-glucosides are competitive inhibitors of β-glucosidase activity, hydrolysis may be inhibited by excess of substrate (Marchin and Duerksen, 1969). The substrate specificity of these enzymes is fairly narrow: their activity is restricted to substrates of the β configuration, and affinity depends on steric characteristics of the non-glucose moiety. Activity has been demonstrated in some instances against aryl-β-D-xylosides and aryl-β-D-mannosides, for instance with the exocellular enzymes of *Candida wickerhamii* and *Candida molischiana* (M. Leclerc, A. Arnaud and P. Galzy, unpublished data). The enzyme of *C. molischiana* possesses a wider specificity: it is effective against α-glucosides such as maltose and maltotriose (Gondé *et al.*, 1985). β-Thioglucosides, which are structural analogues of β-glucosides, are not cleaved by yeast β-glucosidases, but they do form complexes with these enzymes and inhibit their activity (Duerksen and Halvorson, 1958). Similarly, yeast β-glucosidases are competitively inhibited by glucose, and $K_i$ are very low, except that for the exocellular enzyme of *C. wickerhamii* (230 mM instead of 3–10 mM for other enzymes). D-Glucono-δ-lactone powerfully inhibits β-glucosidases, because of its structural analogy with an intermediate product of the enzymatic reaction (Conchie, Gelman and Levvy, 1967; Legler, 1975). Other β-glucosidase inhibitors include heavy metals (such as $Hg^{2+}$, $Pb^{2+}$, $Co^{2+}$) and *p*-chloromercuribenzoate (pCMB) (Duerksen and Halvorson, 1958; Blondin *et al.*, 1983). Ethanol at high concentrations also inhibits yeast β-glucosidases; however, some authors have described activation of the hydrolysis of pNPG or cellobiose by ethanol (Blondin *et al.*, 1983). This is attributable to glucosyltransferase activity of the enzyme: ethanol competes with water, and leads to the formation of ethyl-β-D-glucoside (Pemberton, Brown and Emert, 1980):

$$\text{Cellobiose} + \text{H–OH} \rightarrow \text{Glucose} + \text{Glucose.}$$
$$\text{Cellobiose} + \text{C}_2\text{H}_5\text{–OH} \rightarrow \text{Glucose–O–C}_2\text{H}_5 + \text{Glucose.}$$

## Genetic improvement of yeast strains producing β-glucosidases

### THE AIMS OF GENETIC IMPROVEMENT

The production of β-glucosidases by yeasts must be improved if potential applications are to be achieved. Most yeast β-glucosidases are inducible, which is inconvenient for industrial enzyme production, while the biosynthesis of the constitutive enzymes is repressed by glucose. These characteristics hinder the use of the strains both for enzyme production and for fermentation of cellobiose and cellodextrin because of the cost of a non-repressive growth substrate and the problem of overcoming glucose repression. These difficulties may be overcome:(1) by obtaining repression-insensitive mutant strains (such organisms generally also hyperproduce their enzyme); (2) by increasing β-glucosidase production by cloning and amplification of the genes controlling synthesis of these enzymes.

### DEREPRESSED AND HYPERPRODUCING MUTANT STRAINS

Reports concerning production of glucose-insensitive β-glucosidase-producing strains of yeasts are sparse, but such mutants have been obtained in filamentous fungi synthesizing β-glucosidases, for example in *Trichoderma reesei* (Beja da Costa and Van Uden, 1980), and *Fusarium graminearum* (Loureiro-Dias, 1982). The method used by these teams was based on 2-deoxyglucose, which was also utilized by Van Uden *et al.* (1980) for obtaining derepressed mutants of yeast for α-amylase. 2-Deoxyglucose is a non-metabolizable analogue of glucose which causes catabolic repression of β-glucosidase biosynthesis without allowing growth. Strains plated on agar media containing cellobiose as the sole carbon source and low amounts of 2-deoxyglucose thus cannot grow unless they are insensitive to catabolic repression: such mutant strains have been obtained in *Dekkera intermedia* and *Candida wickerhamii* following mutagenesis and enrichment (Leclerc *et al.*, 1985).

The *C. wickerhamii* mutant was derepressed for the biosynthesis of endocellular β-glucosidase and hyperproduced this enzyme up to four times its normal level; *C. wickerhamii* also produces an extracellular β-glucosidase, the synthesis of which was switched from constitutive to inducible in the mutant strain: this enzyme is ten times hyperproduced when induced (Leclerc *et al.*, 1987). Similar mutants were formed from *D. intermedia*, and in both *C. wickerhamii* and *D. intermedia* the mutation led to inability to produce β-glucosidase under anaerobic conditions, thus preventing the strains from fermenting cellobiose (Leclerc *et al.*, 1987).

### IMPROVEMENT BY GENETIC ENGINEERING

When high enzyme production is required, gene cloning and amplification are powerful methods for obtaining hyperproducing strains. Thus Raynal and Guérineau (1984) and Kohchi and Toh-e (1985, 1986) cloned β-gluco-

sidase genes of *Kluyveromyces fragilis* and *Candida pelliculosa*, respectively, into *Saccharomyces cerevisiae*, in the hope of obtaining a yeast strain capable of the rapid and highly productive fermentation of cellobiose. This did not occur in such species as *Dekkera intermedia* and *Candida wickerhamii* (Leclerc *et al.*, 1985). In *S. cerevisiae* transformed with the *K. fragilis* gene, β-glucosidase is synthesized to an extent up to 400 times its original level, but remains intracellular. This strain does not ferment cellobiose and it is probable that cellobiose does not enter the cells (Leclerc *et al.*, 1986). On the other hand, *S. cerevisiae* transformed with the *C. pelliculosa* gene secretes its β-glucosidase into the periplasmic space. The expression of the gene is inhibited by glucose.

## Possible industrial uses of yeast β-glucosidases

Interest in the industrial use of β-glucosidases is based upon the fact that these enzymes are the limiting factor in conversion of cellulose to glucose: they are repressed and inhibited by glucose, thermally unstable, and poorly synthesized by cellulose-degrading fungi. The addition of exogenous β-glucosidase to the hydrolysis media, or the use of hyperproducing mutants or transformed strains, would overcome these problems, as would the utilization of a cellobiose-fermenting yeast strain where ethanol production from cellulose is concerned. The derepressed and hyperproducing mutant of *C. wickerhamii*, as well as the transformed *S. cerevisiae* secreting the β-glucosidase from *C. pelliculosa*, would be convenient for this type of application.

Unfortunately, yeasts seem to be poor candidates for the production of β-glucosidases designed for use as such because the enzymes generally are not excreted into the culture media (although *C. wickerhamii* and *C. molischiana* excrete small amounts of enzyme) and are thermolabile. Solutions to these problems do exist: enzyme immobilization or modification by protein design could be applied to increase activity and thermostability, and incorporation of certain sequences into cloned genes might permit excretion of the enzymes into the culture medium. The main use of yeast β-glucosidases would be the simultaneous saccharification and fermentation of cellulose, and cellulase genes already have been cloned into fermenting yeasts (Van Arsdell *et al.*, 1987). However, yeast possessing β-glucosidases could also be used, together with cellulolytic fungi, for the partial conversion of cellulosic wastes into animal fodder for single-stomached animals such as pigs and chickens.

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