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12 Measurement of Polysaccharide-degrading Enzymes in Plants Using Chromogenic and Colorimetric Substrates*

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12.1 Introduction

Enzymatic degradation of carbohydrates is of major significance in the industrial processing of cereals and fruits. In the production of beer, barley is germinated under well-defined conditions (malting) to induce maximum enzyme synthesis with minimum respiration of reserve carbohydrates. The grains are dried and then extracted with water under controlled conditions. The amylolytic enzymes synthesized during malting, as well as those present in the original barley, convert the starch reserves to fermentable sugars. Other enzymes act on the cell wall polysaccharides, mixed-linkage β-glucan and arabinoxylan, reducing the viscosity and thus aiding filtration, and reducing the possibility of subsequent precipitation of polymeric material (Bamforth, 1982). In baking, β-amylase and α-amylase give controlled degradation of starch to fermentable sugars so as to sustain yeast growth and gas production. Excess quantities of α-amylase in the flour result in excessive degradation of starch during baking which in turn gives a sticky crumb texture and subsequent problems with bread slicing. Juice yield from fruit pulp is significantly improved if cell-wall-degrading enzymes are used to destroy the three-dimensional structure and water-binding capacity of the pectic polysaccharide components of the cell walls. Problems of routine and reliable assay of carbohydrate-degrading enzymes in the presence of high

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levels of sugar compounds are experienced with such industrial processes.

Enzyme activities present in cereal and fruit products, or added during processing, are ideally assayed using the natural substrate and under conditions which simulate processing conditions. Polysaccharide *endo*-hydrolases aid processing by catalysing a viscosity reduction, and the ideal assay format would follow the reduction in viscosity with a natural substrate (Hardie and Manners, 1974; Bathgate, 1979). However, viscosity measurements are tedious and require considerable skill. Another routine assay for polysaccharide-degrading enzymes is the measurement of the increase in the reducing sugar levels as the substrate is hydrolysed by the appropriate enzyme. The methods to estimate reducing sugars include dinitrosalycilic acid (DNSA) (Bailey, 1988), Nelson-Somogyi (Somogyi, 1960), *p*-hydroxybenzoic acid hydrazide (PAHBAH) (Lever, 1972) and ferricyanide procedures (Park and Johnson, 1949). However, with cereal and fruit extracts these procedures cannot be used to measure enzymatic activity because of the very high levels of reducing sugars present.

To overcome these problems, assays which exploit a specific reaction characteristic or a solubility property of a particular polysaccharide have been developed. For example, the reaction of starch with iodine (to give a purple colour), and the decrease in this colour as the starch is depolymerized by α-amylase, is commonly employed to measure the activity of this enzyme. The reaction of mixed-linkage β-glucan with Congo Red stain and Calcofluor have been used to assay mixed-linkage β-glucanase activity in malt (malt β-glucanase) (Martin and Bamforth, 1983).

12.2 Chromogenic Substrates

Many of the problems experienced in the assay of polysaccharide *endo*-hydrolases can be resolved by the use of chromogenic or dye-labelled substrates. Such substrates may be soluble (Babson *et al.*, 1970; McCleary, 1978) or rendered insoluble (Ceska *et al.*, 1969) through covalent crosslinks, and have the following major advantages:

1. They allow measurement of enzyme activity in extracts containing high levels of reducing sugars, e.g. the measurement of α-amylase, limit-dextrinase and malt β-glucanase in malted barley extracts.
2. They allow specific measurement of polysaccharide *endo*-hydrolases in the presence of high concentrations of *exo*-acting enzymes, e.g. the measurement of α-amylase in the presence of β-amylase in cereal flours, or the measurement of α-amylase in the presence of amyloglucosidase in industrial microbial enzyme preparations.
3. They form the basis of assays which are simple, quantitative and reproducible. In a Royal Australian Chemical Institute evaluation of azo-

barley glucan for the assay of malt β-glucanase, which involved 18 laboratories and five malt samples, inter-laboratory coefficient of variation values of less than 6% for each of the samples were obtained (Buch, 1986).

4. They relate directly to assays based on viscosity reduction and thus more accurately reflect the likely significance of a given concentration of a particular type of enzyme. This means that these assays can be used to compare directly the activity of the same enzyme from different sources (i.e. different microbial sources) in a particular industrial application. One such example of this is a comparison of the different β-glucanase enzymes which are used in the brewing industry to destroy the viscosity of β-glucan. Bacterial and fungal β-glucanases are employed, but for a given activity based on a reducing-sugar assay, bacterial enzymes are far more effective in viscosity reduction than are the fungal enzymes and this is reflected in assays based on the use of soluble-dye-labelled barley β-glucan (azo-barley glucan) (McCleary and Shameer, 1987). Similar observations have been made in the evaluation of β-glucanases as supplements in chicken-feed diets (Rotter *et al.*, 1990) containing high levels of barley (and thus of β-glucan).

5. They can be used in assays over a wide range of temperature and pH conditions.

The single major limitation with these substrates is that the chemistry of dyeing of the polysaccharides cannot be accurately controlled, thus each production batch of the substrate must be standardized with a particular enzyme before use.

12.2.1 Soluble chromogenic substrates

Soluble chromogenic substrates are produced by the controlled dyeing of highly purified soluble polysaccharides. Dyed polysaccharide is usually separated from unreacted dye by precipitation or by gel permeation chromatography. Assay formats employing these substrates generally involve the incubation of an aliquot of soluble dyed substrate with enzyme solution under defined conditions of pH and temperature, with termination of the reaction and precipitation of non-depolymerized substrate by addition of an organic solvent or an organic solvent-salt solution (Friend and Chang, 1982) (Fig. 12.1). Precipitated material is removed by centrifugation and the colour in the supernatant solution measured. This colour can be directly related to the amount of enzyme in the assay mixture by reference to a standard curve.

The major requirements in the production and use of soluble chromogenic substrates are the following:

1. Availability of highly purified, soluble polysaccharides of moderate viscosity (i.e. a viscosity which allows preparation of solutions of at least 1% (w/v) of polysaccharide in water).

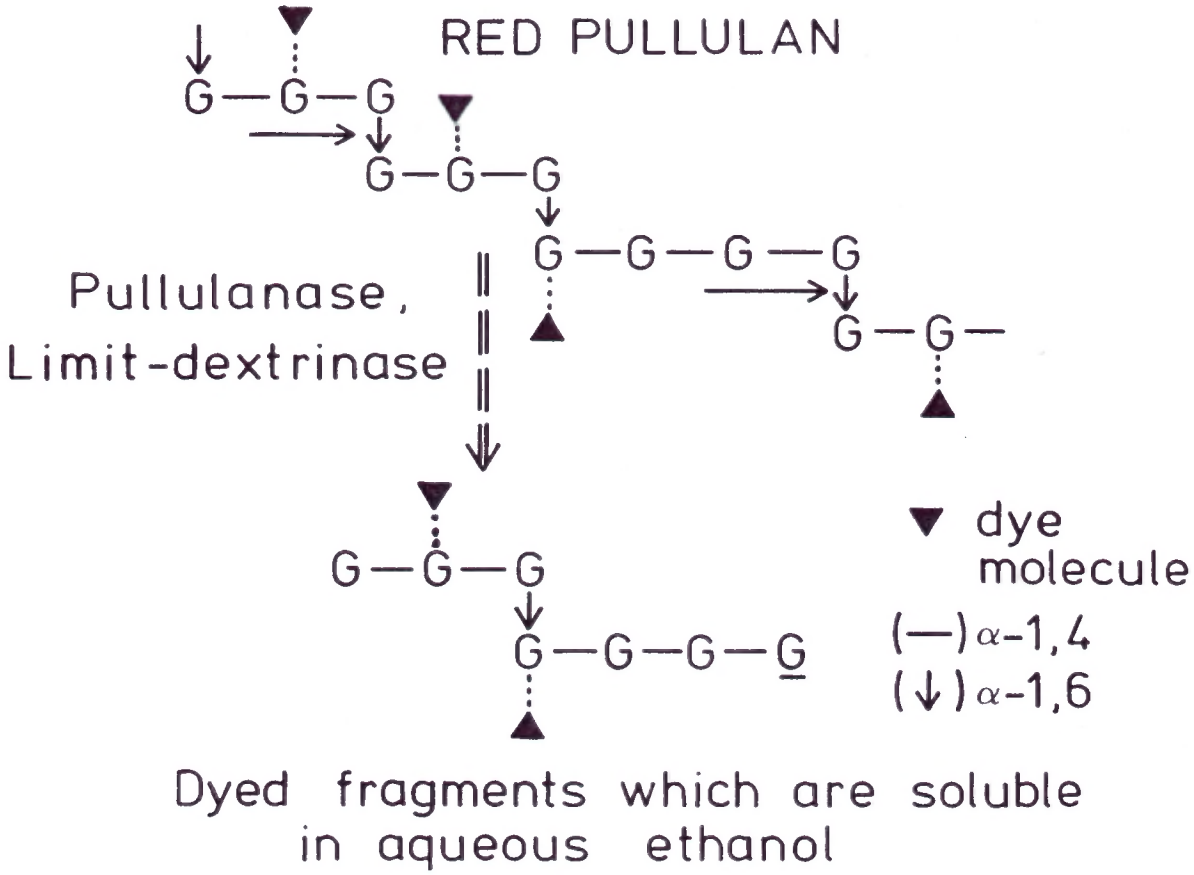

Fig. 12.1. Assay of limit dextrinase using dyed pullulan.

2. Physical, chemical and microbiological stability of the polysaccharide in solution over extended periods of storage.
3. A simple assay format and an effective procedure to quantitatively separate hydrolysed and non-hydrolysed dyed polysaccharide fragments.
4. Adequate dyeing of polysaccharide substrate to give linear, or near-linear, release of dye-labelled fragments over the absorbance range 0.1–1.0 absorbance units.
5. A final dye-labelled polysaccharide substrate which is very susceptible to enzyme attack.

12.2.2 Insoluble chromogenic substrates

The substrates referred to here as insoluble chromogenic substrates are those which have been produced from soluble polysaccharides or dye-labelled, soluble polysaccharides and rendered insoluble by covalent crosslinking with a particular crosslinking agent such as epichlorohydrin. These substrates are far superior to, and should not be confused with, substrates which are prepared by dyeing insoluble polysaccharides such as cellulose. These latter

substrates are very resistant to enzyme attack and have little analytical use. With covalently crosslinked substrates, individual polysaccharide molecules are 'locked' into a three-dimensional conformation as a result of the crosslinking. The susceptibility of such substrates to enzyme attack is influenced by the concentration of the crosslinks, the size (or length) of the crosslinking agent, the concentration of the dye molecules attached to the polysaccharide and the degree of natural substitution of the native polysaccharide. The major requirements for useful and effective insoluble (covalently crosslinked), dye-labelled substrates are very similar to those outlined for soluble dye-labelled substrates.

Insoluble substrates have the inherent disadvantage that they must be weighed accurately into each assay tube, whereas soluble substrates are readily and accurately dispensed with commercially available liquid handling equipment such as the Eppendorf Multipette®. The problem of dispensing the insoluble substrate can, however, be overcome by providing the substrate in a tablet form. Such a substrate is available commercially for the assay of α-amylase (Phadebas tablets; Pharmacia Diagnostics) and for the assay of a range of polysaccharide *endo*-hydrolases, including α-amylase, limit-dextrinase, xylanase and *endo*-arabinanase, from Megazyme (Australia) Pty Ltd.

In the preparation of such tablets there are several essential requirements:

1. The tablet must be sufficiently rigid to cope with normal handling and shipping requirements.
2. On addition to buffer or buffered enzyme solution, it must disintegrate rapidly (i.e. within 20s) and preferably without mechanical agitation (which leaves a varying percentage of the substrate attached to the walls of the test tube).
3. Finally, bulking agents used in conjunction with the active component must not interfere in the assay, i.e. by acting as alternative substrates.

With the tablet substrates produced by Megazyme, the basic assay format involves the addition of the tablet to an aliquot (1ml) of pre-equilibrated and correctly buffered enzyme solution. The tablet is designed to completely disintegrate without agitation within 20s. The reaction is terminated after 10min by addition of 10ml of an alkaline solution (Trizma base, Sigma Chemical Co.) with stirring; the slurry is filtered and the absorbance of the filtrate (at 590nm) is measured. Enzyme activity is determined by reference to a standard curve.

The major advantage of dyed, crosslinked substrates in tablet form is that they are stable indefinitely and, unlike liquid substrates, there is little chance of back-contamination of the bulk substrate preparation. Another advantage of these substrates is that, if prepared correctly and used in a good assay format, they are considerably more sensitive than their soluble counterparts.

All reactions with the insoluble substrates are performed in aqueous solutions, whereas with soluble substrates, the reaction is generally terminated by the addition of an organic solvent to precipitate unhydrolysed material, and this format gives a different molecular size cut-off and a diminished sensitivity. For example, in the measurement of β-xylanase, assays based on the use of Xylazyme tablets (Megazyme Pty Ltd), containing crosslinked and dyed birchwood xylan, are about ten times more sensitive than assays employing soluble dyed arabinoxylan from wheat or soluble dyed oat-spelt xylan.

12.3 Colorimetric Substrates

Colorimetric substrates are based on the use of a defined oligosaccharide which is covalently linked to *p*-nitrophenol through the reducing D-glucosyl residue of the oligosaccharide. At present, such substrates are available for the assay of α-amylase, β-amylase and amyloglucosidase. The action of these enzymes and of limit-dextrinase on starch is shown in Fig. 12.2.

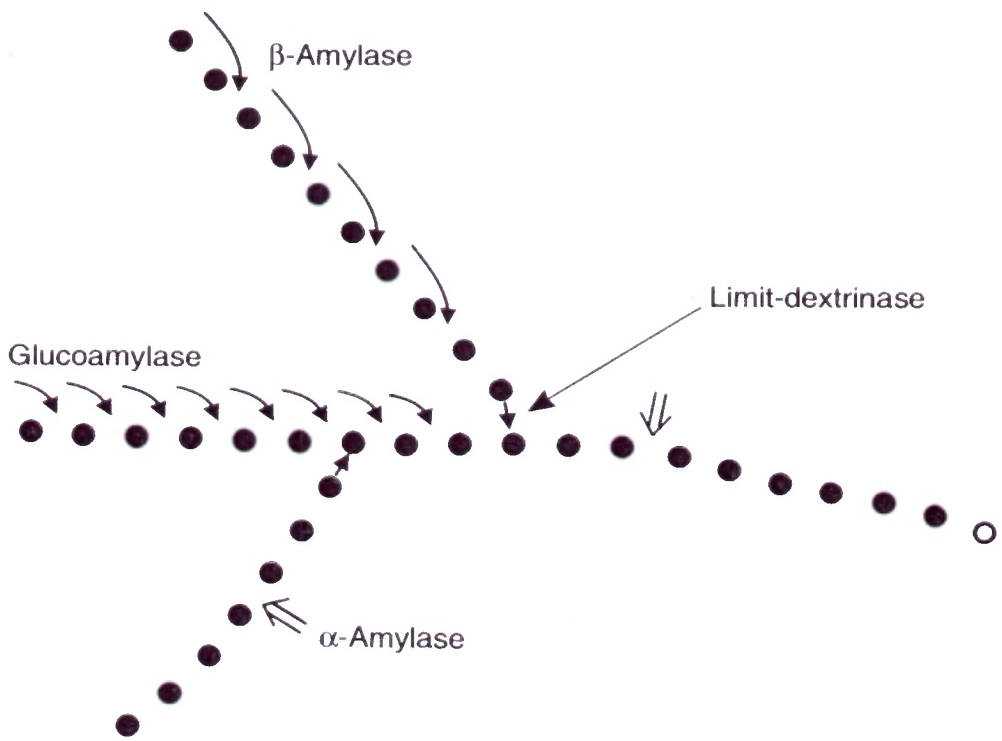

Fig. 12.2. The action of starch-degrading enzymes on amylopectin. ●●, α-1,4-linked D-glucosyl residues; ●⌒●, α-1,6-linked D-glucosyl residue; ○, terminal, reducing D-glucosyl residue.

12.3.1 α-Amylase

For the measurement of α-amylase, the substrate employed is 'non-reducing-end blocked *p*-nitrophenyl maltoheptaoside' (BPNPG7) (Blair, 1989). This substrate is used in conjunction with excess quantities of amyloglucosidase and α-glucosidase (which have no action on the native substrate due to the presence of the 'blocking group'). On hydrolysis of the oligosaccharide by *endo*-acting α-amylase, the excess quantities of amyloglucosidase and α-glucosidase which are present give essentially instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol (McCleary and Sheehan, 1987). The assay format is shown in Fig. 12.3. A further advantage of this type of substrate is that, with minor modifications, it can be used to assay for different forms of the same enzyme in a mixture. A particular application of this technology is the ability to measure relative proportions of fungal and cereal α-amylases in bread improver mixtures.

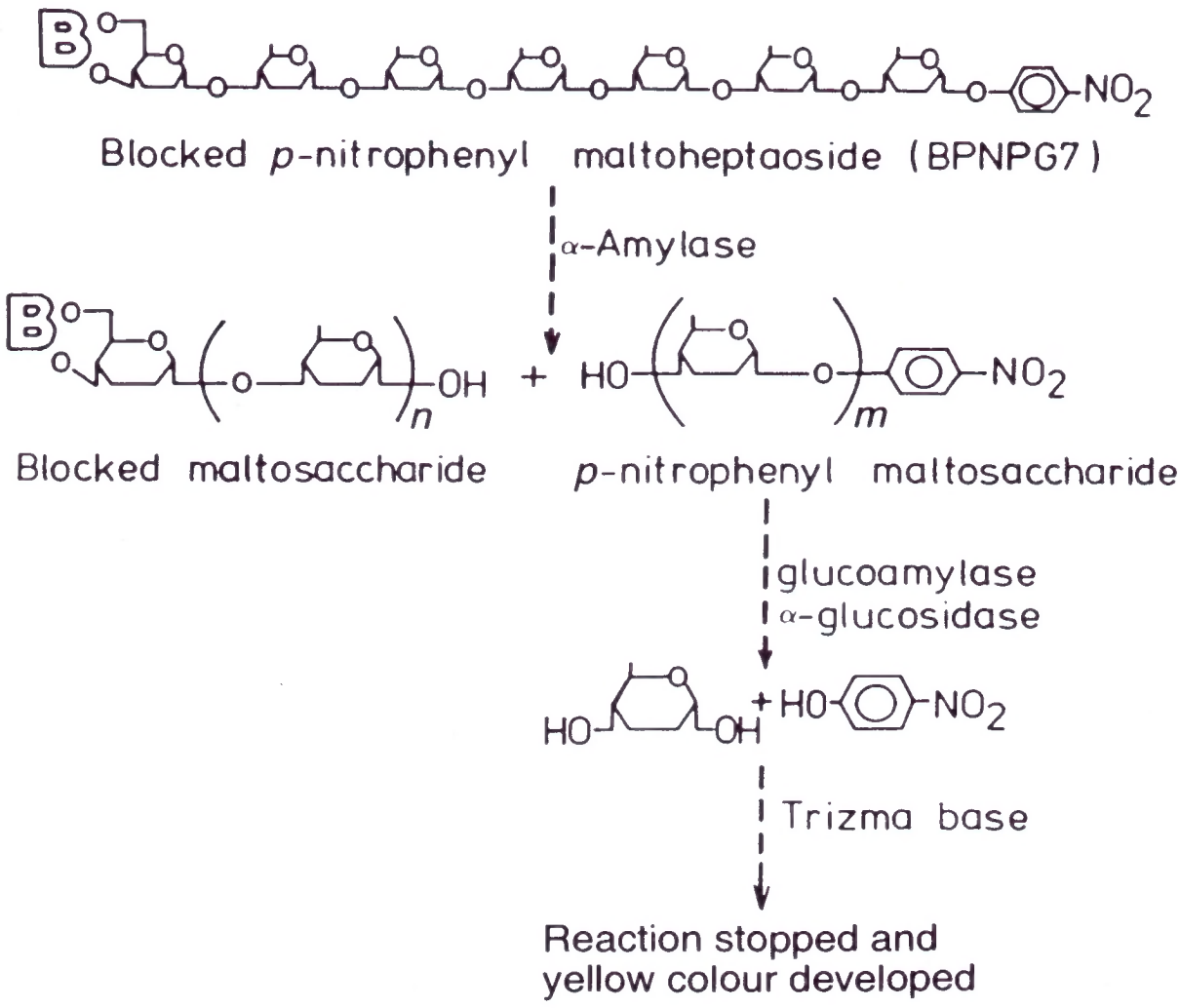

Fig. 12.3. Assay of α-amylase using blocked *p*-nitrophenyl maltoheptaoside.

Cereal α-amylase rapidly hydrolyses blocked *p*-nitrophenyl maltoheptaose, but hydrolysis of the pentasaccharide substrate (blocked *p*-nitrophenyl maltopentaose) is slow. In contrast, fungal α-amylase hydrolyses both substrates rapidly, as a consequence of its different subsite binding requirement at the active site of the enzyme (Megazyme data sheet). Fungal α-amylase is added to bread-making flours to assist the hydrolysis of gelatinized starch to fermentable sugars during the bread proofing stage which assists in improving loaf volume and keeping quality. The enzyme is inactivated at temperatures above 50°C, so it is effectively inactivated during the bread-baking process and thus cannot cause excessive degradation of starch at this stage of the process (which would result in the production of a sticky crumb, and subsequent bread-handling problems). Cereal α-amylases, being more thermostable, would have deleterious effects on bread quality if present in excess quantities in dough.

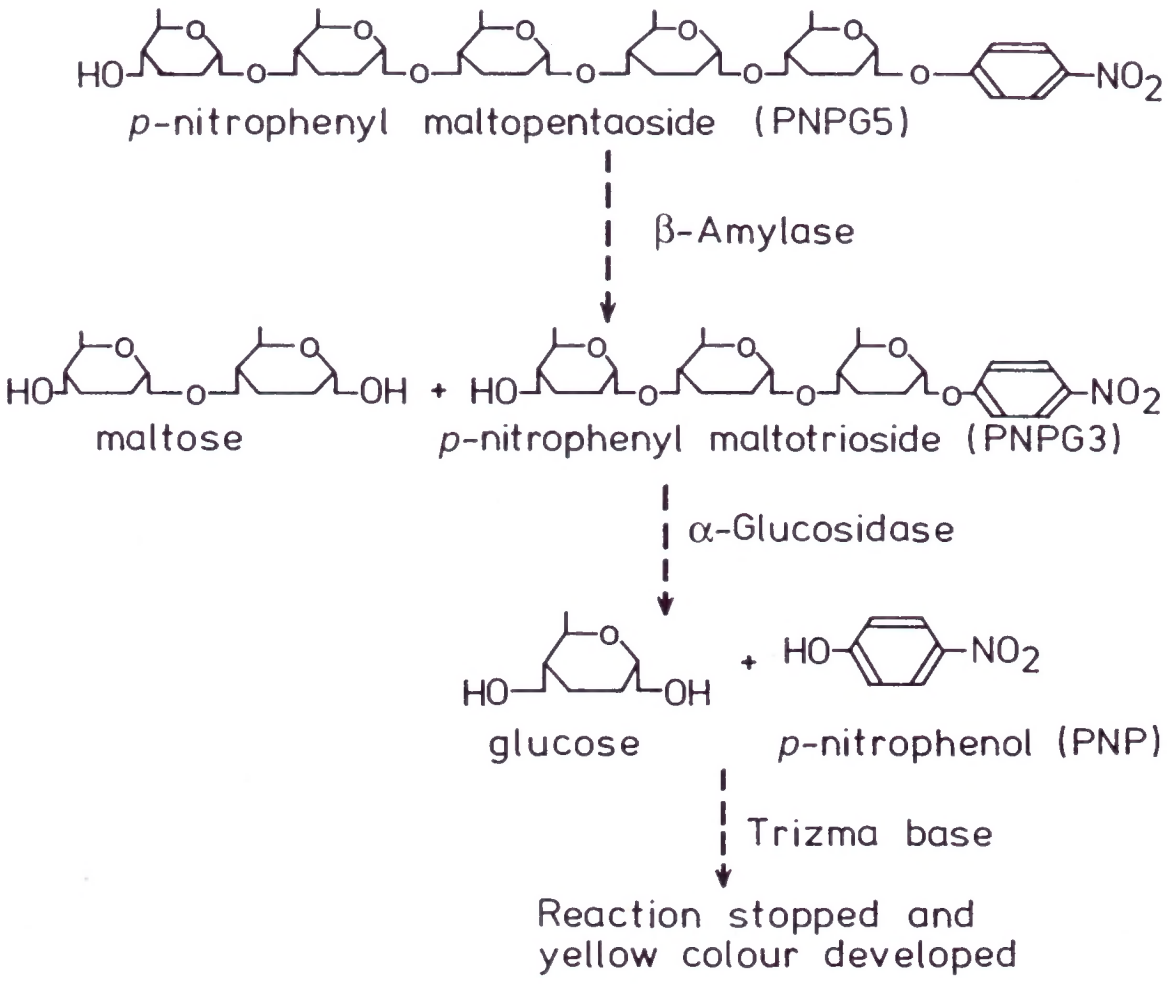

Fig. 12.4. Assay of β-amylase using *p*-nitrophenyl maltopentaoside.

12.3.2 β-Amylase

β-Amylase is an *exo*-acting enzyme which sequentially releases maltose units from the non-reducing terminus of amylose and amylopectin molecules. This enzyme can be effectively assayed in cereal extracts using *p*-nitrophenyl maltopentaose in the presence of yeast α-glucosidase (Fig. 12.4) (Mathewson and Seabourn, 1983; McCleary and Codd, 1989). This assay takes advantage of the fact that cereal α-amylases have very limited action on maltopentaose (oligosaccharides containing at least seven 1,4-α-linked D-glucosyl residues are required for rapid hydrolysis). The assay also takes advantage of the fact that yeast α-glucosidase rapidly hydrolyses maltosaccharides having two or three D-glucosyl residues, but acts extremely slowly on maltopentaose. Thus, when β-amylase removes the terminal maltosyl unit from *p*-nitrophenyl maltopentaose, the resultant *p*-nitrophenyl maltotriose is rapidly hydrolysed by the yeast α-glucosidase, releasing free *p*-nitrophenol.

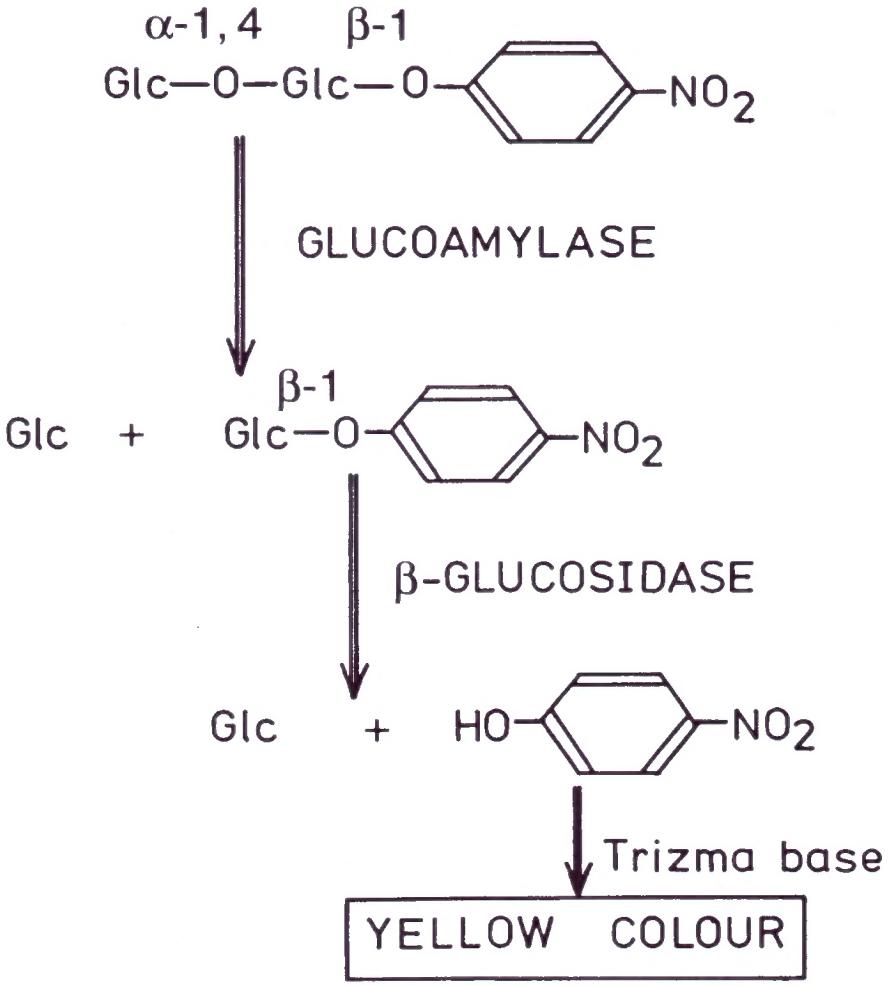

Fig. 12.5. Assay of amyloglucosidase using *p*-nitrophenyl β-maltoside.

12.3.3 Amyloglucosidase

Another *exo* enzyme active on starch fractions is the fungal enzyme amyloglucosidase which finds widespread industrial application. This enzyme is usually assayed with maltose as substrate, rather than starch, because the latter substrate is also rapidly hydrolysed by fungal α-amylase (which occurs at significant but varying levels in amyloglucosidase preparations). As an alternative to maltose, we have employed the substrate *p*-nitrophenyl β-maltoside in the presence of excess quantities of β-glucosidase (Fig. 12.5). (McCleary *et al.*, 1991). When the terminal α-linked D-glucosyl residue is removed by amyloglucosidase, the resultant *p*-nitrophenyl β-glucoside is instantaneously cleaved to glucose and free *p*-nitrophenol by the β-glucosidase. This assay format allows the measurement of amyloglycosidase in 10min instead of 60min required with conventional assay procedures.

12.4 Substrate Preparation and Some Applications of Chromogenic and Colorimetric Substrates

12.4.1 α-Amylase

Because of its industrial significance in the brewing and baking industries, numerous methods have been developed for the assay of α-amylase.

Colorimetric substrates

α-Amylase assay procedures employing the colorimetric substrate end-blocked *p*-nitrophenyl maltoheptaose (McCleary and Sheehan, 1987; Sheehan and McCleary, 1988) have many advantages over most other α-amylase assay formats. The substrate is available commercially in a ready-to-use form as Ceralpha α-amylase assay reagent (Megazyme Pty Ltd). The assay simply involves the incubation of an aliquot (0.2ml) of enzyme preparation with 0.2ml of the substrate mixture for 10min at 40°C, termination of the reaction and colour formation by the addition of an alkaline solution (1%, w/v, Trizma base), and measurement of absorbance at 410nm. In the clinical diagnostics field, this assay format is rapidly replacing most other procedures. However, in some laboratory situations (e.g. in flour mills, in industrial enzyme production facilities and in factories involved in enzyme blending) problems of contamination of the stock substrate solution with trace quantities of α-amylase (possibly from flour dust or aerosols) may be experienced.

Chromogenic substrates

Chromogenic, crosslinked substrates form the basis of standard procedures for the assay of α-amylase in Australia (Barnes and Blakeney, 1974), USA (AACC Method 22–06) and Europe (EBC Method 4.12.3). In Europe (E.B.C. Method 4.12.3) and Australia, Phadebas tablets (Pharmacia Diagnostics AG) are used, whereas in the USA (AACC Method 22–06), special unbuffered Amylochrome tablets (Roche Diagnostics) were employed. These latter tablets are no longer available but can be substituted by Amylazyme tablets (Megazyme). In AACC Method 22–06, the test tablet is added directly to a flour–buffer slurry with filtration after a defined incubation period. In the Australian standard method (Barnes and Blakeney, 1974), the flour is first extracted with buffer and an aliquot of this extract is incubated with the tablet under defined conditions.

Two quite distinct assay formats have been developed for measurement of α-amylase using Amylazyme tablets. With preparations containing high levels of enzyme activity, Format 1 is employed. In this procedure, an aliquot (1.0ml) of suitably diluted extract is pre-equilibrated at 40°C for 5min. An

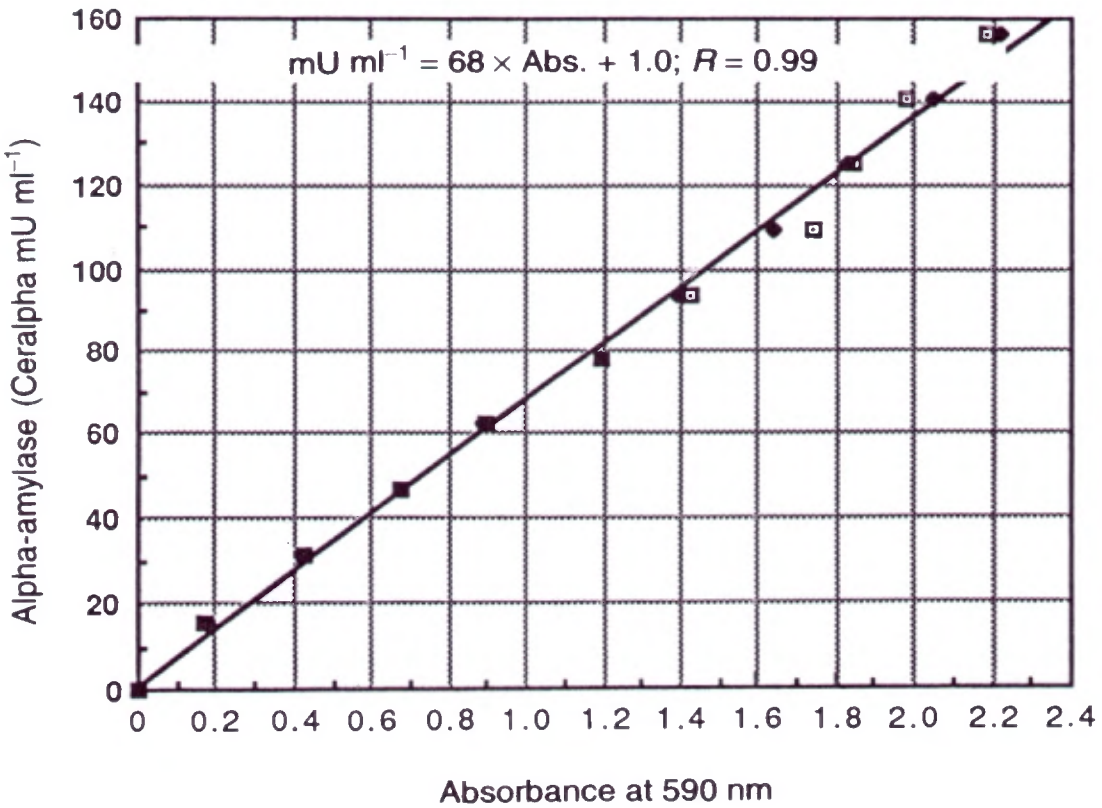

Fig. 12.6. Amylazyme (Lot 11201) standard curve for malt flour α-amylase with assay Format 1. Amylazyme absorbance values (at 590nm) are converted via a standard curve to α-amylase activity in Ceralpha milliunits (mU) ml^{-1} (or per assay).

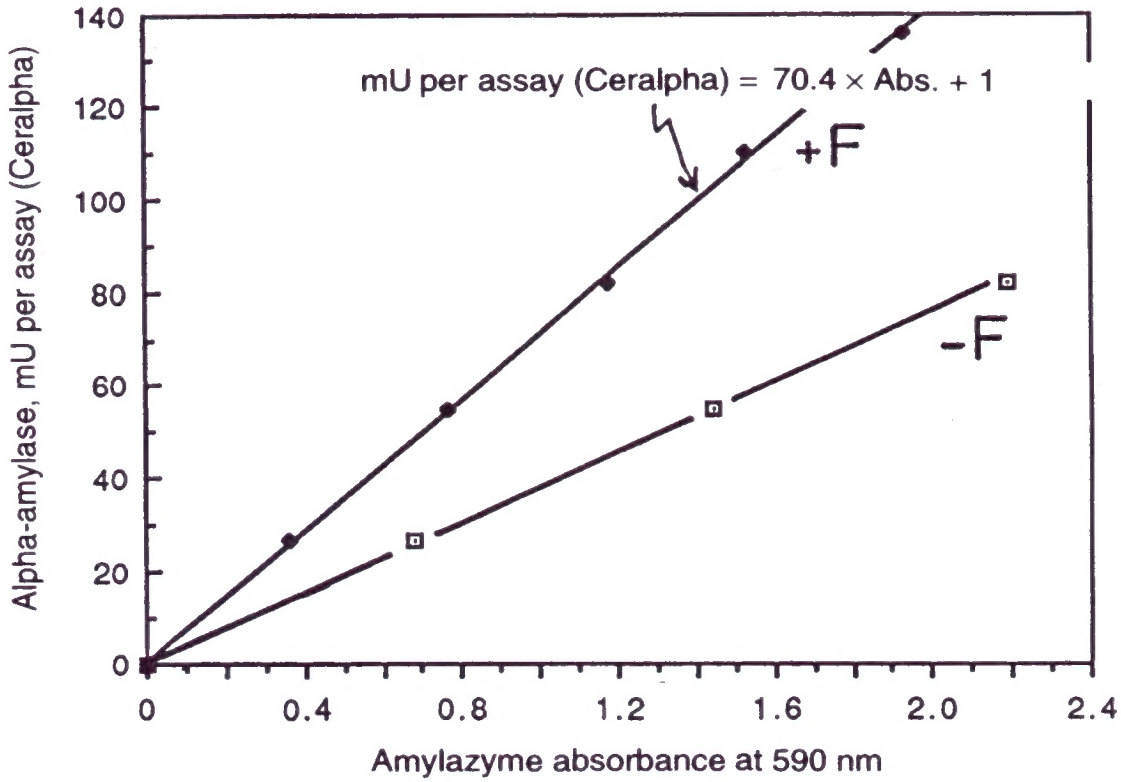

Fig. 12.7. Standard curves relating Amylazyme (Lot 11201) absorbance values to Ceralpha mU per assay (i.e. per 5 ml). An Amylazyme tablet was added to 5 ml of buffer containing Ceralpha 0–140 mU of α-amylase in the presence (+F) or absence (–F) of wheat flour (devoid of additional α-amylase). Stirring and incubation were performed according to assay Format 2.

Amylazyme tablet is added (without stirring) and this rapidly hydrates and absorbs most of the sample volume (i.e. 1.0 ml). After 10 min the reaction is terminated by the addition of Trizma base (a mild alkaline solution) and the slurry is filtered. The absorbance of the filtrate is measured and converted to α-amylase activity via a standard curve (Fig. 12.6). A different assay format (Assay Format 2) is employed for flour samples containing very low levels of α-amylase, e.g. flours from weather-damaged wheat. In this procedure, flour (0.5 g) is suspended in buffer at 55°C and equilibrated with continual stirring. After 5 min, an Amylazyme tablet is added to the slurry and stirring is continued for a further 5 min (exactly). The reaction is terminated with Trizma base and the slurry is filtered. The absorbance of the filtrate is measured at 590 nm. This assay format is about ten times more sensitive than the Ceralpha method and allows the accurate measurement of α-amylase in cereal flour with falling number values as high as 500. Under identical assay conditions, the Amylazyme test tablets are about two to three times more sensitive than Phadebas tablets (Pharmacia Diagnostics AG).

Extraction of α-amylase from cereal flours is likely to be affected by several factors including: the time required to wet the sample; the nature of

the buffer employed; affinity binding of α-amylase to starch granules and, possibly, physical entrapment of α-amylase within starch granules. Activity values will be influenced by each of the above factors as well as by the level of starch in the assay mixture (which may act either as an alternative substrate or may physically bind the α-amylase and potentially render it inactive towards the added substrate). Several of these problems are experienced in the direct assay of α-amylase in flour slurries (Format 2). The effect of wheat flour on the measured α-amylase activity using Amylazyme tablets is shown in Fig. 12.7. Enzyme solution (5ml) was incubated with an Amylazyme tablet in the absence or presence of wheat flour (0.5g; essentially devoid of endogenous α-amylase). It is apparent that addition of flour to the incubation mixture (+F) results in a reduced rate of hydrolysis of the Amylazyme substrate (reduced rate of increase in absorbance at 590nm). This reduction in rate is probably due to a combination of affinity binding of α-amylase to granular starch as well as to substrate competition effects (by soluble starch in the flour sample). Thus to obtain an estimate of the absolute

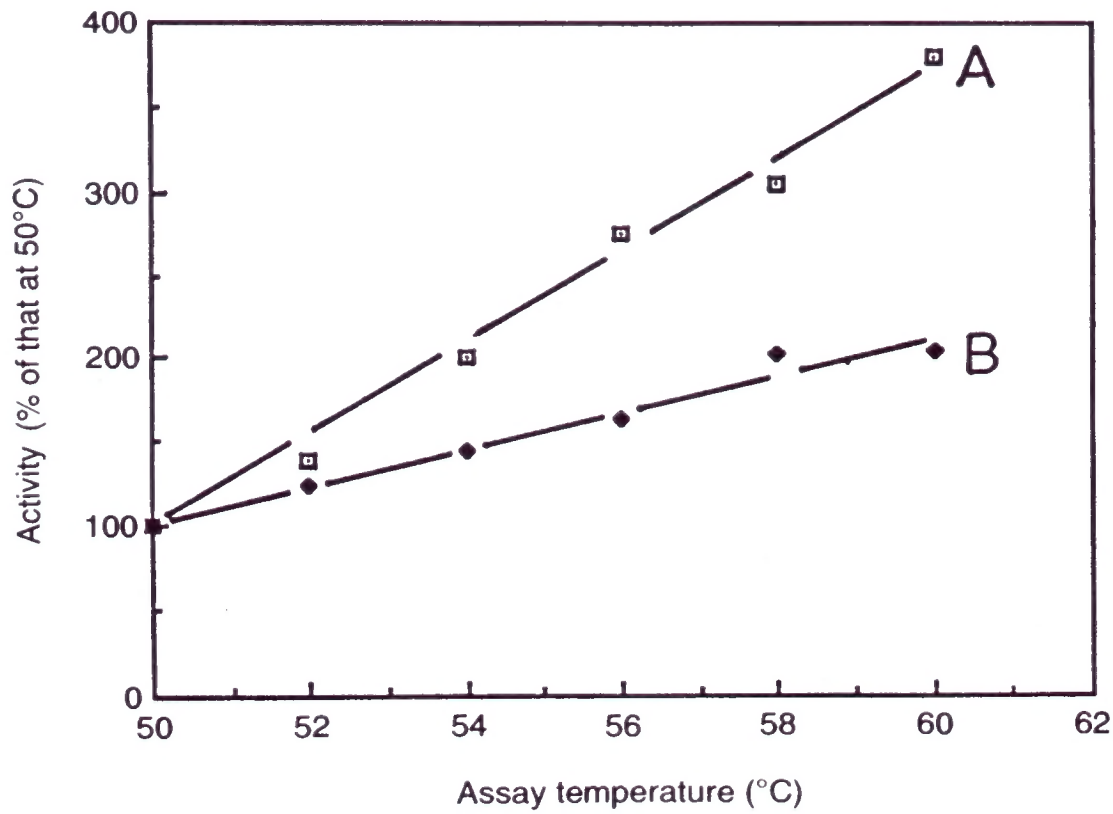

Fig. 12.8. Effect of incubation temperature on Amylazyme values using assay Format 2 with barley α-amylase. Incubations were performed on an aliquot (5 ml) of enzyme extract (B) or a slurry (5 ml) of whole milled barley (A) for 10 min. Activity is presented as a percentage of that at 50°C.

level of α-amylase in flour samples, standard curves should be prepared in the presence of added flour.

A second interesting complication experienced in the measurement of α-amylase, according to assay Format 2, in flour slurries, is the effect of incubation temperature on measured activity. Temperature has a much greater than expected effect on the Amylazyme absorbance values for whole flour slurries; i.e. there is a 3- to 3.5-fold increase in measured activity in the temperature range 50–60°C (Fig. 12.8) (McCleary, 1993). This compares to an increase of just 1.5- to 2-fold for enzyme extracts. This phenomenon may be due to a physical entrapment of the enzyme within starch granules. Heating the granules to temperatures approaching those of starch gelatinization appears to allow the enzyme to diffuse from the granule into the solution, and thus have access to the Amylazyme tablet.

12.4.2 β-Xylanase

Xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) is finding widespread application in chicken feed supplements, in bread improver mixtures and in research on the enzymatic bleaching of wood pulp (Nissen *et al.*, 1992). Historically, xylanase activity has been measured with the DNS reducing sugar assay (Bailey, 1988) with a purified xylan from oat-spelts, larchwood or birchwood as substrate. These assays are limited by their lack of sensitivity and linearity and the fact that they cannot be used to assay activity in materials containing high levels of reducing sugars, e.g. chicken feeds. Alternative assay procedures involve the use of high viscosity wheat-flour arabinoxylan in viscometric assays or of soluble or insoluble, dye-labelled, xylan-based substrates. Commercially available soluble substrates include Azo-xylan (birchwood (McCleary, 1992a), beechwood (Sigma M5019) (Bieley *et al.*, 1985) and oat (McCleary, 1992a)) and Azo-wheat arabinoxylan (McCleary, 1992a). These substrates are prepared by dyeing the polysaccharide with Remazo Brilliant Blue. Of these substrates, those of most practical use are Azo-xylan (birchwood) and Azo-wheat arabinoxylan as they dissolve rapidly in water and are stable in solution (both chemically and physically) for extended periods at room temperature and 4°C and give a linear standard curve over the absorbance range of 0.1 to 1.0 absorbance units (590nm).

The only commercially available insoluble (crosslinked) substrates for the measurement of *endo*-xylanase are AZCL-xylan (birchwood) and AZCL-wheat arabinoxylan which are supplied in powder form or as tablets (Xylazyme or Xylazyme AX). The powdered substrates are useful for incorporation in agar plates or gels for location of xylanolytic microbial cultures and xylanase activity in electrophoretic gels. Xylazyme and Xylazyme AX tablets are used in test-tube assays for the quantitative measurement of xylanase in microbial fermentation broths and in various feed

materials (e.g. chicken feed pellets). Assays employing Xylazyme tablets have ten times the sensitivity of soluble chromogenic substrates (McCleary, 1992a) and about the same sensitivity as viscometric assays employing wheat arabinoxylan as substrate.

Xylanase and cellulase-type enzymes are added to wheat- and barley-based chicken and pig diets to depolymerize arabinoxylan and β-glucan. This results in a decrease in viscosity of the gut contents and an improved absorption of nutrients, with resulting increases in feed conversion ratios. In studies of enzymatic methods for use as predictors of *in vivo* response to enzyme supplementation of barley-based diets when fed to young chicks, Rotter *et al.* (1990) found that the most reliable assay was based on the use of Azo-barley glucan (dyed barley β-glucan). Similar studies have not, as yet, been performed on xylanase assay procedures. However, in preliminary studies we have shown that Xylazyme tablets have the required sensitivity (refer to the assay described in Fig. 12.9). With this assay it has been possible

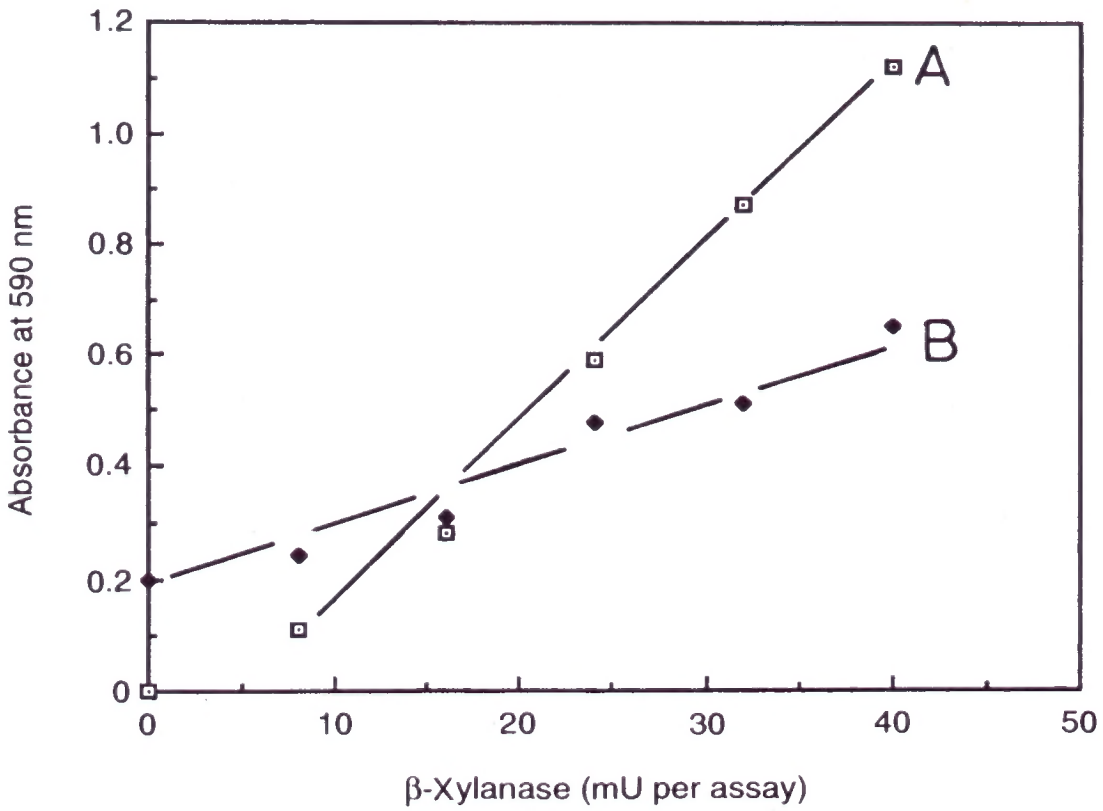

Fig. 12.9. Effect of feed material on the assay of *Trichoderma* sp. xylanase using Xylazyme tablets. Enzyme solution (2.0ml, 0–50 mU) in 25mM solium acetate buffer (pH 4.7) was incubated with stirring at 50°C in the presence (B) or absence (A) of feed material (0.2g) for 10min. The reaction was terminated after 10min by the addition of 6ml of 1% Trizma base. The solution was filtered and the absorbance at 590nm measured. The absorbance of ~0.2 for the feed sample with no exogenous xylanase added in the assay procedure was due to the presence of xylanase in this feed sample.

to demonstrate some of the problems associated with the measurement of trace levels of enzyme activities in feed samples. The relationship between xylanase concentration (*Trichoderma* sp.) and absorbance, in the presence (B) and absence (A) of added feed sample (0.2g) is shown. It is evident that in the presence of the feed material, the slope of the standard curve is significantly reduced. This could be due either to non-specific binding of the enzyme to feed components or alternatively to the competitive action of alternative substrate material in the feed mixture. It is essential that the nature of this effect is identified and, if possible, that assay strategies to remove it be developed. The need for accurate and reliable assays for the measurement of xylanase, cellulase, β-glucanase and α-amylase in animal feeds pre- and post-pelleting is essential to allow the control of processing conditions and for regulatory purposes.

12.4.3 Limit-dextrinase

Limit-dextrinase, also called debranching enzyme or pullulanase, is the enzyme which cleaves the 1,6-α-branch linkages in the amylopectin fraction of starch. This enzyme is thought to be the limiting activity in malt flour which dictates the degree of conversion of starch to fermentable sugars. Yeast cells readily absorb glucose, maltose and maltotriose, but are unable to absorb branched maltosaccharides. It is thought that the fermentability of wort (the malt extract) may be directly related to the level of limit-dextrinase in the malt. Microbial pullulanases, in combination with β-amylase, are also finding increasing application in the starch-syrup industry in the production of high maltose syrups.

Measurement of limit-dextrinase in malt extracts using reducing sugar methods is difficult, time consuming and at best semi-quantitative. The enzyme has to be partially purified before it can be assayed. However, with dye-labelled pullulan as substrate, activity in malt extracts can be assayed directly (Serre and Lauriere, 1990; McCleary, 1992b). A soluble, red-dyed pullulan is finding widespread use in this area. A linear reaction curve in the range 0.1–1.4 absorbance units (at 510 nm) can be obtained, but the substrate is not as stable as would be preferred. An alternative substrate is dyed and crosslinked pullulan in tablet form (Limit-Dextrizyme tablets) which is both more stable and more sensitive than red pullulan (McCleary, 1992b).

12.4.4 Malt β-glucanase

Malt β-glucanase is a specific *endo* (1–3) (1–4)-β-D-glucanase which cleaves the 1,4-β-glycosidic bond of the 3-linked D-glucosyl units in mixed-linkage barley β-glucan. This enzyme is synthesized by barley during the malting process, but is very susceptible to thermal inactivation during kilning. Malt β-glucanase level is considered to be an important quality parameter of malt.

With judicious mashing temperature profiles, this enzyme can be employed to partially depolymerize the mixed linkage β-glucan extracted from the malt (which causes filtration problems). This enzyme has traditionally been assayed by a viscometric procedure using barley β-glucan of intermediate viscosity as the substrate (Bourne and Pierce, 1970). In recent years a method based on the use of the soluble Azo-barley glucan (McCleary and Shameer, 1987), has gained favour and is routinely used in many breweries and malt houses and by industrial enzyme manufacturers. Dye-labelled, crosslinked barley β-glucan in tablet form is now available (Megazyme). This substrate is more convenient to use than the soluble substrate, Azo-barley glucan, but inter-laboratory evaluations have shown that it is not quite as reliable.

12.4.5 Endo-*arabinanase*

To obtain maximum juice extraction from apple and pear pulp, it is common practice to employ pectinase enzymes to destroy the gelatinous, water-binding properties of the pulp pectic polysaccharides. These pectic polysaccharides have a modified polygalacturonic acid backbone with highly substituted regions to which 1,4-β-D-galactans and α-L-arabinans (or L-arabans) are attached (Schols *et al.*, 1990). The arabinans have a 1,5-α-linked L-arabinan backbone to which single unit L-arabinosyl residues are linked α-1,3 and α-1,2. Pectinase enzyme mixtures are usually deficient in *endo*-1,5-α-L-arabinanase (EC 3.2.1.99) and thus high concentrations (about 3–5 mg ml^{-1}) of linear 1,5-α-L-arabinan can occur in the juice concentrate. This arabinan self-associates and crystallizes in the juice, leading to haze formation. The problem is most readily resolved by ensuring that adequate levels of *endo*-arabinanase (which depolymerizes the linear arabinan) are present in the enzyme preparation employed. However, this has been complicated by the lack of a specific assay procedure for *endo*-arabinanase and even the non-availability of the basic polysaccharide substrate. This problem was partly resolved by major enzyme manufacturers by removing the linear arabinan from 'hazy' apple or pear juice concentrates by filtration, and using the arabinan (after purification) as a substrate for *endo*-arabinanase.

An alternative solution, and that adopted by us (McCleary, 1989), was to purify arabinan from sugarbeet pulp and treat this with α-L-arabinofuranosidase (EC 3.2.1.55) to remove all the 1,3- and 1,2-α-linked arabinofuranosyl residues, leaving linear 1,5-α-L-arabinan (Fig. 12.10). This polysaccharide still contains a small percentage of galacturonic acid, galactose and rhamnose (6%, 4% and 2%, respectively), but is resistant to attack by polygalacturonanase and *endo*-1,4-β-D-galactanase (EC 3.2.1.89). This 'linear' or 'debranched' arabinan was dyed with Reactive Red 120 to produce a soluble, red-dyed 1,5-α-L-arabinan, or was dyed and crosslinked to produce AZCL-arabinan (a black-coloured, crosslinked and

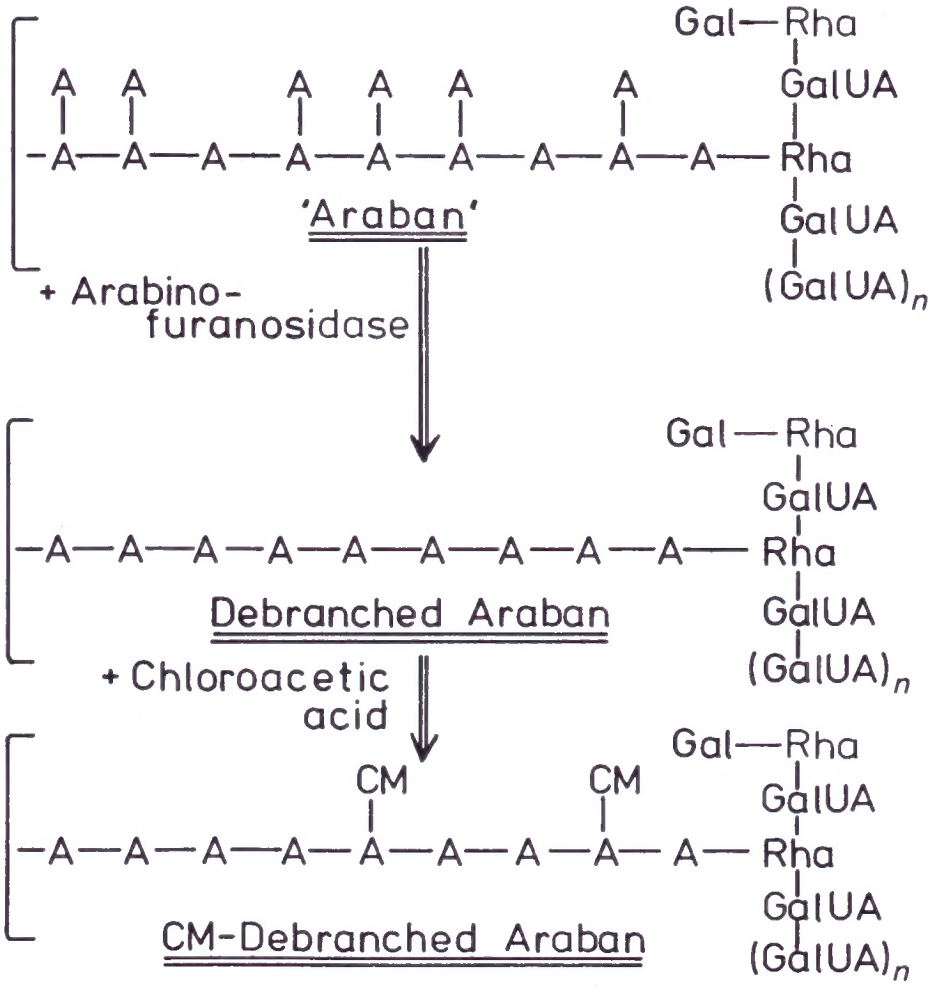

Fig. 12.10. Preparation of debranched araban for use in *endo*-arabinase assays.

debranched arabinan). The latter substrate has been incorporated into tablets (Arabinazyme).

Arabinazyme tablets form the basis of a simple, very specific and highly sensitive assay for the measurement of *endo*-arabinanase in crude enzyme mixtures. This substrate is in routine use in several major enzyme manufacturing companies.

12.4.6 Cellulase (endo-*1,4-β-D-glucanase)*

Cellulase (EC 3.2.1.4) catalyses the *endo*-depolymerization of cellulose (1,4-β-D-glucan). Cellulose, and dyed celluloses, are highly insoluble. Solubility and thus enzyme susceptibility can be greatly increased by chemical modifications which reduce the degree of self-association between cellulose chains, e.g. carboxymethylation or hydroxyethylation. However, the degree of modification of the glucan chains must be sufficient to produce solubility without interfering with the ability of cellulase to bind to, and hydrolyse, the polymer.

A range of chemically modified celluloses are commercially available. Of these, carboxymethylcellulose (CMC) has the greatest solubility with the minimum degree of substitution. CMC with a degree of substitution of 0.7 is completely soluble, but with this degree of substitution it is resistant to cellulase hydrolysis. CMC of degree of substitution 0.4 is only partially hydrated in water. This substrate is readily hydrolysed by cellulases, and RBB-CMC (Azo-CMC) (McCleary, 1980) produced on the dyeing of this with Remazo Brilliant Blue is a useful substrate for the specific assay of *endo*-cellulase in crude microbial mixtures, with a linear standard curve in the absorbance range 0.1–1.4 absorbance units. The substrate is highly specific for *endo*-1,4-β-glucanase (cellulase) and is totally resistant to interference by β-glucosidase (microbial or plant). Assays employing Azo-CMC (Megazyme Pty Ltd) are approximately 50 times more sensitive than those employing Cellulose Azure (cellulose dyed with Remazo Brilliant Blue) (Sigma C8647) and about 15 times more sensitive than TNP-cellulose [*O*-(2,4,6-trinitrophenyl) carboxymethyl cellulose] (an insoluble dyed substrate) (Huang and Tang, 1976).

High sensitivity, insoluble dyed substrates can be prepared by dyeing and crosslinking soluble cellulose derivatives. In this case, hydroxyethyl propyl cellulose (a neutral polysaccharide) was used because the carboxymethyl groups in CMC interfere with the crosslinking reaction. Alternatively, a cellulase substrate can be prepared by dyeing and crosslinking xyloglucan from tamarind seed. Xyloglucan has a 1,4-β-D-glucan backbone which is partially and regularly substituted by D-xylose residues linked α-1,6. Some of the xylose residues are further substituted. AZCL-HE-Cellulose is readily hydrolysed by a range of cellulase enzymes, whereas AZCL-Xyloglucan (tamarind) is attacked by only selected cellulases. *Trichoderma* sp. *endo*-cellulases act on AZCL-Xyloglucan, but *Aspergillus niger* cellulases do not. In tablet form, AZCL-HE-Cellulose (Cellazyme C) is useful for the routine assay of cellulase in microbial enzyme preparations (Megazyme data sheet EZC 7/92). The standard curve is relatively linear in the absorbance range 0.2–2.6 absorbance units, and the sensitivity of the assay is twice that based on Azo-CMC.

12.4.7 β-Mannanase

β-Mannanase (EC 3.2.1.78) catalyses *endo*-hydrolysis of 1,4-β-D-mannans, galactomannans and glucomannans. β-Mannanase activity in mixtures can be specifically assayed viscometrically using carob galactomannan (23% D-galactose). In most cases glucomannan has been employed as substrate, but this substrate is also depolymerized by cellulase (endo-1,4-β-glucanase). Reducing sugar methods employing 1,4-β-D-mannan and carob galactomannan as substrate have been described, but with these assay formats it is necessary to assay separately for β-mannosidase (EC 3.2.1.25) and/or α-galactosidase (EC 3.2.1.22).

An alternative assay for β-mannanase employs carob galactomannan dyed with Remazol Brilliant Blue as substrate (McCleary, 1978). The assay system measures the hydrolysis of RBB-carob galactomannan in terms of the rate of release of fractions soluble in 66% (v/v) aqueous ethanol. The rate of release of dyed fragments on hydrolysis of RBB-carob galactomannan is a function of the dye to anhydrohexose ratio. Maximal sensitivity in the assay system is obtained with a dye to anhydrohexose ratio in RBB-carob galactomannan of 1 : 15 to 1 : 50. Standard curves for the conversion of absorbance values (at 590nm) to enzyme units (on carob galactomannan at 40°C) for RBB-mannan, RBB-carob galactomannan and RBB-guar galactomannan are shown in Fig. 12.11. The low rate of hydrolysis of RBB-mannan is due to the highly insoluble nature of this substrate. The low relative rate of hydrolysis of RBB-guar galactomannan (a soluble substrate) is due to a high degree of D-galactose substitution (38%) of this galactomannan.

In the use of RBB-carob galactomannan to measure β-mannanase in crude enzyme mixtures, an enzyme which could potentially interfere is α-galactosidase. There are two possible mechanisms by which α-galactosidase might increase the rate of release of dyed fragments from RBB-carob galactomannan: the enzyme either might release galactosyl units dyed with RBB or, by releasing galactosyl residues, might make the substrate more susceptible to β-mannanase hydrolysis (McCleary, 1978). Figure 12.12 shows the effect that the release of D-galactose (by purified lucerne

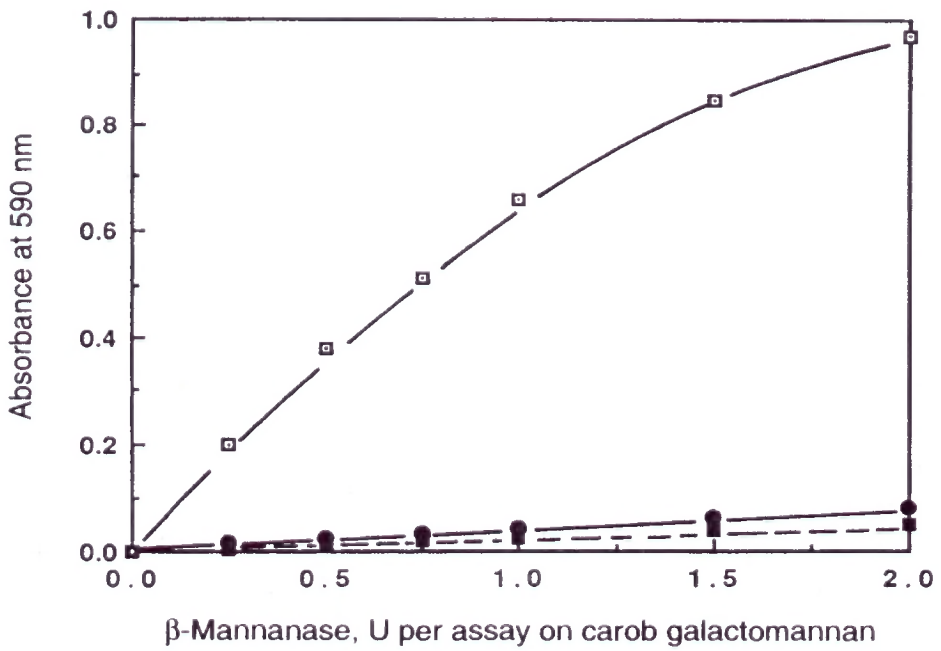

Fig. 12.11. Standard curves relating β-D-mannanase enzyme units on carob galactomannan to absorbance increase (at 590nm) on hydrolysis of RBB-carob galactomannan (1 : 50) ⊡; RBB-guar galactomannan (1 : 50) ●; and RBB-mannan (1 : 35) ■.

Fig. 12.12. Release of dyed fragments on hydrolysis of RBB-carob galactomannan by α-D-galactosidase (●); and on hydrolysis of α-D-galactosidase-prehydrolysed substrate by β-D-mannanase (⊡).

α-galactosidase A) from RBB-carob galactomannan (1 : 50) has on absorbance increase at 590nm, and the effect it has on the susceptibility of the substrate to β-mannanases hydrolysis. Since 70% of the D-galactosyl residues were removed with no release of ethanol-soluble, dyed fragments, it can be concluded that α-galactosidase is unable to remove D-galactosyl residues substituted with Remazo Brilliant Blue dye. Furthermore, removal of up to 70% of the D-galactosyl residues gave only a very slight (≈4%) increase in the susceptibility of the substrate to β-mannanase hydrolysis. In agreement with this, Marshall (1970) has shown that although the *exo*-hydrolase, amyloglucosidase, can remove D-glucose from Cibacron-Blue-dyed amylose, it cannot concurrently release Cibacron-Blue-dyed fragments.

Thus, RBB-carob galactomannan forms the basis of a simple, reliable, relatively sensitive and highly specific assay for β-mannanase. Alternative substrates for the assay of β-mannanase have been prepared by dyeing and crosslinking carob galactomannan to produce AZCL-galactomannan. This substrate in tablet form (β-Mannazyme) is currently under evaluation.

12.4.8 Endo-*1,4-β-D-galactanase*

Endo-galactanase (EC 3.2.1.89) is one of several enzymes involved in the depolymerization of pectic polysaccharides. Galactan in the pectic molecule is attached to rhamnosyl residues in the highly branched ('hairy') rhamnogalacturonosyl regions. The contribution of the galactan chains to

the physiochemical properties or suspensions of solutions of pectin is not known; however, it would appear that galactose substitution interferes with hydrolysis of the rhamnogalacturonan regions of pectins by the enzyme rhamnogalacturonanase (Schols *et al.*, 1990).

Endo-galactanase is usually assayed with a partially purified galactan from potato fibre with a reducing sugar method (Rombouts *et al.*, 1988). However, galactan also contains high levels of arabinofuranosyl residues and thus can be hydrolysed by *endo*-1,5-α-L-arabinanase and arabinofuranosidase.

AZCL-Galactan (dyed, crosslinked) is a sensitive and specific substrate for *endo*-galactanase. The source of the galactan employed can be either potato fibre or lupin-seed fibre. The galactan from lupin-seed fibre contains 12% arabinose, but because this is present as short chains, it is not susceptible to hydrolysis by *endo*-arabinanase. The sensitivity and specificity of AZCL-galactan (lupin) is not altered by pretreatment of the galactan with a mixture of *endo*-arabinanase and arabinofuranosidase (to reduce the arabinose content from 12% to 2%) before dyeing and crosslinking (B.V. McCleary, unpublished). This finding is consistent with the arabinan existing as multiple, short chains.

12.4.9 Endo-*1,3-β-glucanase*

Endo-1,3-β-glucanase (laminarinase; EC 3.2.1.39) is one of the enzymes involved in degradation of fungal cell-wall material. It has been assayed with laminaran, pachyman and curdlan, but a more convenient substrate is carboxymethyl pachyman (which is soluble) (Clarke and Stone, 1962). *Endo*-1,3-β-glucanases can also be assayed with AZCL-curdlan or -pachyman (Megazyme data sheet). The latter substrate, available in tablet form as 1,3-beta-glucazyme substrate, is extremely sensitive (assay range 4–24 milliunits (mU) of 1,3-β-glucanase/assay) and highly specific for *endo*-1,3-β-glucanase. *Exo*-1,3-β-glucanase (EC 3.2.1.58) from *Trichoderma* sp. is unable to release any soluble dyed fragments from this substrate.

12.4.10 Endo-*polygalacturonanase*

A dyed substrate for the measurement of *endo*-pectinase (*endo*-polygalacturonanase) was prepared from polygalacturonic acid by coupling the dye N{1-[4-(3,6-disulpho-1-naphthyl)-azo] naphthyl}ethylenediamine (DISANED) using a water-soluble carbodimide (Friend and Chang, 1982). Pectin methylesterase does not release measurable levels of DISANED from the substrate.

Conclusion

With continued pressure on cereal and fruit processors for increased throughput, improved yields and simpler processing formats, the efficient and effective exploitation of both endogenous and industrial enzymes is imperative. This is only possible if rapid, reliable and specific enzyme assay procedures are available. Many of the procedures currently in use in the cereals and industrial microbiology industries are based on outdated and tedious technologies. There is an immediate requirement for further research, development and education in this area.

It is interesting to note that with the advent of genetic engineering, the technology available for the large-scale production of specific enzymes has actually outpaced the development of technologies for the accurate and reliable measurement of the enzyme activity.

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