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# Pullulan from Agro-industrial Wastes

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## Introduction

Extracellular polysaccharide production is encountered in many microorganisms. Many strains of bacteria, yeasts and fungi have been selected and are used commercially because they have been found to produce enough extracellular polysaccharides in broth culture to be of economic interest. There are two different types of extracellular polysaccharides, homopolysaccharides such as dextrans and levans which contain one type of monomeric unit, and heteropolysaccharides which contain two or more different monomeric units such as hyaluronic acid.

Pullulan is a homopolysaccharide of industrial interest and economic importance produced by the yeast-like fungus *Aureobasidium pullulans*. It was first reported by Bernier (1958) and its structure was elaborated by Bender *et al.* (1959). It is a glucan composed mainly of maltotriose units linked by α-1,6-glycosidic linkages (Le Duy *et al.*, 1983; Shin *et al.*, 1989). The presence of maltotetraosyl units has also been reported (Catley and Whelan, 1970; *Figure 1*). The molecular weight varies greatly from 50,000 up to $40x10^6$ in different strains (Fujii and Shinohara, 1987; Pollock *et al.*, 1992).

Pullulan has many industrial applications (Yuen, 1974; Hayashibara bulletin, 1990; Krochta and De Mulder-Johnston, 1997). It can be used as a partial replacement for starch ingredients as a low calorie viscosity enhancer, binder and quality improver of foods. It finds uses as an oxygen-impermeable food packaging material preventing oxidation and retaining the aroma, flavour and freshness of food and prolonging shelf life.

Pullulan dissolves readily in cold water and forms a viscous solution, but does not gel. By drying in hot air, the solution forms transparent water soluble films which are odourless, tasteless and oxygen impermeable. The strength of pullulan films compares

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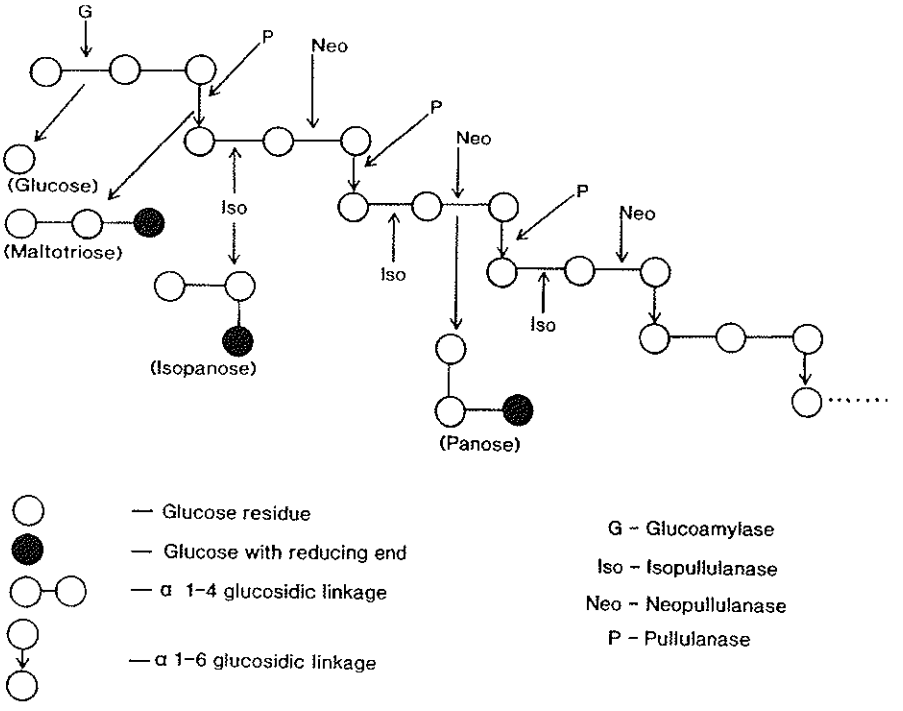


**Figure 1.** Structure of pullulan and sites of enzymatic hydrolysis.

with polystyrene, but with the advantage that they are edible and biodegradable (Kaplan *et al.*, 1993). They are also heat sealable, oil resistant and printable. Due to these properties, pullulan can help solve the recycling problem by replacing the aluminium foil layer in paperboard drinks cartons. Additionally, pullulan-film used for packaging meat or vegetables can be readily dissolved on addition of hot water. Another similar application is a water-soluble sugar packet for coffee and tea bags. In the pharmaceutical industry, pullulan is used as a bulking agent for tablets and as an encapsulating agent for soft and hard capsules. The solubility of capsules can be retarded by chemical modification of pullulan so as to release their content in different gastro-intestinal locations.

Pullulan can also be used as an adhesive, as a gelling agent, moisture retainer and stabilizer in cosmetics, as a strong film former in paints, as a coating material to prevent glass bottles shattering and as a contruction material. It can also be formed into a fibre. Pullulan has some uses in the fertilizer industry. It (or its derivatives) is added in amounts up to 50% (w/w) to the fertilizers to provide a higher water solubility (Matsunaga *et al.*, 1976).

The market price of food and pharmaceutical grade pullulan is about 50 Pounds Sterling per kg. The current world supply (circa 10,000 tonnes pa) is produced almost exclusively by the Japanese company Hayashibara Co. Ltd., Okayama, Japan.

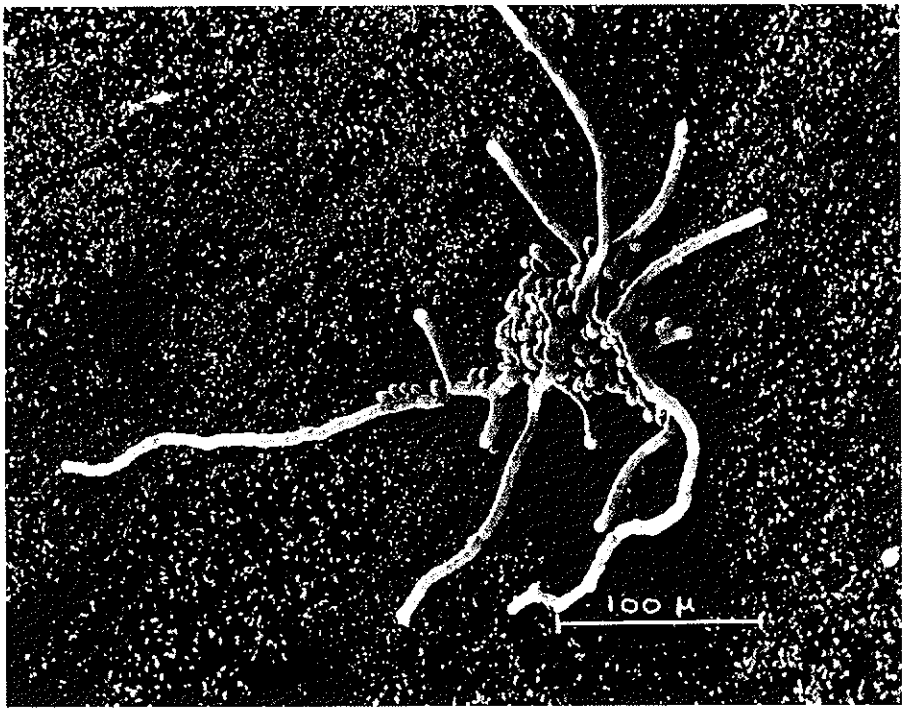


**Figure 2.** *Aureobasidium pullulans.* Scanning electron micrograph showing chlamydospores originating from the hyphal wall.

## Pullulan Production by *Aureobasidium pullulans*

The production of pullulan depends on the fermentation parameters, the morphological state and the fungal strains. *A. pullulans* (de Bary) has been placed in the *Deuteromycota* (Fungi Imperfecti) as its sexual stage has not been established (Cooke, 1959; Hudson, 1965). It is considered to be a 'black yeast' (Hermanides-Nijhof, 1997; de Hoog and McGinnis, 1987). The existence of a perfect stage of *A. pullulans* has been a matter of controversy, and the organism is placed by some in the *Ascomycetes* and by others in the *Basidiomycetes.*

It is polymorphic in that it generally appears as a unicellular yeast-like organism in liquid media and as a mycelium on solid media. The *Aureobasidiaceae* are characterised by the presence of thick-walled spores (chlamydospores or radulospores) produced on minute spicules projecting from the hyphal wall. (*Figures 2* and *3*). Furthermore, there is more than one spore type, namely blastospores (conidia), and resting forms, swollen cells and chlamydospores some of which occur in pairs (*Figure 4*). A pH range of 3.6–5.2 and a low nitrogen to carbon ratio facilitates the conversion of blastospores to swollen cells and chlamydospores (Kockova-Kratochilova *et al.*, 1980).

Although there is no general agreement as to the role that the various morphological stages play in pullulan production, there seems to be a consensus that the main

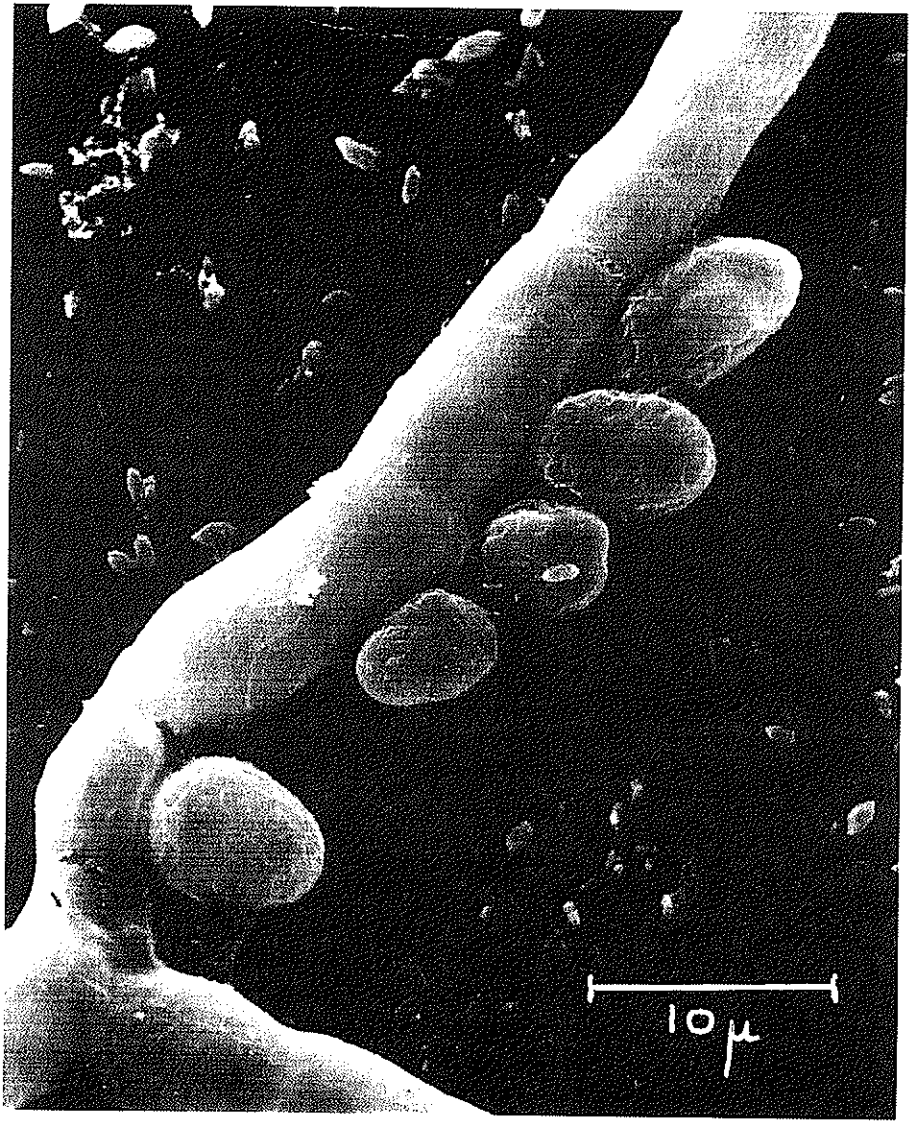


**Figure 3.** *Aureobasidium pullulans.* Hypha with chlamydospores (close up).

morphological types responsible for the elaboration of pullulan are the chlamydospore and swollen cell (Simon *et al.*, 1993; Simon *et al.*, 1995). The factors affecting shifting from one morphological type to another are not quite clear but there are reports that it can be related to inoculum size; a low inoculum yielding exclusively mycelium and a high inoculum giving rise to spore types (Catley, 1980; Ramos and Garcia-Acha, 1975).

*A. pullulans* produces a black pigment, melanin, with a characteristic absorption peak at between 400–600 nm. It is a secondary metabolite produced at the end of exponential growth.

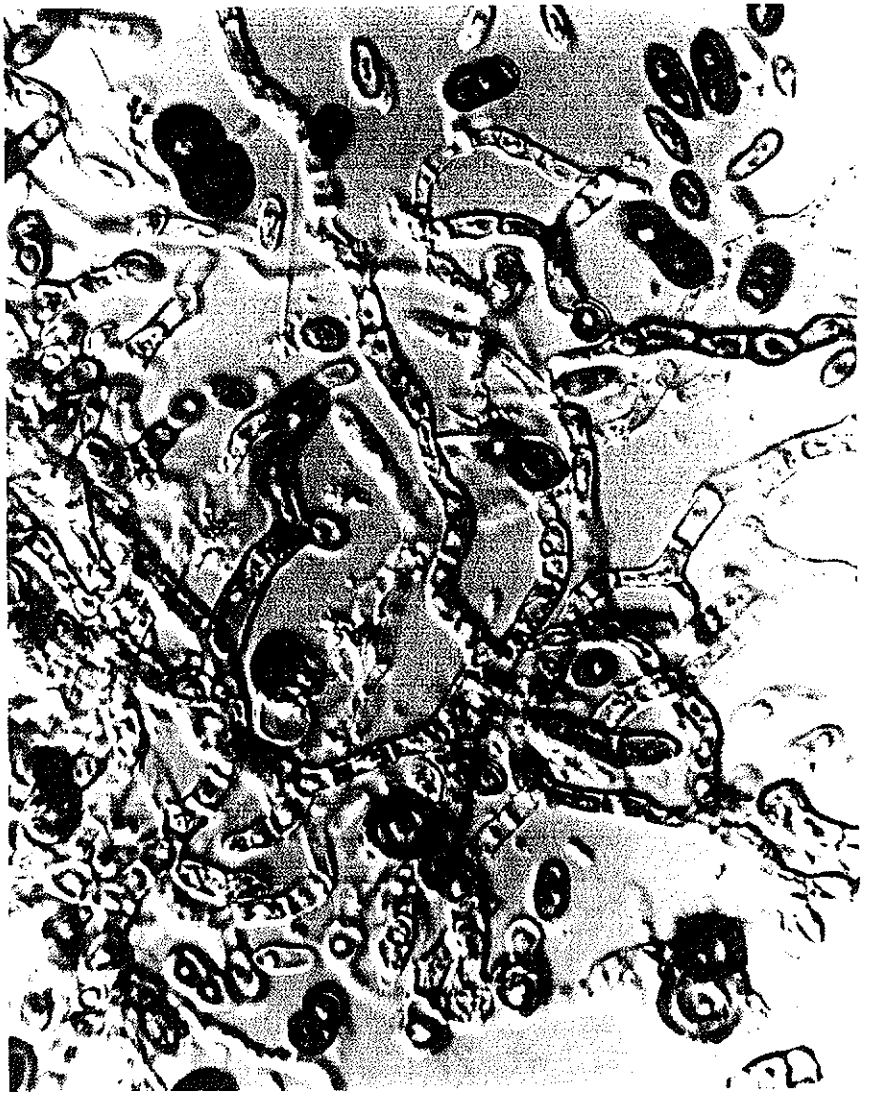

**Figure 4.** *Aureobasidium pullulans*, under Nomarski microscopy (x 1600), showing hyphae, blastospores, swollen cells and chlamydospores mostly in pairs.

The pigment is released into the growth medium where it covalently binds to pullulan, which is undesirable industrially. The role of melanin is not clear, but it may have a protective function against UV radiation, heavy metal toxicity, free radical formation and enzymatic attack. Melanisation is also thought to increase the susceptibility of plants to attachment and penetration by fungi. Fungal melanin differs from animal melanins in that it is not synthesised from tyrosine but from acetate via the polyketide biosynthetic pathway (Siehr, 1981). This pathway may be blocked by several antifungal compounds.

The formation of melanin and the morphology of the organism depends strongly on

cultural conditions used (medium composition, temperature, light, etc.) (Lingapa *et al.*, 1963; Wickerham and Kurtzman, 1975). Current studies also indicate that the production of melanin in the fermentation broth is related to dissolved oxygen tension. It has been observed that in stirred tank reactors where the dissolved oxygen is limited, pigment develops very late in fermentation and sometimes not at all (Rho *et al.*, 1988). The production of pigment-free pullulan has also been reported by maintaining the *A. pullulans* cells in the yeast form in a two stage fermentation (Shabtai and Mukmenev, 1995).

*A. pullulans* utilises a variety of monosaccharides as carbon sources (e.g. xylose, glucose, fructose). Among disaccharides, sucrose and maltose were better substrates than trehalose, while lactose gave poor growth (Cooke and Matsura, 1963). *A. pullulans* cannot utilize cellulose. Of the polysaccharides on which it can grow, it grows best on dextrins, followed by starch, pectin and inulin. The organism is capable of utilizing cutin, grows excellently on tannic acid and has enzymatic activity (polyphenol oxidase) against lignin (Cooke, 1958). Furthermore, it has the ability to produce a range of important enzymes, including glucoamylase, sucrase, xylanase, pectolytic enzymes, amylases, urease, DNase and RNase (Deshpande *et al.*, 1992). This makes it a serious threat to paints and plastic surfaces. *A. pullulans* was also shown to be a potential microorganism for the production of single cell protein for animal feed, being classified as risk Group I by the World Health Organisation. The palatability (acceptability) as well as digestibility and protein content of straw was enhanced by acid hydrolysis, followed by ammonification of the acid treated straw and fermenting it with *A. pullulans* (Israilides *et al.*, 1981). *A. pullulans* grew well with asparagine, $(NH_4)_2\ SO_4$, $NH_4NO_3$, $KNO_3$, $NaNO_2$ or urea as a nitrogen source (Israilides, 1979). Pullulan production, however, occurs under nitrogen limiting conditions, and high levels of nitrogen favour the production of biomass. It has been reported that pullulan synthesis proceeded only when all the available $NH_4^+$ had been utilised (Catley, 1971), while the presence of $NH_4^+$ inhibited polysaccharide production independently of pH (Seviour and Kristiansen, 1983).

It can grow over a wide range of pH from 1.9 to 10.1 and temperatures from 14° to 37°C (Han and Anderson, 1975). Furthermore, supplementation of $K_2HPO_4$, $NH_4NO_3$ and yeast extract to a certain level had a favourable effect in pullulan yields (Israilides, 1998). Fermentation parameters which influence pullulan production, (e.g. temperature, pH, $DO_2$ agitation, carbon and nitrogen source, etc.) have been studied extensively by many investigators (Catley, 1971; Imshenetsky *et al.*, 1981; Mc Neil and Kristiansen, 1987; 1990; Madi *et al.*, 1996; Wecker and Onken, 1991; West and Reed-Hamer, 1991; Reeslev *et al.*, 1991; Auer and Seviour, 1990; Gibbs and Seviour, 1996; Lacroix *et al.*, 1985).

In general, pullulan is best produced under nitrogen limiting conditions and at a pH value between 5.0 and 6.5; below pH 3 pullulan production is inhibited (Catley, 1971). Most researchers agree that pullulan synthesis is favoured under aerobic conditions and high oxygen transfer rate (Rho *et al.*, 1988; McNeil and Kristiansen, 1987; Moscovici *et al.*, 1996). It has been shown (Rho *et al.*, 1988) that when aeration was stopped, pullulan production also ceased, but synthesis continued on the resumption of aeration. In the same paper it was reported that levels of 10% dissolved oxygen tension (DOT) gave good yields of pullulan and that with further increases in DOT there was a decrease in pullulan production. There are other reports suggesting that

beyond certain levels of DOT pullulan synthesis may be reduced, although this may be strain specific (Imshenetsky *et al.*, 1981; Wecker and Onken, 1991).

**Pullulan assays**

Pullulan is usually precipitated from the cell-free fermentation medium by the addition of two volumes of ethanol or propanol. The precipitated material is refererred to as the agglutinating substance. Different laboratories have used a variety of analytical methods to assess the purity of pullulan produced in this way. Simply weighing the agglutinating substance or measuring total sugars (Dubois *et al.*, 1956) is very unreliable. Hydrolysing the agglutinating substance with pullulanase and determining the maltotriose produced by chromatography, radiometry or other method (Catley, 1971; Finkelman and Vardanis, 1982) is a far better way, but sometimes can be very time consuming. Good resolution and sensitivity can be achieved using high performance anion exchange chromatography (HPAEC) quantified by a pulsed amperometric detector (PAD) (Israilides *et al.*, 1998) or by capillary electrophoresis, a method which was shown to be very powerful in carbohydrate separations (Hertmentin *et al.*, 1994; Barnett *et al.*, 1998).

Detection of oligosaccharides like maltotriose eluted from HPLC columns is the biggest challenge and weakest link in the analysis of oligosaccharides. Non-specific detectors such as the refractive index (R.I.) are used routinely, but their lack of sensitivity and restriction to isocratic elution severely limits the development of improved techniques. Low wavelength UV detection (<210 nm) has been shown to have comparable sensitivity to HPAEC-PAD whilst allowing the use of limited gradient elution, but requires ultra-pure solvents. The use of HPAEC-PAD offsets the above drawbacks.

A coupled enzyme assay method (CEA) was developed for the estimation of pullulan in ethanol agglutinating substances (Israilides *et al.*, 1994a). The assay involves the concerted action of pullulanase, amyloglucosidase and glucose oxidase. The combined action of these enzymes allows pullulan to be measured spectrophotometrically. The rate of colour development was correlated to the concentration of pullulan. *Figure 5* shows the Lineweaver-Burk kinetic plot which depicts the correlation of the rate of such colour development with the concentration of pullulan. Similar correlations were achieved with other kinetic plots.

The CEA can differentiate between pullulan and highly branched polyglucans by repeating the assay omitting the pullulanase. The reaction rate is now dependent on the number of non-reducing ends, which in turn largely depends on the degree of branching. Thus, linear polysaccharides such as pullulan give very low rates, while highly branched polysaccharides will give high rates. High values (>10) for the ratio of the rate of coupled enzyme reaction to amyloglucosidase reaction rate (A/B) indicate a linear polyglucan with predominately α-1,4- and α-1,6-glycosidic bonds. The reproducibility of the CEA was good with a standard error of ±5%, based on reference pullulan from Sigma.

Simon *et al.* (1993) used the number of residues in repeating units (ratio of glucose produced by total hydrolysis to the reducing sugar produced by hydrolysis with pullulanase) as a means of differentiating pullulan from other polysacccharides produced in the broth. Under various cultural conditions, the number of residues in repeating units ranged from 3.2 to 4.2 for pullulan.

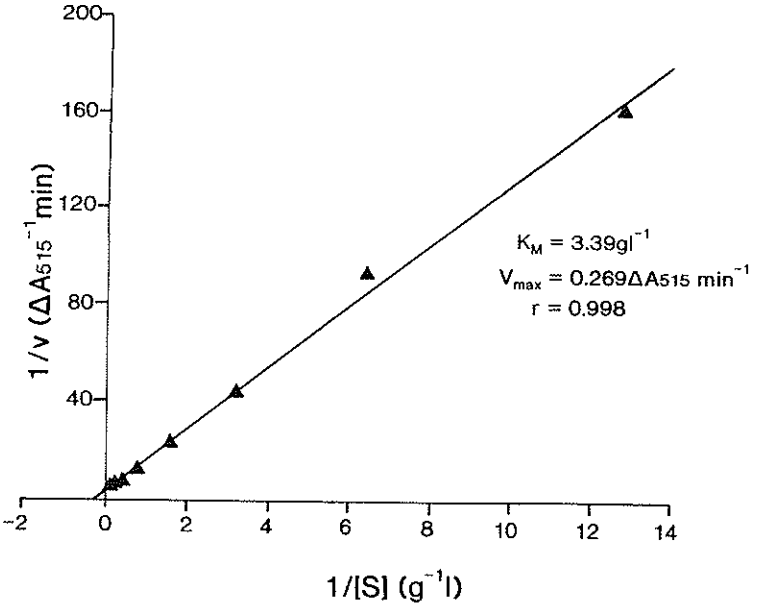


**Figure 5.** Lineweaver-Burk kinetic plot in pullulan hydrolysis with the coupled enzyme assay.

*A. pullulans* elaborates polysaccharides other than pullulan. One of these polysaccharides isolated by Hamada and Tsujisaka (1983), was a β-1,3 polyglucan with single glucose residues attached β-1,6 every third or fourth residue along the backbone of the polysaccharide.

## Molecular weight determination

The molecular weight of pullulan is important in terms of its industrial applications. The molecular weight of pullulan produced by *A. pullulans* is dependent on pH (Lee and Yoo, 1993), phosphate content of the medium (Hayashibara, 1990), impeller speed (McNeil and Kristiansen, 1987) and the carbon source and age of the culture (Israilides *et al.*, 1994b). Furthermore, there are occasional maltotetraose units incorporated randomly within pullulan. These represent sites particularly susceptible to endoamylase catalysed hydrolysis. Hence, there is a relationship between the fine structure of pullulan and the molecular weight (Catley, 1970; Carolan *et al.*, 1983).

When different substrates from various agro-industrial wastes were used, the pullulan produced differed in molecular weight, purity and other physicochemical properties (Israilides *et al.*, 1994b). For example, grape skin pulp, hot water extracts and hydrolysed starch waste proved to be good substrates for pullulan production. Molasses and olive oil wastes produced small amounts of agglutinating substances, heterogeneous in composition and with very small amounts of pullulan.

## Biosynthesis of pullulan

Significant progress has been made in recent years in elucidating the pathways of

polysaccharide biosynthesis. Similar progress has not yet been made in determining the pathway for pullulan biosynthesis in *Aureobasidium pullulans*. Taguchi *et al.* (1972) reported the biosynthesis of pullulan from both cell-free preparations and acetone-dried cells. Frozen cells were disrupted by grinding with alumina, the debris removed by centrifugation and the supernatant precipitated with 80% saturated ammonium sulphate. This protein precipitate catalysed pullulan biosynthesis from uridine-5-diphosphoglucose (UDPG) and ATP giving a yield of 54% of pullulan with respect to the UDPG added. In the absence of ATP, no pullulan was formed and neither sucrose nor ADPG could act as substrates. The acetone dried cells when resuspended were incubated with sucrose in a phosphate buffer produced pullulan. When $^{14}$C-labelled sucrose was used, $^{14}$C activity was found in the lipid fraction (chloroform-methanol extract). An aliquot of this extract was dried under nitrogen, hydrolysed under acid conditions and partitioned between chloroform and an aqueous phase. Most of the radioactivity (85%) was found in the aqueous phase and was shown to be glucose by paper chromatography. The properties of this glycolipid suggested that it was lipid-pyrophosphate-glucose. Under conditions where pullulan synthesis was taking place, the $^{14}$C-labelled glycolipid pool was turned over rapidly. The authors suggested that lipid intermediates were involved in pullulan biosynthesis. Catley and McDowell (1982) also showed the incorporation of $^{14}$C-labelled glucose into glycolipid, pullulan and other extracellular polysaccharides. The glycolipid extract was separated into three fractions by chromatography on DEAE-cellulose. They chacterised the glyco moeity in the second fraction as a mixture of glucose, isomaltose, panose and isopanose. Since cell wall saccharides do not contain α–(1,4)- or α-(1,6)-D-glucosidic bonds and glycogen biosynthesis does not involve a glycolipid intermediate (Finkelman and Vardanis, 1987), it was proposed that these glycolipids were intermediates in pullulan biosynthesis. The lipid involved was assumed to be a polyprenyl phosphate of the dolichol type partly on the basis of its stability in alkaine solution (Hemming, 1974) and the isolation of polyprenyl-linked saccharides from both *Saccharomyces cerevisiae* (Lehle and Tanner, 1978) and mammalian liver (Behrens and Tabora, 1978).

In a study of the oxygen-dependence of growth and pullulan biosynthesis by *Aureobasidium pullulans,* Rho *et al.* (1988) found that both were absolutely dependent on oxygen. In terms of pullulan biosynthesis, oxygen may be required to recycle the UDPG and ATP that Taguchi *et al.* (1973) showed were required for pullulan biosynthesis in their cell-free preparation. Studies by Rho *et al.* (1988) with *Aureobasidium pullulans* in non-growth media (lacking nitrogen) showed that both biomass and pullulan increased with time (biomass increasing from around 3 to 15 $g.l^{-1}$ over 40 hours and up to 10 $g.l^{-1}$ of pullulan was synthesised over the same time). The increase in biomass was accompanied by an increase in the carbon to nitrogen ratio indicating that the cells were storing some carbonaceous compound(s). Based on an analysis of their data, they proposed that there were two pathways involved in pullulan biosynthesis in nitrogen-free media. Part of the glucose taken up by the cell was converted directly to pullulan, the remaining glucose accumulated within the cell and could be converted to pullulan by a different pathway. Simon *et al.* (1995) showed that glycogen and lipidic granules were concentrated at the plasmalemma in swollen cells actively synthesising pullulan. They proposed that glycogen might act as the source of 1,4-linked glucose units and the lipid granules the source of the lipid for glycolipid synthesis. These glycolipids would then be transported across the plasma membrane

and act as precursors for pullulan biosynthesis.

Catly and McDowell (1982) and Barnett (1997) were unable to repeat the work of Taguchi *et al.* (1972). Like Finkelman and Vardanis (1987), we found that the 100,000g pellet from cell-free preparations of *Aureobasidium pullulans* was active in glycogen synthesis but there was no evidence for pullulan synthesis in this membrane-enriched preparation. Cell-free preparations of *Aureobasidium pullulans* can be prepared that are active in pullulan biosynthesis but efforts to purify this activity have so far failed (Barnett, 1997).

Given that the role of lipids in peptidoglycan, glycopeptides and oligosaccharides is well established (Bugg and Brandish, 1994), the genetics and enzymology of polysaccharide biosynthesis have been studied extensively (Glucksman *et al.*, 1993; Keller *et al.*, 1995; Villar-Palasi and Guinovart, 1997) and transformation of *Aureobasidium pullulans* reported (Cullen *et al.*, 1991; Thornwell *et al.*, 1995), our understanding of pullulan biosynthesis should improve significantly in the near future.

## Production of pullulan from agro-industrial wastes

Pullulan can be synthesised from a variety of carbohydrate substrates incorporated into either defined (synthetic) or non-defined media. Within the latter are several agro-industrial wastes which have been shown to be suitable for pullulan production (Israilides *et al.*, 1993; Le Duy and Boa, 1982; Le Duy *et al.*, 1983; Shin *et al.*, 1989; Zajic *et al.*, 1979). Utilisation of these substrates would seem to be ecologically sound and economically advantageous as they have low or even negative costs. In this way, regional development seems to be feasible with the creation of small or medium enterprises (SME's), which will give rise to new jobs and protect the environment from pollution.

Among the substrates reported as potentially suitable for pullulan production were hemicelluloses in waste water from the production of viscose fibres (Biely *et al.*, 1978), peat hydrolysate, which is the liquid resulting from the heat treatment of raw peat in an acid solution (Le Duy and Boa, 1983), spent sulphite liquor (Zajic *et al.*, 1979), starch waste and corn glucose syrup (Hayashibara Inc., 1990), olive oil wastes, grape skin pulp extract, molasses (Israilides *et al.*, 1993, 1994b; Bambalov and Jordanov, 1993), carob pod extract (Roukas and Biliaderis, 1995).

Although fermentation parameters for pullulan production have usually been studied with defined substrates such as glucose and sucrose, the results from agro-industrial wastes as substrates have shown that pullulan can be produced in yields similar or better to those obtained with conventional substrates (Israilides *et al.*, 1998). However, it has been shown (Israilides *et al.*, 1994a; 1994b) that the purity of the pullulan in the crude precipitated substance may vary widely with the different carbon substrate and fermentation conditions. Furthermore, pullulan produced in such fermentations is often characterised by heterogeneity of both composition and molecular weight (Israilides *et al.*, 1994b).

### Starch waste

Waste potato starch from the manufacture of potato crisps or from other potato processing industries (e.g. frozen pre-fried potatoes) is a fairly homogeneous substrate

and relatively free from extraneous material. Certain strains of *A. pullulans* possess starch degrading enzymes (Saha and Bothast, 1993). However, this activity was greater against linear α-1,4-glucans, but very little if any against polysaccharides with α-1,6-linkages. Therefore, in order for starch waste to be considered a good substrate for pullulan fermentation, it must be partially hydrolysed.

Studies using starch waste as a substrate revealed that *A. pullulans* showed preference for oligosaccharides over glucose when it was grown for pullulan production; in fact, the yield of pullulan was dependent on the degree of hydrolysis of starch or dextrose equivalent (DE). This is an indication of total reducing sugar as a percentage of glucose. Unhydrolysed starch has a DE of zero and D-glucose has a DE of 100. The optimum DE was around 55% for pullulan yield, (Barnett *et al.*, 1998). Using hydrolysed starch waste as a substrate, it was also found that the pullulan content of the agglutinating substance increased during the course of fermentation and reached more than 90% (w/w) on the sixth day. This becomes important in determining the optimum time for the production of the highest amount of pullulan.

### Grape skin pulp

Grape skin pulp is a by-product of the wine industry and amounts to thousands of tonnes in wine producing countries. Some of this waste material goes for animal feed but most of it is disposed of as waste. Hot water extracts of the pulp can serve as a good substrate for the production of pullulan. Pullulan has been produced in high yield, with a high molecular weight ($4.22 \times 10^6$) and was pure as assessed by its gel elution profile, glucose content and the number of residues in repeating units (Israilides *et al.*, 1994b). Hence, this is a good substrate for pullulan production.

### Olive oil wastes

Olive oil wastes are effluents from the mills producing olive oil. It is considered a major pollutant and causes great problems in olive tree cultivation areas in many Mediterranean countries. Fresh waste is particularly phytotoxic, mainly due to the presence of phenolic compounds (Israilides *et al.*, 1997). At present, there is not an ecological or economic solution for the problem of these wastes. Therefore, many biological and physichochemical methods are used for its treatment, depending on local conditions and various other factors.

Although many microorganisms are growth-inhibited by oil waste because of the presence of phenols, *Aureobasidium pullulans* is able to grow in it to produce pullulan (Iniotakis *et al.*, 1991; Israilides *et al.*, 1993). It has been observed that *A. pullulans.* when grown in the effluent, can reduce the phenolic content by 41% in six days. The pullulan concentration of the ethanol agglutinating substance, however, increased when phenols were removed, indicating that their presence plays an inhibitory role in pullulan production.

The yield of pullulan was very low, 2–3% (w/w) of the agglutinating substance, based on the coupled enzyme and HPAEC (PAD) assays (Israilides *et al.*, 1994b).

### Molasses

Fermentation using molasses as a substrate produced mainly low molecular weight

($0.55 \times 10^6$ Da) material and the amount of pullulan produced was about 5–8% (w/w) of the agglutinating substance (Israilides *et al.*, 1994b). Given the fact that molasses is used widely as a substrate for many industrial fermentations for the production of high value compounds, the potential use of it for the production of pullulan would depend on purification and separation costs, as well as other marketing factors.

## Carob pod

Carob tree (*Ceratonia siliqua*, L.) grows naturally on barren soils which are unproductive for most crops. It is found in most warm regions of the Mediterranean area and other countries having a similar climate (Rhodesia, parts of USA, Australia, South America, India and Philippines). The ripe de-seeded carob pod, which contains high levels of tannins, is partially utilised for the production of health confections as a cocoa substitute. It is also used as animal feed, although its high tannin content minimizes the pod's nutritional value. Due to difficulties and high harvesting costs, most carob beans are left unutilised.

The carob pod contains high amounts of water soluble sugars (40–60%) and can serve as a substrate for pullulan production. The yields and fermentation efficiencies reported were 30% and 89% respectively (Roukas and Biliaderis, 1995). However, the pullulan concentration based on the coupled enzyme assay, HPAEC-PAD assay, was low and, in most cases, under 2%. Further, the low A/B ratio in the CEA and the unfavourable number of residues in the repeating units is not in accord with the polysaccharide being pullulan (unpublished data).

## Concluding remarks

Pullulan is an edible and biodegradable polysaccharide with numerous commercial applications and relatively high market value. Current research shows that the production of pullulan from agro-industrial wastes is feasible and ecologically sound. Utilization of agro-industrial wastes for the production of an industrially important biopolymer, pullulan, is both challenging and reasonable. Improvements in both yields and purity of pullulan from fermentations from agro-industrial wastes could be achieved by admixture of various wastes in an effort to improve the C/N ratio of the substrate. Improvements in production practices, economies of scale and selection of the proper substrate and strain of *A. pullulans* could all be developed to produce a more favourable product, while protecting the environment from pollution.

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