65th JECFA - Chemical and Technical Assessment (CTA) 2005

PULLULAN

Chemical and Technical Assessment (CTA)

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1 Summary

Pullulan is a polysaccharide produced by a yeast like fungus *Aureobasidium pullulans*. Pullulan is an essentially linear glucan consisting mainly of 1,6-linked maltotriose and some interspersed maltotetraose units.

Pullulan has not previously been evaluated by JECFA but has been recommended for priority evaluation by CCFAC.

The commercially available pullulan (Pullulan PI-20) has a purity of more than 90 %. Its average molecular weight (at peak of a gel permeation chromatogram) is about 200 kD. The main impurities are mono-, di- and oligosaccharides, which are carried over from the raw material (hydrolysed starch) into the final product. The specifications for Pullulan include standard parameters for identification and for chemical and microbiological purity.

The film-forming properties of pullulan are the basis for its proposed use as a substitute for gelatin in the production of capsule shells (for dietary supplements), as an ingredient of coated tablets (dietary supplements), and as an ingredient of edible flavoured films (breath fresheners). It has been used as an additive and as a food ingredient in Japan since 1976.

Specifications for Pullulan were prepared at 65th JECFA (2005) and will be published in FNP 52 Add 13.

2 Description

2.1 Chemistry and nature of the product

The name "pullulan" was proposed by Bender, who was the first to describe the formation of this extra-cellular polysaccharide by *Aureobasidium pullulans* (syn. *Pullularia pullulans*) (Bender *et al*., 1959). It is essentially a linear polymer of repeating maltotriose units linked by α-1,6 glycosidic bonds. Depending upon the culture conditions (duration, pH, phosphate concentration, etc.) under which this extra-cellular glucan is elaborated by *Aureobasidium pullulans*, the molecular weight varies from about 10 to 3 000 kDa (Sugimoto, 1978; Wiley *et al*., 1993; Gibbs & Seviour, 1996; Madi *et al*., 1997; Lazaridou *et al*., 2002).

“Pullulan PI-20” is the brand name where "P" stands for "pullulan", "I" for "deionized" and the figure 20 designates the number-average molecular weight (M_n) of about 200 kDa (Okada *et al*., 1990; Nakamura, 1984). The subject of the present CTA is pullulan (Pullulan PI-20) with a number-average molecular weight (M_n) of about 100-200 kDa and a weight-average molecular weight (M_w) of about 362-480 kDa (Okada *et al*., 1990).

The INS No. of pullulan is 1204, the CAS No. is 9057-02-7, and its chemical formula is $(C_6H_{10}O_5)_n$. For Pullulan PI-20, n corresponds to about 1250 glucose units on the basis of M_n. The structural formula of pullulan corresponds to:

$[\alpha\text{-D-Glc}_p\text{-}(1\rightarrow4)\text{-}\alpha\text{-D-Glc}_p\text{-}(1\rightarrow4)\text{-}\alpha\text{-D-Glc}_p\text{-}(1\rightarrow6)]_n$

2.2. Natural vs. synthetic origin

Pullulan is a naturally occurring, fungal exopolysaccharide produced by *Aureobasidium pullulans.* The organism is ubiquitous. It is found in soil, lake water, on the surface of latex paint films, synthetic plastic materials, shared-used cosmetic and foods such as cereals, fruits, cheese and tomato (Vadkertiova 1964, Zabel *et al*., 1980, Webb *et al.*, 1999, Mislivec *et al.*, 1993). Because it forms a black pigment (melanin), this organism is also known as "black yeast" (Cooke, 1961, Durrell, 1967, Domsch *et al*., 1993; Gibbs & Seviour, 1996). Pullulan is produced on an industrial scale by fermentation of liquefied starch under controlled conditions using a specific, not genetically modified, non-pathogenic and non-toxigenic strain of *Aureobasidium pullulans*.

3 Method of manufacture

3.1 Principle

Pullulan is produced commercially by mesophilic (22-30°C) fermentation of hydrolysed starch with a selected non-toxigenic strain of *Aureobasidium pullulans* (Yuen, 1974; Leathers, 2003; Shingel, 2004) and further purification of the product. Pullulan is formed extracellularly when the cells are in the late log phase and stationery phase of growth (Catley, 1971) and its formation is dependent on a various factors including pH, temperature, substrate and strain (Catley, 1971, Yuen, 1974, Madi *et al*, 1997, Gibbs and Seviour, 1996, Lazaridou *et al*. 2002, Sugimoto, 1978, Ueda *et al.* 1963, 1966). The yield and molecular weight of pullulan can be adjusted by manipulation of the substrate and fermentation conditions.

3.2 Detailed description

The manufacturing process is conducted under conditions of good manufacturing practices and uses raw materials and processing aids that comply with food grade specifications. Pullulan is produced by mesophilic fermentation of starch syrup by the selected non-toxigenic strain of *Aureobasidium pullulans.* The strain has been selected by traditional techniques, i.e. the strain is not the product of genetic modification using recombinant technologies. The production strain has a high yield of pullulan, low production of melanin and does not produce aureobasidin A.

After completion of the fermentation, the fungal cells are removed by microfiltration. The cell-free filtrate is heat-sterilized and treated with activated carbon to remove pigments and other impurities by adsorption. The decolourized filtrate is cooled and deionized using cation and anion exchange resins. The deionized solution is concentrated to a solids content of about 12 %, treated a second time with activated carbon, and filtered using diatomaceous earth as a filter aid. The filtrate is concentrated by evaporation to a solids content of about 30 % and dried in a drum dryer. The dried pullulan is pulverized to a specified particle size and packed in sterilized polyethylene bags.

4 Chemical characterization

4.1 CComposition of pullulan

Pullulan is an essentially linear polysaccharide (glucan) consisting predominantly of repeating maltotriose units. The maltotriose units, which consist of three 1,4-linked glucose molecules, are linked by α-1,6-glycosidic bonds. This repeating sequence forms a stair-step-type structure (Figure 1).

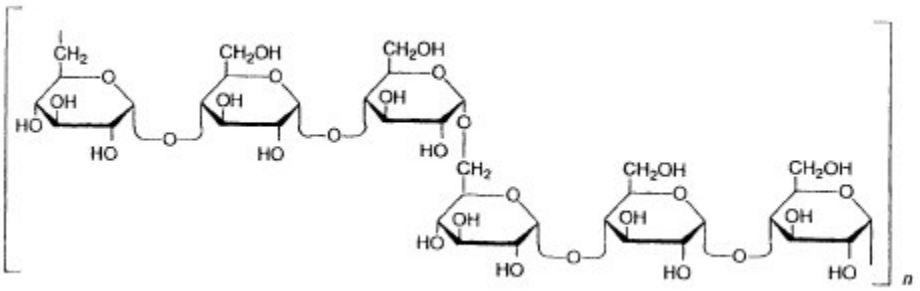

Figure 1. Structural formula of pullulan

Maltotetraose units consisting of four 1,4-linked glucose molecules also occur, probably randomly, but are rarer (about 6 %) (Wallenfels *et al*., 1965, Catley, 1971; Carolan *et al*., 1983). There is also evidence for a rare occurrence of branching points where poly-maltotriosyl side-chains are attached to the main chain by a 1,3-glycosidic bond (Catley *et al*., 1986; Sowa *et al*., 1963).

4.2 Physicochemical properties

Pullulan PI-20 is a white to off-white, tasteless and odourless powder. It is not hygroscopic. According to the specifications provided by the sponsors it contains less than 6 % water. Pullulan dissolves readily in cold or hot water, but is insoluble in organic solvents, except dimethylformamide and dimethylsulfoxide (Sugimoto, 1978; Tsujisaka & Mitsuhashi, 1993). Aqueous solutions of pullulan are viscous but do not form gels. The viscosity (10% w/w, 30°C) of ten batches of pullulan PI-20 was 132-179 mm^2/s. The viscosity of pullulan solutions resembles that of gum acacia (gum arabic) solutions, i.e. the viscosity of pullulan is rather low in comparison with that of other soluble polysaccharides, such as guar gum (Sugimoto, 1978; Tsujisaka & Mitsuhashi, 1993). Differences in the pH or salt content do not substantially affect the viscosity of pullulan solutions (Sugimoto, 1978). The viscosity of an aqueous solution of Pullulan PI-20 decreases upon incubation with pullulanase (EC 3.2.1.41). An aqueous solution of Pullulan PI-20 (10% w/w) has a pH of 5.0-7.0.

Molecular weight gel permeation chromatograms of three batches of Pullulan PI-20 showed a molecular weight at the peak of the chromatogram of 173000-186000 Da with a number-average molecular weight (M_n) of 96900-101000 Da and a weight-average molecular weight (M_w) of 433000-479000 Da.

Pullulan is structurally closely related to starch amylopectin and maltodextrin. All three carbohydrates consist of glucose units linked by α-1,4 and α-1,6-glucosidic bonds. Maltodextrin contains approximately 20% α-1,6-glucosidic bonds and pullulan approximately 30%. In comparison, corn starch contains 95% α-1,4-glucosidic bonds and 5% α-1,6-glucosidic bonds. Differences between pullulan and these glucans, besides the relative proportions of alfa-1,4 and alfa-1,6 bonds, are the tertiary structure of the molecule and the extent and mechanism of degradation of the materials in the human gut. Pullulan can be classified in the group of soluble fibres.

4.3 Possible impurities (including degradation products)

Pullulan PI-20 has a purity of more than 90% on a dry substance basis. Since the purity of this polymeric substance cannot be determined directly, it is calculated as the difference between 100% and the sum of the percentages of analytically determined known impurities, i.e. mono-, di- and oligosaccharides (determined with anthrone-sulfuric acid reagent (Morris, 1948)) and water. The purity of 10 batches of Pullulan PI-20 was in the range of 91.2- 95.0 %.

The analysis of 10 lots of Pullulan PI-20 reveals a combined content of mono-, di- and oligosaccharides of between 5.0 and 8.7%. Mono- and disaccharides constitute about 30-40 % of these non-pullulan carbohydrates. About 37-42% of the molecules have a degree of polymerization (DP) between 3 and 10. These carbohydrates are derived from the food-grade corn syrup, which is used as the raw material for the production of pullulan.

The analysis of 10 lots of Pullulan PI-20 reveals ash contents of 0.00-0.16 %. The sources of minerals are the food-grade corn syrup raw material and the inorganic salts, which are added to the culture medium (ammonium sulfate, calcium hydroxide, sodium chloride, diammonium phosphate, dipotassium phosphate, sodium glutamate, magnesium sulfate).

The analysis of 10 lots of Pullulan PI-20 using the semi-micro-Kjeldahl method reveals a nitrogen content of 0.002-0.004% (limit of detection: 0.001%). Applying the standard conversion factor of 6.25, this corresponds to a protein content of about 0.01-0.03 %.

Specific analyses for lead revealed values of less than 1 mg/kg, i.e. lower than general JECFA limit of 2 mg/kg. Additional analyses of two different batches of Pullulan PI-20 for Cd, Pb, Hg and As confirmed the high purity of the product.

Other impurities in pullulan could stem either from the starting material (food-grade corn syrup), the fermentative action and metabolism of *Aureobasidium pullulans*, or the cell wall of this microorganism. The purification steps that are included in the manufacturing process of Pullulan PI-20 ensure that such by-products are eliminated. Thus particulate materials from the cell walls are removed by micro filtration and treatment with activated carbon; ionic compounds (e.g. fermentatively produced organic acids) are removed during the deionization step; organic compounds (e.g. melanin, protein) are absorbed during the treatment with activated carbon; and volatile products (e.g. fermentatively formed ethanol) disappear during the final evaporation and drying.

Two batches of Pullulan PI-20 were subjected to detailed microbiological analysis. With the exception of the test for so called 'flat sour spores', none of the applied tests gave a result above the threshold of detection. Flat sour spores were found in one of the two tested batches only at 2 CFU/g. Flat sour spores represent mainly *Bacillus stearothermophilus* and *Bacillus coagulans*. It is likely that these heat-resistant, spore-forming microorganisms originate from the raw material, i.e. corn syrup. The canning industry, for example, accepts sugar with flat sours of up to 7.5 CFU/g.

There was no evidence for the presence of *Aureobasidium pullulans* in ten examined batches. This microorganism would be detected in the tests for yeast and moulds. Analyses for total mesophilic bacteria demonstrate the high microbiological purity of the product.

Some strains of *Aureobasidium pullulans* produce aureobasidin A, which is toxic to fungi and yeast at low concentrations (0.1-0.5 µg/ml). The strain used for the production of pullulan has been checked for aureobasidin production. Using *Saccharomyces cerevisiae* as a sensitive tester strain, it was determined that neither pullulan (samples from six commercial batches) nor the unpurified filtrate of the pullulan culture medium contained aureobasidin-like activity (limit of detection 2 ppm) (Hasimoto & Fukuda, 2002). There are no observations that would indicate that *Aureobasidium pullulans* produces mycotoxins other than aureobasidin. Two batches of Pullulan PI-20 were analysed for the presence of aflatoxins (B_1, B_2, G_1, G_2), zearalenone, sterigmatocystin and ochratoxin. Using standard analytical methods, all batches tested negatively for these mycotoxins.

Tests of two batches of Pullulan PI-20 with a number of tester strains for antibacterial activity gave negative results.

4.4 Analytical methods

The analytical methods for the proposed specifications of pullulan are based on general tests for identity and purity published in Food and Nutrition Paper 5, Rev 2 (FAO, 1991) (solubility, pH, sulfated ash, loss on drying, lead, nitrogen determination, microbiological criteria and spectrophotometry), as well as the determination of mono-, di- and oligosaccharides with Dreywood's anthrone reagent (Morris, 1948) and determination of kinematic viscosity using a Ubbelohde-type (falling-ball) viscometer.

4.5 Rationale for proposed specifications

The specifications proposed for Pullulan are based on the manufacturing process and raw materials and define the composition of the material of commerce. The parameters tested include the identified components of pullulan. Specifications of polymeric carbohydrates take into consideration the heterogeneous composition of these products with respect to chain length, degree of branching, etc. Pullulan is a glucan, i.e. a homopolysaccharide consisting solely of glucose molecules, and the definition of the purity of pullulan as 'not less than 90% of glucan'" is, therefore, consistent with current practice. Batches containing less than 90% of glucan on an anhydrous basis would not meet the proposed specifications. Levels of possible impurities are also included in the specifications to ensure that these levels remain at a minimum and that the article of commerce is identical to that evaluated in the toxicological tests. The lead limit is included in the specifications for safety purposes and is lower than the general limit adopted by JECFA. In addition, analytical data for 10 different manufacturing batches of Pullulan PI-20 indicate that the method of manufacture produces a consistent product and suggests that the finished product produced by the described manufacturing process complies with the proposed specifications.

5 Functional uses

Pullulan is used as a glazing agent, as a film forming agent, as a thickener or as a carrier in the production of capsules for dietary supplements (substitute for gelatin), coatings for coated tablets (dietary supplements), for production of edible flavoured films (breath fresheners), jams and jellies, confectionery and some meat and fruit products. It is also used as a texturizer in chewing gum and as a foaming agent in milk based desserts.

5.1 Technological function

Pullulan forms transparent, water-soluble, fat-resistant, antistatic films of low oxygen permeability (Tsujisaka & Mitsuhashi, 1993; Sugimoto, 1978; Yuen, 1974). Films are usually prepared by rapid evaporation of a 5-10% aqueous pullulan solution applied to a smooth surface and dried. Very thin films (down to 0.01mm) can be made (Yuan, 1974). By admixture of other components, the relevant properties of these films can be modified (Yuen, 1974; Shih, 1996; Biliaderis *et al*., 1999; Diab *et al*., 2001). By compression moulding or extrusion at elevated temperature, pullulan films can be formed to shaped bodies (Hijiya & Shiosaka, 1974). Because of these properties, Pullulan PI-20 can be used as a substitute for gelatin in the production of capsule shells for dietary supplements and medicinal products.

Pullulan-based edible films can also serve as a matrix to hold flavours. Pullulan films with, for example, menthol dissolve quickly on consumption, releasing the bound flavour and thus acting as an instant breath freshener. Because pullulan-based films have a low permeability to oxygen and humidity, pullulan may also be used for the coating of foods in tablet form (dietary supplements). In this application, it protects susceptible ingredients (nutrients, colours, flavours) from deterioration and thus preserves the nutritional and organoleptic quality of the products.

5.2 Food Categories and use levels

Pullulan-based hard or capsule shells may contain 15-90% pullulan. Pullulan-based, flavoured, edible films consumed as breath fresheners may contain up to 90% pullulan. The coating of tablets with pullulan results in products with a content of up to 2% pullulan. Use levels of pullulan in jams and jellies, chewing gum, confectionery and some meat, milk and fruit products are in the range of 0.2 to 5%.

6 Reactions and Fate in Foods

6.1 Stability

The chemical structure and thus the reactivity of pullulan resembles that of maltodextrin and starch, both of which are common constituents of food. Having a large molecular weight, Pullulan PI-20 is essentially non-reducing. It is stable in aqueous solution over a wide pH range (3-8) (Wallenfels *et al*., 1965). Only prolonged heating at pH< 3 leads to a decrease of viscosity which is indicative of hydrolytic depolymerization (Nakamura, 1984).

On dry heating, pullulan decomposes and carbonizes at 250-280°C (Tsujisaka & Mitsuhashi, 1993).

6.2 Chemical interactions with nutrients

Because it does not contain any chemically reactive group, pullulan is not expected to interact chemically with other nutrients in foods. As it is not degraded by the digestive enzymes of the human alimentary tract to a significant extent, pullulan remains intact in the small intestine. Having a low viscosity and a chemical structure lacking anionic or cationic groups at recommended levels of intake, pullulan is not expected to impair the small-intestinal absorption of essential nutrients such as vitamins and minerals (Gordon *et al*., 1995, Gorman & Bowman, 1993, Rossander *et al*., 1992, Kelsay, 1990).

7 References

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