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MINI-REVIEW

Pullulan: biosynthesis, production, and applications

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Abstract Pullulan is a linear glucosic polysaccharide produced by the polymorphic fungus *Aureobasidium pullulans*, which has long been applied for various applications from food additives to environmental remediation agents. This review article presents an overview of pullulan's chemistry, biosynthesis, applications, state-of-the-art advances in the enhancement of pullulan production through the investigations of enzyme regulations, molecular properties, cultivation parameters, and bioreactor design. The enzyme regulations are intended to illustrate the influences of metabolic pathway on pullulan production and its structural composition. Molecular properties, such as molecular weight distribution and pure pullulan content, of pullulan are crucial for pullulan applications and vary with different fermentation parameters. Studies on the effects of environmental parameters and new bioreactor design for enhancing pullulan production are getting attention. Finally, the potential applications of pullulan through chemical modification as a novel biologically active derivative are also discussed.

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Introduction

Bauer (1938) first reported pullulan, which is a water-soluble, neutral polysaccharide and obtained from the fermentation broth of *Aureobasidium pullulans*. Elemental analysis revealed that the content of carbon and hydrogen atoms in this exopolysaccharide have the chemical formula $C_6H_{10}O_5$. This polymer can form complexes with Cu^{2+} and gives no color reaction with I_2 (Ueda et al. 1963). Pullulan is produced by *A. pullulans* as an amorphous slime matter consisting of maltotriose repeating units joined by α-1,6 linkages (Catley et al. 1986; Sutherland 1998). The internal glucose units within maltotriose are connected by a α-1,4 -glycosidic bond (Fig. 1). The molecular weight of pullulan has considerable variety, ranging from 4.5×10^4 to 6×10^5 Da and greatly affected by cultivation parameters (Lee and Yoo 1993). The average molecular weight and molecular weight distribution are both crucial for its bioactivities, such as chemical releasing capability and immuno-modulatory activity (Shu et al. 2007).

With its unique linkage pattern, pullulan demonstrates distinctive physical properties, such as adhesive ability, the capacity to form fibers, and thin biodegradable films, which are transparent and impermeable to oxygen (Yuen 1974). As a result, pullulan has long been used in various applications such as blood plasma substitutes, food, adhesive and cosmetic additives, and flocculants (Rekha and Sharma 2007; Leathers 2003). Although the relatively high price (ca. US $25/kg) may constrain its use in the past, the patent numbers are increasing recently due to the new applications of pullulan related to human health (Leathers 2003).

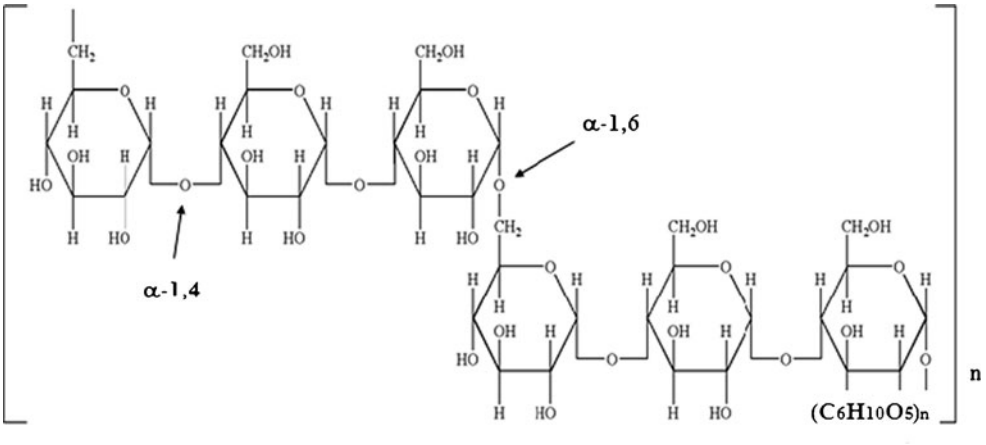

Fig. 1 Structure of pullulan (Yuen 1974)

Current studies concerning the fundamental findings of a pullulan-producing strain, pullulan synthesis, and genetic regulations have been described by Leathers (2003). Shingel (2004), Singh et al. (2008), and Cheng et al. (2009a) also summarized state-of-the-art pullulan production and applications. However, a comprehensive review of pullulan production concerning the upstream genetic regulations, molecular properties, and downstream processes including cultivation parameters, new bioreactor design, and its applications is still needed.

In this article, the main focus is to present concepts, methods, and strategies on pullulan biosynthesis, production, and applications and also to propose future prospects on its possible applications through chemical modification as a novel bioactive derivative for future work.

Pullulan biosynthesis

Mechanism of pullulan biosynthesis

Most of the functions of exopolysaccharides produced by microorganisms are related to the protection of producer microorganism (Kumar et al. 2007). Microorganisms would like to surround themselves with a highly hydrated exopolysaccharide layer, which may protect itself from desiccation and against predation by protozoans. The presence of exopolysaccharide around the cell surface may also affect its diffusion properties (Dudman 1977).

Pullulan is synthesized intracellularly at the cell wall membrane and secreted out to the cell surface to form a loose, slimy layer (Simon et al. 1993). Despite an intensive investigation on the cytological and physiological characteristics of *A. pullulans*, the mechanism of pullulan biosynthesis is not fully understood yet. Duan et al. (2008) have proposed the possible pathway for pullulan synthesis. The obtained glucose units needed the presence of three key enzymes to be converted into pullulan, and they are α-phosphoglucose mutase, uridine diphosphoglucose pyrophosphorylase (UDPG-pyrophosphorylase), and glucosyltransferase. Besides glucose, *A. pullulans* also consumes sucrose, mannose, galactose, maltose, fructose, and even agricultural wastes such as carbon sources (Catley 1971; Leathers 2003; Madi et al. 1996). The presence of hexokinase and isomerase is necessary for *A. pullulans* to convert different carbon sources into the pullulan precursor, UDPG.

The pullulan precursor, UDPG, is an important medium for pullulan production (Catley and McDowell 1982). UDPG initiates the attachment of a D-glucose residue to the lipid molecule, lipid hydroperoxides with a phosphoester bridge. Subsequently, a further transfer of the D-glucose residue from UPDG gives lipid-linked isomaltose. In the next step, isomaltosyl participates in the reaction with lipid-linked glucose to yield an isopanosyl residue as a precursor. Finally, isopanosyl residues are polymerized into the pullulan chain. The proposed pathway of pullulan synthesis is summarized in Fig. 2.

Pullulan can also be synthesized from sucrose with cell-free enzymes of *A. pullulans* when both ATP and UDPG are present in the reaction mixture (Ono et al. 1977). UDPG cannot be replaced by ADPG, proving that the pullulan chains or pullulan precursors originated from UDPG. Unfortunately, the formation pathway of these media is not clear. It is only known that, in the case of maltose-containing media, the carbohydrate metabolites needed for the polymer formation, which are panose [α-Glc-(1fi6)-α-Glc-(1fi4)-α-Glc] and/or isomaltose [α-Glc-(1fi6)-α-Glc], can be synthesized via a glucosyl transfer reaction in *A. pullulans* (Hayashi et al. 1994).

LeDuy et al. (1988) suggested that *A. pullulans* does not directly convert glucose residues into polysaccharide and may instead be involved in the polymerization of carbohydrate precursors stored inside the cells (Fig. 2). It is believed that cells first accumulate sugars and use this carbohydrate reserved for pullulan production in the late

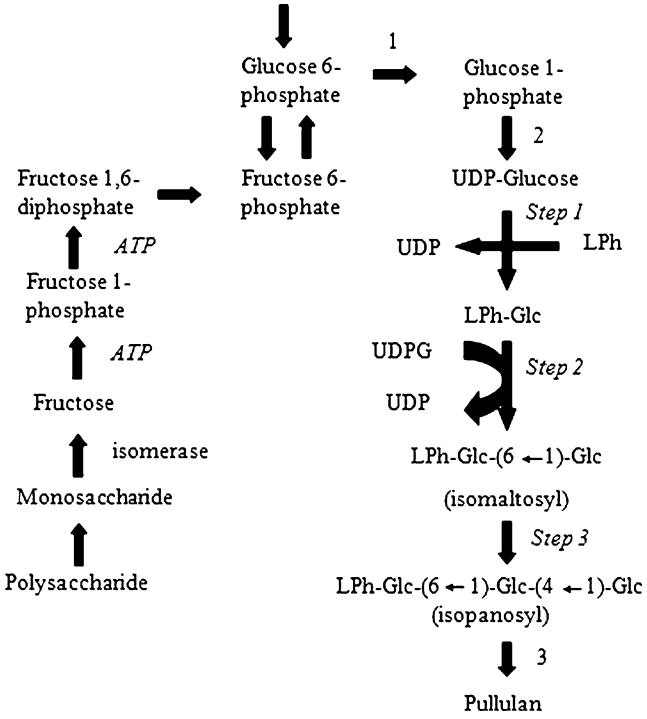

Fig. 2 Putative biosynthesis of pullulan (*1*, α-phosphoglucose mutase; *2*, UDPG-pyrophosphorylase; *3*, glucosyltransferase). Summarized from the studies of Catley and McDowell (1982) and LeDuy et al. (1988)

stages of their life cycle. This hypothesis was later proved by Simon et al. (1998) who found an inverse correlation between the concentration of pullulan and the content of intracellular glycogen.

Pullulan-producing strains

Many microorganisms were reported as pullulan producers, including *Aureobasidium* spp. (Bauer 1938), *Tremella mesenterica* (Fraser and Jennings 1971), *Cytaria* spp. (Waksman et al. 1977; Oliva et al. 1986), *Cryphonectria parasitica* (Forabosco et al. 2006; Delben et al. 2006), *Teloschistes flavicans* (Reis et al. 2002), and *Rhodototula bacarum* (Chi and Zhao 2003). However, most studies of pullulan production and its material property analysis mainly focused on the strain of *A. pullulans* due to its high production yield (Singh et al. 2008).

A. pullulans

A. pullulans (a.k.a. *Pullularia pullulans*) is widely spread in all ecological niches including forest soil, fresh and sea water, plant and animal tissues, etc. Generally, the culture of *A. pullulans* is classified as nonpathogenic; however, some strains may cause human health problems (World Health Organization 1979) and is pathogenic to plants (Cooke 1959). The name "black yeast" was given due to the production of a black melanin pigment; however, brightly pigmented variants have been reported from tropical latitudes, which produce reduced amounts of melanin (Leathers et al. 1988). The produced melanin provides the microbe resistance to phagocytosis in the host (Chabasse 2002). A detailed review of the bioproducts produced by *A. pullulans* and its biotechnological importance has been done by Chi et al. (2009).

Pullulan was the major exopolysaccharide (EPS) produced by *A. pullulans* (Bauer 1938). Some findings suggested that different strains of *A. pullulans* produce nonidentical pullulans with respect to their structure and composition (Gorin et al. 1968; Catley et al. 1966). However, Taguchi et al. (1973) presented the evidence of structure uniformity of pullulans produced by different strains of *A. pullulans*. The authors developed a purification process to remove pullulan from the water-insoluble jelly-like polysaccharide. It is now widely accepted that pullulan is a linear polysaccharide with maltotriosyl repeating units joined by α-1,6-linkages.

Although the large-scale production of pullulan has been developed, the major problem with pullulan production is the discoloration of the polysaccharide resulting from the pigment melanin, which is also synthesized simultaneously by the fungus (Pollock 1992; Simon et al. 1995). Melanin removal includes adsorption of activated charcoal; the use of solvent/solvent blends or solvent/salt combinations may increase the capital investment of pullulan production (Kachhawa et al. 2003). Fortunately, several pigment-reduced strains have recently been identified, which retain their ability to produce pullulan without dramatic changes in their physical properties, such as viscosity and distribution of molecular weight (West and Reed-Hamer 1993; West and Stohfus 2001a; Singh and Saini 2007).

Cell morphologies of *A. pullulans*

The yeast-like fungi *A. pullulans* has five different cell morphologies: yeast-like cells, young blastospores, swollen blastospores, chlamydospores, and mycelia (Ronen et al. 2002). Although a large number of studies have been focused on the relationship between pullulan production and the morphological form of *A. pullulans*, this issue is still under debate. Catley (1973, 1980) reported that the blastospore cells are the main pullulan producers. Some researchers reported that the other unicellular forms are the major producers. Yeast-like cells are claimed as main pullulan producers by Heald and Kristiansen (1985) as well as by Ronen et al. (2002). Evidence also supports that chlamydospores as well as swollen cells are responsible for pullulan production (Campbell et al. 2004; Simon et al.

1995; Li et al. 2009). The hyphal forms of *A. pullulans* may produce exopolysaccharides other than pullulan (Simon et al. 1995). In our recent study, the ratio of hyphal cells of *A. pullulans* increased when attached on a solid support during cultivation though it did not affect the total productivity (Cheng et al. 2011a).

Pullulan fermentation medium

Many studies have illustrated that *A. pullulans* has the capability to grow on a variety of substrates, even the agricultural waste with its multiple enzyme system that saccharifies plant fibers into glucose units as its carbon source (Leathers and Gupta 1994; Leathers 2003; Duan et al. 2008). This leads to many investigations concerning medium optimization for growth and pullulan production.

Carbon and nitrogen source

Pullulan can be synthesized from diverse carbon sources including glucose, sucrose, mannose, galactose, fructose, and agriculture waste and has been well documented (Duan et al. 2008; Singh and Saini 2007).

Bender et al. (1959) first reported that a wild strain of *A. pullulans* consumed 22 g/L of glucose and 20 g/L of sucrose during 5 days for EPS production and yielded 1.3 g of EPS per gram of dry cells. Sucrose is mostly used as a carbon source on pullulan production medium (Singh and Saini 2007). Sucrose has demonstrated itself superior to glucose on the basis of yield and titer of pullulan produced (Gibson and Coughlin 2002; Cheng et al. 2011b). Medium containing xylose or lactose as carbon source may result in both less cell growth and low pullulan-producing activity (Duan et al. 2008; Cheng et al. 2011b).

Corn syrup, a liquid start hydrolysate, has also been utilized as a carbon source in pullulan fermentation medium (West and Stohfus 1996, 2001a). Leathers and Gupta (1994) reported an *Aureobasidium* sp. color variant strain NRRL Y-12974 growing well on basal medium containing wet-milled corn fiber or corn condensed distiller's solubles, but not on thin stillage (TS), which are by-products from sugar and starch processes. Leathers (2003) later concluded that an agriculture waste, maize residue, can also be used as carbon source for pullulan production by *A. pullulans*. Other wastes from the agriculture and food industries, such as deproteinized whey, beet molasses, sugar cane juice, sweet potato, and peat hydrolysate are also considered as economical substrates for pullulan production (Roukas 1999; LeDuy and Boa 1983; Wu et al. 2009).

Excess carbon source (i.e., above 5%) was reported as exhibiting an inhibition effect on pullulan production (Kim et al. 2000; Shin et al. 1987). The reason could be due to the suppression effect of sugars on enzymes related to pullulan production, such as α-phosphoglucose mutase, UDPG-pyrophosphrylase, and glycotransferase (Duan et al. 2008). This suppression effect can be overcome by using fed-batch and continuous fermentation or through strain selection and improvement of cultivation methods (Shin et al. 1987; McNeil and Kristiansen 1987; Reeslev et al. 1997). For example, Cheng et al. (2010a) optimized the concentration of carbon and nitrogen sources for pullulan production. A final pullulan concentration of 60.7 g/L was obtained when 100 g/L of sucrose was applied.

Pullulan production can also be achieved by co-culturing fermentation. Shin et al. (1987) co-cultured both a pullulan-producing strain, *A. pullulans* SH 8646, and an insulin degradation strain, *Kluyveromyces fragili* ATCC 52466. The inulin, which contains D-fructopyranosyl residues with a terminal D-glucose residue, can be hydrolyzed by the inulase produced by *K. fragili* and serves as the carbon source for *A. pullulans*.

Nitrogen source, usually ammonium ion (NH_4^+), plays a significant role in pullulan production. The depletion of nitrogen is regarded as a signal for the exopolysaccharide formation of *A. pullulans* fermentation (Bulmer et al. 1987; Gibbs and Seviour 1996). Catley et al. (1986) examined the nitrogen limitation effect when producing pullulan by *A. pullulans*. The results indicated that, with similar rates of carbon utilization, the diversion of glucose from incorporation into cellular material to the elaboration of polysaccharide is dependent on ammonium ion concentration. Campbell et al. (2003) also reported that NH_4^+ might exert its influence as an effector of pullulan-degrading enzyme activity, controlling carbon flow within the cell. Additionally, excess nitrogen supply could contribute to the higher level of biomass but not enhance polysaccharide production (Orr et al. 2009).

Some studies also reported that the activity of a pullulan-degrading enzyme may be detected at the late stage of pullulan fermentation, which results in a decrease of pullulan yield (Catley 1971; Pollock 1992; Zheng et al. 2008; Cheng et al. 2011c). However, this phenomenon is only the consequence of the exhaustion of the carbon source and with a high initial nitrogen concentration (Campbell et al. 2003). Therefore, pullulan-degrading enzyme activity can also be used as an indicator to explain the pullulan production profiles.

It was also reported that a 10:1 carbon/nitrogen ratio is the most favorable condition for exopolysaccharide production (Morin 1998; Kumar et al. 2007). The concentration of mineral salts in medium should be adjusted accordingly (Gao et al. 2010). This combination is also supported by our study on the medium composition of sucrose, ammonium sulfate, and yeast extract (Cheng et al. 2011b). A combination of 75 g/L of sucrose, 3 g/L of YE, and 5 g/L of ammonium sulfate was recommended for

pullulan production. A 25.8-g/L of pullulan with 94.5% of purity was obtained after 7 days of cultivation.

pH and temperature

The optimal pH for pullulan production has been suggested to be in the range of 5.5 to 7.5 by many studies (Lee and Yoo 1993; Shingel 2004; Cheng et al. 2009b; Li et al. 2009). A relatively low pH suppresses the synthesis of pullulan but stimulates the production of insoluble glucan (Madi et al. 1997a).

Catley (1971) first illustrated the pH effect of pullulans on production. The results showed that the optimal pH for pullulan synthesis and cell mass growth is different. Lacroix et al. (1985) proposed a two-stage pH profile, which produces a biomass concentration with a low initial pH of 2.0. The pH was later adjusted to a higher value (5.0) for promoting the synthesis of pullulan. With this fashion, pullulan production increased from 17 to 26 g/L after 13 days of cultivation.

Although the exact cellular type responsible for pullulan synthesis is still a matter of debate, there is a convincing agreement that pH stimulates the morphological changes of cells. The yeast-like cells at neutral pH produce pullulan at a very high molecular weight (MW > 2,000,000) (Lee et al. 2001), while the combined cultivation of mycelial and yeast-like cellular forms gives a high pullulan concentration (Roukas and Biliaderis 1995).

The optimal temperature for pullulan production is different from strains in the range of 25 to 30 °C. Finkelman and Vardanis (1982) reported that *A. pullulans* (ATCC 42023) produced the highest concentration of pullulan at 30 °C. However, different optimal cultivation temperatures were also indicated among research groups (Gibson and Coughlin 2002; Prasongsuk et al. 2007; Cheng et al. 2011b). Wu et al. (2010) reported a two-stage temperature on pullulan production. The optimum temperature for pullulan production (27.4 g/L) was 26 °C, while the optimal temperature for cell growth (10.0 g/L) was 32 °C. In their study, the morphology of the *A. pullulans* (CGMCC 1234) was also affected by temperature; the lower temperature (26 °C) supported unicellular biomass growth.

Oxygen profile

Aeration is a critical parameter for cells to produce pullulan. Under anaerobic conditions, the cell population neither grows nor produces pullulan (Leathers et al. 1988). An intense aeration (up to 2.0 volume of airflow per volume of medium per minute (vvm)) during fermentation leads to an increase in pullulan concentration due to the accumulation of pullulan-producing cells (Roukas and Mantzouridou 2001). However, Audet et al. (1996) suggested that an intense aeration should be used with care since the pullulan produced will decrease its molecular weight under well-aerated conditions. The reason could be due to the change of α-amylase activity within the fermented broth, which was reported as the key enzyme to determine the molecular weight of pullulan (Leathers 1987). Contrarily, Wecker and Onken (2005) reported that pullulan production increased at decreased constant dissolved oxygen in connection with decreased shear rate. They proposed a possible reason that most cells were in their yeast form as influenced by the lower shear rate.

Dufresne et al. (1990) increased the partial air pressure to enhance the oxygen transfer rate, and the polysaccharide-producing activity of *A. pullulans* was improved. However, when pressure exceeds the critical values of 0.5–0.75 MPa, the cells aggregate spontaneously, and a cessation of pullulan production was observed. Another concern is that excess aeration will result in the adverse foam issue due to the high viscous nature of pullulan. From our recent study, *A. pullulans* produced the highest amount of pullulan (25.8 g/L) when aeration was at 1.5 vvm and resulted in both higher pullulan production rate and pullulan yield (Cheng et al. 2011b).

Light intensity

Chang (2009) reported that the low density of blue light (470 nm with 100- and 200-lx intensity) can promote the productivity of pullulan, and they are 57% and 90% higher than the results obtained for fermentation in dark. However, the side effect is that the production of melanin was also increased in dark conditions.

Fermentation methods and bioreactor design

Production of pullulan using submerged fermentation has been successfully implemented for commercial market (Yuen 1974). The concentration of pullulan produced by *A. pullulans* depends on the cultivation parameters as well as the bioreactor design.

Batch

Pollock (1992) used a mutated strain with ethidium bromide, which exhibited an increased tendency toward yeast-like cell growth and reduced pigmentation. Moreover, some of the new isolates and mutant derivatives produced pullulan of relatively high molecular weight. Tarabasz-Szymanska et al. (2000) also conducted a massive run of experiments including eight parameters. The pullulan yield with optimum condition was 3.5 times higher than before

(from 3.5 to 12.2 wt%). Singh et al. (2009) evaluated five cultivation parameters (sucrose, ammonium sulfate, yeast extract, dipotassium hydrogen phosphate, and sodium chloride) by using response surface methodology. The results demonstrated that 44.2 g/L of pullulan was produced with the optimal condition (5.31%, 0.11%, 0.07%, 0.05%, and 0.15% (w/v), respectively). We also performed a thorough evaluation of medium and cultivation parameters of the pigment-reduced strain *A. pullulans* ATCC 201253 (Cheng et al. 2011b). The results demonstrated that, with 75 g/L of initial sucrose, pullulan production was 25.8 g/L (94.5% purity) after 7 days of cultivation with a 0.68-g/L/h maximum production rate. There is one report which claimed that they can obtain 80 g/L of pullulan. However, the result appeared to be erroneous since the initial carbon source was only 50 g/L (Thirumavalavan et al. 2008).

Fed-batch

In order to overcome the sucrose suppression effect of pullulan production, Shin et al. (1987) performed fed-batch fermentation and 58 g/L exopolysaccharide was obtained though the exact pullulan content was not determined. Later, Youssef et al. (1999) performed pullulan fermentation using fed-batch fermentation method. However, the results indicated that maximum pullulan concentration decreased from 31.3 to 24.5 g/L after 7 days of cultivation. Cheng et al. (2011b) also examined the effect of fed-batch fermentation. However, the result demonstrated that pullulan production increased with time until the 10th day, and there was no significant increase of production rate after the addition of sucrose. The maximum production rate was constant at about 0.65 g/L/h.

Continuous fermentation

Several attempts have been made to enhance pullulan production by using continuous fermentations. Schuster et al. (1993) first tried to produce pullulan in a continuous culture. The production rate of exopolysaccharide increased from 0.16 to 0.35 g/L/h with a dilution rate at 0.05 h^{-1}. The culture was maintained over 1,000 h without any problems. Reeslev et al. (1997) also performed continuous fermentation and the results demonstrated that the optimal pH was 4.0 under zinc-limited condition. Seo et al. (2006) reported that 78.4 g/L of pullulan was obtained using continuous fermentation. However, the dilution rate was extremely as low as 0.015 h^{-1}.

A more feasible way for long-term production is the combination of increased cell population and continuous fermentation. We reported a continuous pullulan production process by using a biofilm reactor, which provided higher cell density. Pullulan concentration and production rate reached a maximum (8.3 g/L and 1.33 g/L/h) at 0.16 h^{-1} when 15 g/L of sucrose, 0.9 g/L of ammonium sulfate, and 0.4 g/L of yeast extract were applied in the medium (Cheng et al. 2011a). In our study, the residual inorganic nitrogen (NH_4^+) in the medium reached 0 at an optimal condition (0.16 h^{-1} when 15 g/L of sucrose, 0.9 g/L of ammonium sulfate, and 0.4 g/L of yeast extract were applied in the medium). However, some organic nitrogen compounds (i.e., red blood cells and defatted soybean) were slowly released from the solid support, which can maintain *A. pullulans* biofilm growth. The role of nitrogen limitation here becomes complicated. Further studies on the relationship between nitrogen limitation, cell growth, and pullulan production are therefore needed.

Immobilized and biofilm systems

In order to increase the total biomass in the bioreactors, many cell immobilization studies for pullulan production have been carried out by using agar, calcium alginate, and carrageenan in a batch fermentation (West and Strohfus 2001b; West 2000).

West (2000) successfully immobilized *A. pullulans* cells using agarose and carrageenan. Later, West and Strohfus (2001b) immobilized *A. pullulans* cells within agar and alginate beads. The results showed that alginate-entrapped cells produced higher polysaccharide levels during the second cycle than the first one. However, the pullulan yield from immobilized cells is relatively low. Only 0.43 mg of polysaccharide per gram of cells per hour with 36% purity was reported. The reason is probably due to the relatively small pore size of these materials, which hindered the release of pullulan produced. Ürküt et al. (2007) optimized three fermentation parameters (initial pH, agitation speed, and incubation time) and further improved pullulan yield to 19.5 g/L. However, the gel beads began to lose their activity after four consecutive batch fermentations. A concomitant decrease of pullulan productivity was also observed. A possible reason is that the fungus was also able to produce other polysaccharides with the presence of calcium ions (Madi et al. 1997b)

Instead of artificial immobilization, biofilm grows on the solid support, which is a natural form of cell immobilization (Characklis and Wilderer 1989). For *A. pullulans* strain, this attachment mechanism may be due to the self-secreted uronic acid polymer (Pouliot et al. 2005). Mulchandani et al., (1988) employed polyurethane foam as the solid support of *A. pullulans* cells, and the setup yielded around 18 g/L of pullulan after 96 h of cultivation.

Another type of solid support is plastic composite support (PCS), an extrusion product of a mixture of polyprolylene and nutritious compounds which provides an ideal surface for biofilm formation (Pometto et al. 1997;

Cheng et al. 2010c). Polypropylene acts as a matrix and integrates agricultural mixtures, such as ground soybean hulls, soybean flour, and microbial nutrients (i.e., bovine albumin, red blood cells, yeast extract, peptone, and mineral salts). Therefore, PCS not only provides an ideal physical structure for biofilm formation but also releases nutrients slowly during fermentation (Ho et al. 1997).With the implementation of PCS, Cheng et al. (2009b, 2010a, 2011a) adopted biofilm reactors for a series studies, and the yield of pullulan reached around 60 g/L after the optimization of cultivation parameters in a repeated batch system. Continuous pullulan production was also applied as described earlier (Cheng et al. 2011a).

Other bioreactor designs

Audet et al. (1996) proposed a new bioreactor design, which is called reciprocating plate bioreactor (a cylinder 420 nm in height and 206 mm in diameter with a 13-L working volume) and is particularly well suited for conducting fermentations which give high viscous broth. The mixing mechanism was composed of six perforated plates mounted on a central shaft. Later, Audet et al. (1998) also evaluated four different mixing devices in the bioreactor for pullulan production. The results demonstrated that a bioreactor implemented with reciprocating plates (RPB2; a mixing plate stack consisted of six perforated stainless steel plates) gave the highest pullulan yield. Although the pullulan produced from helical ribbon impeller (HR; a 1.73-m-long and 30-mm-wide stainless steel ribbon winded and mounted about the central shaft) reactor exhibited highest viscosity, the overall productivity was extremely low. Table 1 summarizes the progress of pullulan production in the past 5 years.

Modeling of pullulan fermentation

Mathematical models for pullulan fermentation (a type II fermentation) not only provide information about the kinetic and metabolic nature of pullulan but also facilitate the control and optimization of pullulan production during fermentation. Predictions of pullulan production using biomass production would be valuable for a practical purpose because the biomass can be measured much faster or even in a continuous manner. Several attempts have been made to develop a model that would describe the growth of pullulan producers and pullulan production (Mohammad et al. 1995; Boa and LeDuy 1987; Klimek and Ollis 1980; Thomson and Ollis 1980).

Mohammad et al. (1995) used a logistic model to predict biomass production at different sucrose concentrations. Mulchandani et al., (1988) also adopted a logistic equation to calculate the batch kinetics of microbial growth for

Table 1 Summary of pullulan production by *A. pullulans* reports in the past 5 years

Strains	Fermentation type	Production	Purity	Reference
P56	Batch	17.2 g/L	NA	Göksungur et al. 2005
	Batch	54.2 g/L	NA	
ATCC 42023 and ATCC 62921	Batch	22.6 g/L	NA	Lin et al. 2007
P56	Immobilization/batch	21.1 g/L	64–84%	Ürküt et al. 2007
NRM2	Batch	25.1 g/L	97%	Prasongsuk et al. 2007
FB-1	Batch	23.1 g/L	NA	Singh and Saini 2007
Y68	Batch	52.5 g/L	NA	Duan et al. 2008
MTCC 2195	Batch	80.0 g/L[a]	NA	Thirumavalavan et al. 2008
ATCC 9348	Batch	7.02 g/L	NA	Zheng et al. 2008
AP 329	Batch	29.4 g/L	95.2%	Wu et al. 2009
ATCC 9348	Airlift/batch	18.0 g/L	~90%	Orr et al. 2009
	Continuous	NA	60–80%	
FB-1	Batch	45.0 g/L	94.3%	Singh et al. 2009
FB-1	Batch	44.2 g/L	NA	Singh et al. 2009
CGMCC 1234	Two-stage temp.	27.4 g/L	94.0%	Wu et al. 2010
ATCC 201253	Batch	25.8 g/L	94.5%	Cheng et al. 2011b
ATCC 201253	Immobilization/batch	60.7 g/L	95.2%	Cheng et al. 2010a
ATCC 201253	Immobilization/continous	1.33 g/L/h	93.0%	Cheng et al. 2011a
ATCC 42023	Immobilization/batch	21.9 mg/g cells/h	59.0%	West 2011

[a] The results are dubious since the sugar input was only 50 g/L

polysaccharide production. The model describes the growth of the microbial population as a function of maximum population density, lag time, specific growth rate, and time. On the other hand, since the logistic model is symmetrical at the time when maximum growth rate is t_m, the Gompertz function is usually chosen for generating an asymmetrical growth curve (Pouliot et al. 2005).

Pullulan was reported as a secondary metabolite and begun to be produced in late exponential phase; it can be produced more when growth becomes limited or as the cells approach a stationary phase (Pouliot et al. 2005). Mohammad et al. (1995) used Luedeking–Piret equation to calculate the batch kinetics of pullulan production at different sucrose concentrations. They also used modified Luedeking–Piret to predict sucrose consumption. Bi-stage pH profile has been reported as a favored pH environment to enhance pullulan production (Bulmer et al. 1987; Cheng et al. 2010b). However, the two-stage profiles of pullulan production and sucrose consumption increase the difficulty of modeling. In our recent study, the proposed modified Gompertz model was used to predict both biomass and pullulan production and sucrose consumption. Later, the re-modified Gompertz equation was reported to describe pullulan fermentation profile under different ammonium ion concentrations even with the presence of pullulan-degrading enzymes at the late cultivation stage (Cheng et al. 2011c). When the re-modified Gompertz equation was applied, the values of root mean square errors and mean absolute errors of pullulan production with the presence of pullulan-degrading enzymes were only 0.88 and 0.59 g/L. Meanwhile, the R^2 value and slope were 0.992 and 0.98, respectively.

Quality analysis

In optimization studies of extra-polysaccharide production by *A. xylinum*, it was usually mistakenly assumed that all polysaccharides were pullulan. However, it has been reported that other polysaccharides were produced as well during cultivation (Simon et al. 1993; Lee et al. 1999), and the pullulan content ranged from 27% to 100% (West and Strohfus 2001a). To further illustrate the exact pullulan yield, several analyses including purity content, molecular weight distribution, viscosity, and structural determination are needed.

Pullulan content of EPS produced by *A. pullulans*

Polysaccharides produced by *A. pullulans* were assayed for their sensitivity to pullulanase, which specifically digests α-1,6 bond and releases maltotriose units, to determine the pullulan content (Leathers et al. 1988). These maltotriose units were further hydrolyzed and determined using dinitrosalicylic acid method (Miller 1959). FTIR spectrometry is often adopted as supporting data to prove the presence of α−(1, 4) and α−(1, 6) linkages, which are the two distinct linkages within pullulan from other EPS of *A. pullulans* (Cheng et al. 2009b). Thin-layer chromatography was also adopted for the development of pullulanase-digested EPS to identify the repeating unit of pullulan (Orr et al. 2009; Zheng et al. 2008).

MW distribution

The molecular weight of pullulan varies with the culture conditions and strains (Gibson and Coughlin 2002; Wiley et al. 1993; Lee et al. 1999; Pollock 1992; Madi et al. 1996; Seo et al. 2004; Prasongsuk et al. 2007), and higher molecular weight is more desirable for commercial use. The number-average molecular weight (M_n) of pullulan is about 100–200 kDa, and the weight-average molecular weight (M_w) is about 362–480 kDa (Okada et al. 1990). Values of polydispersity (M_w/M_n) have been frequently reported and lie between 2.1 and 4.1 (Roukas and Mantzouridou 2001; Wiley et al. 1993) and are important criteria for application in pharmaceutical industry.

Pollock (1992) reported that a pigment-reduced *A. pullulans* AP27 can produce pullulan with higher molecular weight, and the molecular weight of pullulan produced depends largely on the timing of harvest. Wiley et al. (1993) and Lin et al. (2007) investigated the effects of carbon, nitrogen, and phosphate source on the molecular weight distribution of pullulan. Nitrogen source had the most significant influence on the MW of the products. For the production of high-MW pullulan, NH_4^+ was found to be better than NO_3^-. Madi et al. (1996) investigated the effects of pH and aeration on pullulan production; the results indicated that aeration is a crucial factor since the anaerobic fermentation of *A. pullulans* will accumulate ethanol instead of pullulan. Lee et al. (1999) and Gibson and Coughlin (2002) also reported that pullulan will decrease its molecular weight during cultivation and different carbon sources will result in both different M_w and M_n. One of the undesirable features of pullulan production by *A. pullulans* is the declination of molecular weight of pullulan with the progression of fermentation, which could be due to the presence of α-amylase during cultivation. Prasongsuk et al. (2007) added an α-amylase inhibitor, acarbose, to the fermentation medium. The results demonstrated that, with the addition of acarbose, a higher molecular weight of pullulan was obtained after 7 days of cultivation. Manitchotpisit et al. (2010) studied on the role of α-amylase in the reduction of pullulan molecular weight at the gene level. They proposed that the low level of α-amylase in the fermentation broth attacks the minor maltotetraose

subunits of pullulan, resulting in the reduction of molecular weight.

The viscosity of pullulan solution corresponds to the weight-average molecular weight (Buliga and Brant 1987). Therefore, the viscosity test of pullulan produced in a water solution was often carried out as supporting data (Cheng et al. 2009a). However, further studies on the molecular weight distribution of pullulan produced can be achieved by using high-performance size exclusion chromatography (Pollock 1992; Prasongsuk et al. 2007; Seo et al. 2004; Wiley et al. 1993).

Harvest and downstream processing

Downstream processing is necessary to obtain the target exopolysaccharide produced by microorganisms from the fermentation broth. For pullulan, the process comprises of the removal of cells and melanin pigments produced during the fermentation and precipitation of EPS produced with a suitable solvent. Melanin was one of the obstacles of pullulan production and resulted in the dark green to black color of the broth (Kachhawa et al. 2003). This issue can be solved by the discovery of new strains, which are pigment-reduced or pigment-free pullulan producers. The biomass of *A. pullulans* in the fermented broth can be removed in advance by cross-flow membrane filtration (Yamasaki et al. 1993). Precipitation step is necessary for pullulan recovery and can be accomplished by using organic solvents. A 2:1 (*v*/*v*) combination of 95% of ethanol and fermentation supernatant is commonly used for both safety and economic concerns (Youssef et al. 1999). Further purification of pullulan can be achieved by ultrafiltration and ion exchange resins (Kachhawa et al. 2003). The harvested pullulan will be further dried and mechanically ground into powder. One continuously purifying procedure for pullulan has been published by Kato and Nomura (1976). Serial tanks with an increasing concentration of organic solvent were implemented to precipitate and recover the dehydrated pullulan. The precipitated pullulan can therefore be dried in an oven at 80 °C.

Application of pullulan

Pullulan has been applied in various fields, ranging from food manufacturing to pharmaceutical applications, and even used as a source of monosaccharides. The first commercially produced pullulan was sold in 1959 by the Hayashibara Company in Okayama, Japan (Yuen 1974).

Table 2 Summary of pullulan modifications and applications

Pullulan products/derivatives	Current or potential application(s)	Reference(s)
Biodegradable pullulan		Mayer et al. 1990
Carbonation	Drug carriers	Bruneel and Schacht 1993
Pulluan additives	Blood plasma substitute	Tsujisaka and Mitsuhashi 1993
Palmitoyl and cholesteryl derivatives	Hydrophobic reactant	Nishikawa et al. 1994
Adenine-, thymine-, pyrene-modified pullulan	Tensioactive agents/thickeners/enzyme deposit	Mocanu et al. 1995
Food additive	Dietary and functional food	Oku et al. 1979; Sugawa-Katayama et al. 1994
Siloxane pullulan	Water stable pullulan/silicone composite	Uchida et al. 1996
NPcaps® capsules	Capsules	Kimoto et al. 1997
Pullulan 6-hydroxyhexanoates/6-dilactates		Donabedian and McCarthy 1998
2-Nitroalkyl pullulan ester	EPS with amino functionalities	Heeres et al. 2000
Pullulan blend	Drug/gene delivery and imaging	Kaneo et al. 2001; Suginoshita et al. 2002; Hosseinkhani et al. 2002; San Juan et al. 2009; Boridy et al. 2009
Anionically modified pullulan	Blood plasma substitute	Shingel and Petrov 2002
Poly(L-lactide)-grafted pullulan	Biodegradable polymer	Ouchi et al. 2004
Pullulan gel	Electrophoresis	Nakatani et al. 2006; Olmo et al. 2008
Pullulan cinnamates	Modeling of cell wall biogenesis	Kaya et al. 2009
Methacrylate pullulan gel	Cell proliferation and cluster formation	Bae et al. 2011
Heparin-conjugated pullulan	Cell/tissue engineering	Na et al. 2003
Antibacterial film	Food preservation	Kandemir et al. 2005; Gounga et al. 2008
Carboxymethyl pullulan gel	Antibacterial release wound dressing	Li et al. 2011

Considerable efforts have been directed toward the modification of pullulan to improve its application. Recent literature concerning the modifications and applications of pullulan has been summarized in Table 2 and addressed as follows.

Pharmaceutical applications

Due to a high concentration of hydroxyl groups, pullulan exerts inherent physiological activity and has been used as bioactive polymers, a dextran-based blood plasma substitute, since 1944 (Tsujisaka and Mitsuhashi 1993). However, the use of pullulan with a molecular weight higher than 150 kDa leads to the unwanted rapid increase of venous pressure. As a result, the researchers concluded that pullulan, which is suitable for intravenous injection, should have a narrow molecule weight distribution with Mw/Mn = 1.2 and Mw of ~60 kDa (Seibutsu and Kenkyujo 1983). Shingel and Petrov (2002) developed an anionically modified pullulan, which was used as a blood plasma substitute. Modification with carboxyl and carbonyl groups can increase the resistance of pullulan from enzyme degradation.

Pullulan can also be formed into capsules for use of with pharmaceutical and nutraceutical products. Its non-animal origin ensures that there are no safety concerns with consumers and that it is suitable for all clients. At the same time, pullulan has no mutagenic, carcinogenic, and toxicological activities (Kimoto et al. 1997). A commercialized product, NPcaps® capsules, which are made from pullulan, are an all-natural, two-piece, non-animal capsule suitable for addressing a variety of cultural and dietary requirements, including those of vegetarians, diabetics, and patients with restricted diets.

Pullulan derivatives are promising due to its non-toxic property as conjugates for vaccines, proteins, and interferons. The current and potential applications of pullulan as a biomaterial were summarized by Rekha and Sharma (2007). Pullulan can serve as a carrier for targeted drug/gene delivery and imaging (Kaneo et al. 2001). Pullulan also exhibited its property of accumulation in liver. With this advantage, Suginoshita et al. (2002) used the blend of pullulan/diethylenetriamine pentaacetic acid (DTPA) and successfully accumulated interferon (IFN) in human liver to kill hepatic virus C. The pullulan–DTPA–IFN conjugate also exhibited a higher IFN activity than the free IFN. Akiyoshi et al. (1998) also developed an insulin delivery system using the hydrogel nanoparticle of cholesterol-bearing pullulan. A non-viral vector has been developed by Hosseinkhani et al. (2002) to mix with a plasmid DNA with the presence of Zn^{2+} in aqueous solution and targeting the liver. A cationized pullulan hydrogel also served as coating material which can be loaded with small interfering RNA targeted at matrix metalloproteinase-2 for gene silencing in vascular cells (San Juan et al. 2009). Another cationic pullulan also exhibited its high liver binding affinity and can serve as a non-viral vector-based gene delivery (Rekha and Sharma 2009). Boridy et al. (2009) also reported that nanoparticles (20–30 nm) with pullulan-bearing cholesteryl moieties (CHP) can reduce the cytotoxicity of Abeta (1–42) in primary cortical cells and microglial (N9) cells, which could provide a valid complementary approach to antibody immunotherapy in neurological disorders characterized by the formation of soluble toxic aggregates, such as those in Alzheimer's disease.

Since the ligand molecules that bind to the cell surface are important for cell attachment, the development of novel materials for in vivo and in vitro cell/tissue engineering are mainly focused on new biomaterials which provide coupling ligands through covalent attachment (Na et al. 2003). This heparin-conjugated pullulan can inhibit the proliferation of smooth muscle cells and serve as a good surface for endothelial cells in vitro. Cholesterol-bearing pullulan nanogel-crosslinking hydrogel was used to deliver bone morphogenetic protein, which induced osteoblastic activation and new bone formation in vivo (Hayashi et al. 2009). Nagane et al. (2009) established an efficient induction system of dopamine-producing neuronal cells from bone marrow stromal cells in several species using the spermine–pullulan-mediated reverse transfection technique. The pullulan itself can be used as low-viscosity filler in suspension cell culture to prevent mammalian cells from harsh shear force.

Food applications

Pullulan is an excellent film former, producing a biodegradable film, which is heat sealable with good oxygen barrier properties and can also be printed (Yuen 1974). As a result, pullulan film is sometimes referred to as "edible packing." Coloring, flavoring, and other functional ingredients can be entrapped in the film matrix and are very stable. Yuen (1974) also reported that pullulan can inhibit fungal growth in food. Kandemir et al. (2005) presented antimicrobial films by incorporating lysozyme into pullulan films, which are effective on *Escherichia coli*. Later, Gounga et al. (2008) presented a whey protein isolate pullulan as a coating material to protect fresh harvest chestnut fruits from moisture loss and change of external color. Other functions such as low-viscosity filler for beverage and sauce, stabilizer for mayonnaise, binder and stabilizer for food pastes, anti-sticking substance, cookies, and tobaccos have also been reported (Hijiya and shiosaka 1975; Miyaka 1979; Matsunaga et al. 1978; Hasa et al. 2006).

In addition to food additives, pullulan also served as dietary food as a starch substitute because of its resistance to human intestinal enzymes (Oku et al. 1979). Pullulan also served as prebiotics and promotes the growth of beneficial bifidobacteria in human intestines (Sugawa-Katayama et al. 1994).

Other applications

Environmental remediation Pullulan-producing strains possess heavy metal removal ability due to their adhesive nature. Breierova et al. (2002) reported that *A. pullulans* can remove heavy metal ions from aqueous solution. Radulovic et al. (2008) reported that, during the growth of *A. pullulans* CH-1 on the acid hydrolysate of peat from the Valsina Lake, the content of Cu, Fe, Zn, Mn, Pb, Cd, Ni, and Cr decreased as a result of pullulan production. With its high viscosity, pullulan is also a candidate for conducting microbial-mediated oil recovery from the polluted field (Iyer et al. 2005).

Filtration and chromatography Cross-linked pullulan is water resistant and hence have been commercialized as gel beads for chromatography (Motozato et al. 1986). Pure pullulan samples with accurate sizes of molecular weight have also adopted as chromatography standards (Kawahara et al. 1984). Nakatani et al. (2006) reported that pullulan can be used for capillary electrophoresis of sodium dodecyl sulfate proteins. The separation result was in accordance with the Ogston theory. This capillary electrophoresis technique was also applied for the purification of human histone H4 (Olmo et al. 2008). Pullulan can also involve in concurrent phase separation to harvest oat β-glucans (Lazaridou and Biliaderis 2009).

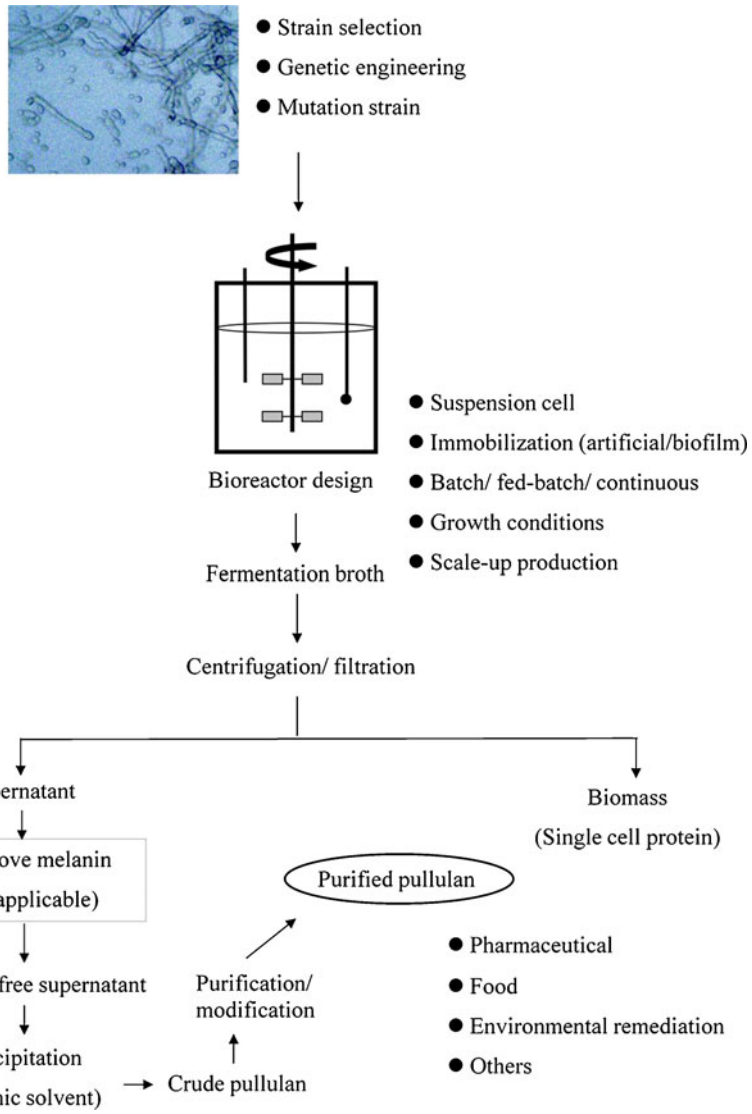

Fig. 3 Strategies of pullulan production, downstream processing, and applications

Preservation of bacteria Pullulan can also be used as preservation agents. Krumnow et al. (2009) reported that pullulan was used to preserve model bacteria *E. coli* and *Bacillus subtilis* via immobilization and storage under various conditions.

Future and prospects

Although the pullulan production yield is considerably stable for years, the clarification of pullulan synthesis pathway is urgently needed to further improve pullulan production through genetic engineering methods and to compensate the increasing demand for pullulan.

The flow diagram showing the various steps for an enhanced pullulan production and its applications were summarized in Fig. 3. The ways for enhanced pullulan production have been illustrated and detailed in the previous sections. The current and potential modification of pullulan and its applications have also been summarized and proposed.

Pullulan, as a member of bacterial exopolysaccharides, demonstrates itself a high potential for applications due to its bioactive property. Since the major limitation of pullulan production, the removal of melanin, has been solved in the early 1990s, the production cost of pullulan can be further reduced and therefore becomes more competitive to similar products, such as dextran, xanthan, and other microbial-produced polysaccharides.

In the past two decades, the major market of pullulan was mainly in food applications. With the advanced technologies of modification and cultivation skills, production of modified pullulan derivatives possessing distinctive material properties and pullulan with specific molecule weight distribution can be achieved. As a result, a boosting number of reports concerning pullulan applications in pharmaceutical, medical, and environmental remediation areas have been published.

An emerging market for pullulan could be cancer therapy. The modified pullulan exhibits high bioactivity with some cytotoxic molecules and will form the conjugates with them. These conjugates can accumulate at target tissue and gradually release those cytotoxic molecules. Another promising market could be bio-remediation since pullulan exhibits strong absorption ability toward heavy metal within the soil and water environment. Many research groups have also been working on the investigations of novel pullulan composites blended with other biodegradable materials which can possess desired properties, such as thermal stability, water impermeability, and high tensile strength.

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