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Review

Production of the Polysaccharide Pullulan by *Aureobasidium pullulans* Cell Immobilization

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Abstract: This review examines the immobilization of *A. pullulans* cells for production of the fungal polysaccharide pullulan. Pullulan is a water-soluble gum that exists structurally as a glucan consisting primarily of maltotriose units, which has a variety of food, non-food and biomedical applications. Cells can be immobilized by carrier-binding or entrapment techniques. The number of studies utilizing carrier-binding as a method to immobilize *A. pullulans* cells appears to outnumber the investigations using cell entrapment. A variety of solid supports, including polyurethane foam, sponge, diatomaceous earth, ion-exchanger, zeolite and plastic composite, have been employed to immobilize pullulan-producing *A. pullulans* cells. The most effective solid support that was used to adsorb the fungal cells was polyurethane foam which produced polysaccharide after 18 cycles of use. To entrap pullulan-producing fungal cells, agents such as polyurethane foam, polyvinyl alcohol, calcium alginate, agar, agarose, carrageenan and chitosan were investigated. Polysaccharide production by cells entrapped in polyurethane foam, polyvinyl alcohol or calcium alginate was highest and the immobilized cells could be reutilized for several cycles. It was shown that the pullulan content of the polysaccharide synthesized by cells entrapped in calcium alginate beads was low, which limits the method's usefulness for pullulan production. Further, many of the entrapped fungal cells synthesized polysaccharide with a low pullulan content. It was concluded that carrier-binding techniques may be more effective than entrapment techniques for *A. pullulans* cell immobilization, since carrier-binding is less likely to affect the pullulan content of the polysaccharide being synthesized.

Keywords: pullulan; polysaccharide; applications; cell immobilization; carrier-binding; entrapment; *Aureobasidium pullulans*

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1. Introduction

The complex polysaccharide pullulan is a gum synthesized extracellularly by the yeast-like imperfect fungus *Aureobasidium pullulans* [1]. Taxonomically, it has been reported that the synthesis of polysaccharide with high pullulan content is restricted to certain varieties of *A. pullulans* within the genus *Aureobasidium* [2,3]. The biopolymer synthesized is a neutral polysaccharide with nylon-like properties. Pullulan is water-soluble and is insoluble in alcohol [4]. Depending upon the strain of *A. pullulans* utilized to synthesize pullulan, its molecular weight can range from 50,000 to 2,500,000 [5]. Structurally, pullulan exists as a complex linear polysaccharide consisting of maltotriose and maltotetraose units linked through α-D-(1→6) bonds on its terminal glucose residues (Figure 1) [6–8]. The properties of pullulan make it a commercially valuable polysaccharide gum [2,3,9]. Currently, important commercial applications of pullulan include its use in the films in Listerine PocketPaks®, as well as the Listerine Whitening Quick Dissolving Strips® (Warner Lambert Division of Pfizer, Inc., Morris Plains, NJ, USA) that are marketed by Johnson & Johnson (New Brunswick, NJ, USA). A number of other commercial patented applications exist for this fungal gum, including use as a flocculant, a blood plasma substitute, a food additive, an adhesive and a dielectric material [2,10–13].

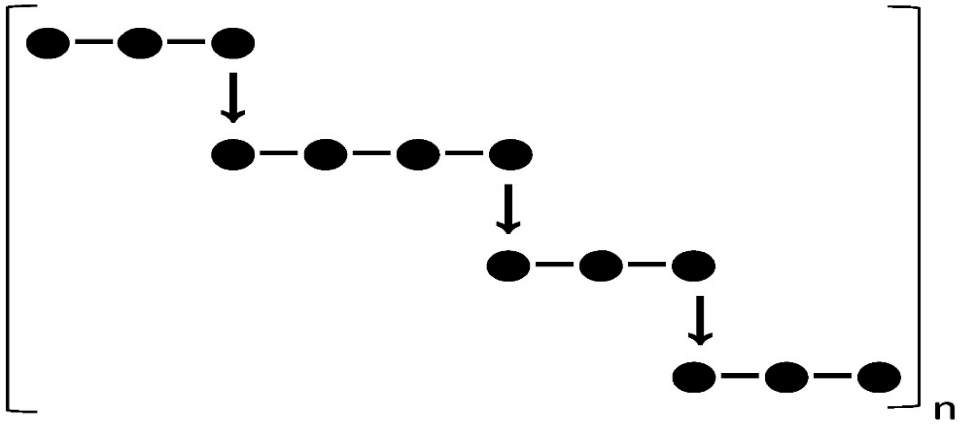

Figure 1. The structure of pullulan consists of maltotriose and maltotetraose units (glucose residue = ●) connected through α-D-(1→6) bonds on its terminal glucose residues.

With the variety of applications that exist for pullulan, several studies have explored its production using free cells of *A. pullulan* strains [14–38]. Several carbon sources can be utilized for pullulan synthesis by *A. pullulans* [15–17]. The carbon source utilized in the culture medium also influences pullulan content. Both glucose and maltose corn syrup as carbon sources have been found to promote fungal synthesis of a polysaccharide with a higher pullulan content than if the fungus was grown on sucrose as a carbon source [18,19]. For each carbon source, its fermentation by the fungus causes the pH of the culture medium to become acidic and this decrease is vital to pullulan elaboration [20,21]. The effect of culture medium pH has been explored with respect to its influence on polysaccharide levels. The optimal initial pH of the culture medium is 6.0–6.5 depending on the carbon source employed [21]. Nitrogen availability is also critical to the rate of pullulan production by the fungus [16,22–28]. The optimal temperature for polysaccharide production by the fungus is 26 °C, independent of carbon source tested [29]. The composition of the culture medium has also been found to influence fungal polysaccharide synthesis [30]. Yeast extract is known to be an important component in the culture medium to promote fungal pullulan synthesis [31]. In the absence of yeast extract, the optimal culture medium pH is 5.5 or below, depending on the carbon source [21]. With yeast extract supplementation being critical to pullulan synthesis, as well as an expensive component of the production medium, vitamin supplementation to the culture medium was studied; however, no specific vitamin supplement could be used to replace yeast extract [32]. It appears that hydrolyzed plant biomass may be able to substitute for yeast extract in the production medium [33–35]. Supplementation of the mineral salt ferric chloride or manganese chloride have been shown to increase fungal pullulan production [32,36]. Other growth factors, such as fungal cell density and oxygen concentration, are also important for pullulan synthesis [31,37,38]. Discoloration of pullulan by melanin is a significant problem during its production since a step involving activated carbon is required for removal of the pigment [18]. A reduced pigmentation mutant of *A. pullulans* ATCC 42023 has been isolated in which the cellular and polysaccharide pigmentation was substantially reduced by several-fold [18]. A pullulan overproducer mutant has also been isolated [19]. Much previous research has focused on making the pullulan production process more economical to lower the price of the biopolymer. Apart from utilizing free cells of known pullulan-producing *A. pullulans* strains to produce the polysaccharide, an alternative method to reduce the cost of synthesizing pullulan could involve the use of cell immobilization technology. The advantage of using cell immobilization technology is that it is possible to use the immobilized fungal cells to synthesize pullulan for more than one production cycle. This review explores how prior studies have immobilized cells of pullulan-producing *A. pullulans* strains to produce authentic pullulan using various immobilization procedures.

2. Types of Cell Immobilization

There are primarily two methodologies for cell immobilization that have been studied: carrier-binding and entrapment [39–44]. With respect to carrier-binding procedures, cells immobilized on diatomaceous earth, porous ceramics, sand, hydrated titanium oxide, wood shavings, sawdust, anthracite, ion exchange resins, polyurethane foam, sponge cubes or alumina have been shown to synthesize products [45–57]. The carrier-binding method usually involves cell adsorption onto a solid support or cell penetration into pores within the solid support. Carrier-binding immobilization procedures are usually used to immobilize high molecular weight products, such as pullulan, since the polysaccharide may not be able to escape from the entrapped cells. The most significant problem encountered when using carrier-binding methods of cell immobilization is the loss of cells from the solid support due to leakage. The loss of cells from the solid support results in a reduction in the product being measured during each subsequent production cycle. The other methodology for cell immobilization is the use of agents that polymerize to form a matrix surrounding the target cells. A number of investigations have explored the entrapment of fungal cells and the ability of the immobilized cells to synthesize a product. Microbial cells have been entrapped in agarose, carrageenan, chitosan, collagen, calcium alginate and gelatin, with all supports being capable of product formation [58–65]. Usually, cells that produce low molecular weight products are entrapped in a polymerizing agent where the product moves freely into the culture medium. Previous studies have explored the effectiveness of using immobilized fungal cells to produce pullulan and have shown varying degrees of success. The use of immobilized *A. pullulans* cells to synthesize pullulan using fungal cell adsorption onto solid supports or using fungal cell entrapment has been compared in a number of studies in which polysaccharide production was explored in batch cultures or in a bioreactor for several cycles.

3. Pullulan Production by Carrier-Binding Cell Immobilization

3.1. Polyurethane Foam, Sponge and Diatomaceous Earth as Solid Supports

A number of investigations have explored the possible adsorption of pullulan-producing *A. pullulans* cells onto solid supports (Table 1). The cells of *A. pullulans* ATCC 42023 were immobilized on polyester fiber mat or fiberglass using culture medium buffered at pH 2.0, since cell loading was maximum at this pH. Using either support, pullulan production was highest after 96 h with aeration at 250 rpm at 28 °C compared to aeration at 100 rpm or 200 rpm [66]. Polyurethane foam was used to adsorb nutrient broth-grown *A. pullulans* wild-type cells at 42 °C for 24 h [67]. It was found that aeration rate was important to pullulan production of the immobilized wild-type cells in a 10 L reactor (with a working volume of 7 L) where sucrose served as the carbon source and ammonium sulfate was the nitrogen source. It was possible to use the polyurethane-adsorbed cells for 18 cycle of pullulan production in culture medium buffered at pH 5.5 and incubated at 42 °C [67]. ATCC 42023 cells were immobilized by adsorption into the pores of sponge cubes. The sponge cubes were inoculated with fungal cells grown in maltose corn syrup, sucrose or glucose as a carbon source, with ammonium sulfate as the nitrogen source [68]. The sponge cubes were initially found to contain 7–9 $\times$ 10^7 fungal cells/g sponge, which was reduced to 3–4 $\times$ 10^7 fungal cells/g sponge following the third cycle of use. Independent of carbon source, polysaccharide production by the fungal cells immobilized on the sponge cubes was found to be higher after 168 h of use for the third production cycle compared to the first production cycle [68]. Relative to the carbon source used, it was observed that the glucose-grown cells immobilized on the sponge cubes synthesized the highest level of pullulan after 168 h of production during the third cycle [68]. Diatomaceous earth was another solid support that was utilized to adsorb ATCC 42023 cells grown on maltose corn syrup as a carbon source and ammonium sulfate as the nitrogen source [69]. The culture medium pH that was most effective in adsorbing a high fungal cell concentration was 2.0. The immobilized ATCC 42023 cells immobilized on diatomaceous earth were investigated for two cycles of pullulan production of 168 h. The findings indicated that pullulan production

decreased by about 25% when comparing polysaccharide levels produced during the second cycle compared with the first cycle polysaccharide levels [69]. It appeared that cell leakage was likely a factor in the decrease in pullulan production between the cycles.

Table 1. Growth conditions and maximum pullulan production by *Aureobasidium pullulans* strains immobilized by carrier binding.

Carrier Support	Strain	Carbon Source	Adsorption pH/Growth Conditions	Cycles of Use	Pullulan (g/L) First Use	Pullulan (g/L) Final Use	Reference
Polyurethane foam	Wild-type	Sucrose	5.5/48 h, 42 °C	18	37.0	37.0	[67]
Sponge	ATCC 42023	Maltose corn syrup	6.0/168 h, 30 °C	3	4.5	5.6	[68]
Sponge	ATCC 42023	Sucrose	6.0/168 h, 30 °C	3	5.4	6.2	[68]
Sponge	ATCC 42023	Glucose	6.0/168 h, 30 °C	3	5.4	6.7	[68]
Diatomaceous earth	ATCC 42023	Maltose corn syrup	2.0/168 h, 30 °C	2	7.0	5.3	[69]
DEAE-cellulose	ATCC 42023	Maltose corn syrup	2.0/168 h, 30 °C	2	4.5	5.8	[69]
DEAE-cellulose	ATCC 201253	Maltose corn syrup	2.0/168 h, 30 °C	2	5.6	3.9	[70]
TEAE-cellulose	ATCC 201253	Maltose corn syrup	2.0/168 h, 30 °C	2	6.4	6.9	[70]
TEAE-cellulose	ATCC 201253	Maltose corn syrup	4.0/168 h, 30 °C	2	5.7	5.1	[71]
TEAE-cellulose	ATCC 201253	Maltose corn syrup	2.0/336 h, 25 °C	2	1.7	2.6	[72]
TEAE-cellulose	ATCC 201253	Maltose corn syrup	6.0/336 h, 25 °C	2	3.6	4.4	[72]
TEAE-cellulose	ATCC 201253	Maltose corn syrup	7.5/336 h, 25 °C	2	6.4	0.6	[72]
Phosphocellulose	ATCC 201253	Maltose corn syrup	2.0/168 h, 30 °C	2	4.6	4.0	[70]
Phosphocellulose	ATCC 201253	Maltose corn syrup	4.0/168 h, 30 °C	2	3.6	4.3	[71]
ECTEOLA-cellulose	ATCC 42023	Maltose corn syrup	2.0/168 h, 30 °C	2	5.5	5.0	[73]
Zeolite	ATCC 201253	Maltose corn syrup	4.0/168 h, 30 °C	2	6.2	7.2	[74]
Zeolite	ATCC 201253	Maltose corn syrup	5.0/168 h, 30 °C	2	6.3	6.9	[74]
Plastic composite	ATCC 201253	Glucose	2.0/168 h, 30 °C	1	0.0	32.9	[75]
Plastic composite	ATCC 201253	Glucose	5.0/168 h, 30 °C	1	0.0	25.2	[75]

3.2. Ion Exchange Resins as Solid Supports

A variety of ion exchange resins have been used to immobilize *A. pullulans* cells (Table 1). Maltose corn syrup-grown ATCC 42023 cells (using ammonium sulfate as the nitrogen source) have been immobilized by adsorption onto the anionic resin diethylaminoethyl (DEAE)-cellulose using a culture medium pH of 2.0 [69]. Pullulan production by the maltose corn syrup-grown ATCC 42023 cells immobilized on the DEAE-cellulose was followed for two production cycles of 168 h at 30 °C. Similar to what was observed for the ATCC 42023 cells adsorbed onto diatomaceous earth, it was observed that there was a decrease in pullulan production of 22% following the second production cycle compared to the initial production cycle [69]. Again, it was likely that cell leakage, or a drop in cell viability, was a factor in the decrease in polysaccharide production observed. The ion exchange resin DEAE-cellulose was also utilized to adsorb the maltose corn syrup-grown cells of the reduced pigmentation mutant strain ATCC 201253 (strain RP-1) in which the medium contained ammonium sulfate as the nitrogen source [70]. This enabled a comparison between pigmented parent strain ATCC 42023 cells and the reduced pigmentation strain ATCC 201253 cells immobilized on DEAE-cellulose to be made. The ATCC 201253 cells were immobilized on the DEAE-cellulose at pH 2.0 since this culture medium pH resulted in the highest adsorption of fungal cells [70]. The DEAE cellulose-immobilized ATCC 201253 cells were shown to produce higher levels of pullulan batchwise during the first production cycle of 168 h relative to the second production cycle of 168 h. There was a 30% decrease in pullulan level produced during the second production cycle compared to the first production cycle. Although the ATCC 42023 cells immobilized on the DEAE-cellulose

produced less pullulan during the first production cycle compared to the immobilized ATCC 201253 cells, the ATCC 42023 cells immobilized on the DEAE-cellulose produced a higher pullulan level during the second production cycle, in contrast to what was observed for the immobilized ATCC 201253 cells [69,70]. It is possible that the presence of the negatively charged melanin pigment in the ATCC 42023 cells increased their interaction with the anionic DEAE-cellulose compared to the reduced melanin pigment-containing ATCC 201253 cells. Another anionic exchanger triethylaminoethyl (TEAE)-cellulose was examined for its ability to be used as a solid support to adsorb ATCC 201253 cells [70]. The highest level of ATCC 201253 cells was adsorbed onto the TEAE-cellulose when the culture medium pH was 2.0 [70]. Using the ATCC 201253 cells immobilized onto TEAE-cellulose in a batch process, pullulan levels were slightly lower after the second production cycle of 168 h compared to the pullulan concentration detected after the initial production cycle of 168 h [70]. When the ATCC 201253 cells were adsorbed onto the TEAE-cellulose at pH 4.0, polysaccharide production during the initial cycle was shown to decrease compared to the second cycle [71]. This contrasted with what was observed for polysaccharide production by the cells adsorbed onto the TEAE-cellulose at pH 2.0 [70]. The pH 2.0-adsorbed cells produced higher pullulan concentrations and exhibited increased pullulan levels following the second production cycle [70]. It was also noted that the cells immobilized on the TEAE-cellulose supported greater pullulan synthesis during both production cycles than did the cells immobilized on DEAE-cellulose [70]. It appeared that TEAE-cellulose was a more effective anion exchanger for immobilization of ATCC 201253 cells than DEAE-cellulose. A bioreactor column with an airflow rate of 300 mL/min containing ATCC 201253 cells immobilized on TEAE-cellulose at pH 2.0, 6.0 or 7.5 was investigated for pullulan production using production cycles of 336 h at 25 °C [72]. The bioreactor containing the pH 2.0-immobilized cells was capable of synthesizing low levels of pullulan for two production cycles of 336 h. There was a 1.5-fold increase in pullulan production after 336 h by the pH 2.0-immobilized cells during the second cycle compared to the initial cycle [72]. When the ATCC 201253 cells were immobilized at pH 6.0, the bioreactor produced higher pullulan levels than the pH-2.0-immobilized cells. It was observed that the pH 6.0-immobilized cells produced 1.2-fold higher polysaccharide levels during the second production cycle compared to the initial production cycle [72]. After the ATCC 201253 cells were immobilized onto TEAE-cellulose at pH 7.5, the concentration of pullulan produced by the column bioreactor after 336 h was higher than the concentration of pullulan produced by the cells immobilized onto TEAE-cellulose at pH 2.0 or 6.0 [72]. Unfortunately, pullulan production by the pH-7.5-immobilized cells dropped by about 90% during the second cycle compared to the first cycle [72]. It appeared that washing of the pH 7.5-immobilized cells resulted in a significant drop in pullulan-producing capacity. The cation exchanger phosphocellulose was tested as a possible solid support for the immobilization of maltose corn syrup-grown ATCC 201253 cells that utilized ammonium sulfate as the nitrogen source. It was determined that the ability of phosphocellulose to adsorb ATCC 201253 cells was much lower than either TEAE-cellulose or DEAE-cellulose [70]. Similar to cell adsorption on TEAE-cellulose or DEAE-cellulose, the culture medium pH used to immobilize the cells onto the phosphocellulose was 2.0. When the ATCC 201253 cells immobilized on the phosphocellulose were tested for two cycles of pullulan production for 168 h in batch processes, polysaccharide production decreased by about 13% during the second production cycle compared to the first production cycle [71]. As might be expected due to fewer ATCC 201253 cells being adsorbed onto the phosphocellulose, polysaccharide production by the immobilized cells was lower during both production cycles relative to the production cycles when the ATCC 201253 cells were immobilized on TEAE-cellulose or DEAE-cellulose [70]. The immobilization of ATCC 201253 cells on phosphocellulose, when the culture medium pH used to adsorb the fungal cells was pH 4.0, was also examined [71]. It was found that the batch polysaccharide production by the pH 4.0-immobilized cells during the initial production cycle of 168 h at 30 °C was lower than when the cells were adsorbed at pH 2.0 [71]. In contrast, the batch polysaccharide production by the pH 4.0-

immobilized cells during the second production cycle of 168 h at 30 °C was higher than when the cells were adsorbed at pH 2.0 [71]. It was apparent that the culture medium pH used to adsorb the fungal cells on the ion exchange resins was critical for the concentrations of pullulan produced. Epichlorohydrin triethanolamine (ECTEOLA)-cellulose has been used to immobilize maltose corn syrup-grown cells of ATCC 42023 cells where ammonium sulfate served as the nitrogen source [73]. As previously observed for all the ion exchange resins tested, this anionic exchange resin adsorbed the highest concentration of ATCC 42023 cells when the culture medium pH was 2.0 [73]. The immobilized cells on the ECTEOLA cellulose were capable of sustaining batch pullulan production for two cycles of use. It was seen that polysaccharide production by the immobilized cells was higher during their first cycle of use for 168 h with polysaccharide production, lowering slightly during their second cycle of use for 168 h [73]. It appeared that cell leakage from the solid support probably occurred during the washing process of the immobilized cells following the initial cycle of polysaccharide production. Overall, the anion exchangers screened as possible solid supports to adsorb the fungal cells seemed to be superior to those using the cation exchanger phosphocellulose to immobilize the fungal cells. It may be that the fungal cells are negatively charged so they are more likely to adsorb to the anion exchangers.

3.3. Zeolite and Plastic Composites as Solid Supports

The aluminosilicate zeolite was studied as a possible solid support to adsorb *A. pullulans* cells (Table 1). It was demonstrated that zeolite could adsorb *A. pullulans* cells, with the level of adsorption being dependent on the culture medium pH utilized during the adsorption process. The ATCC 201253 cells were immobilized onto zeolite using a culture medium pH of 4.0 or 5.0 [74]. When the ATCC 201253 cells were immobilized on zeolite at pH 4.0 or 5.0, polysaccharide production after 168 h by the immobilized cells produced a higher pullulan concentration during the second cycle of use than during their initial cycle of use [74]. It was observed that the increase in pullulan levels by the pH 4.0-immobilized cells was about 1.2-fold higher during the second production cycle relative to the first production cycle [74]. Similarly, the increase in pullulan levels by the pH 5.0-immobilized cells was about 1.1-fold higher during the second production cycle relative to the first production cycle [74]. Apparently, the change in culture medium pH utilized to adsorb the fungal cells onto the zeolite produced a slight change in polysaccharide production during both cycles of use. A plastic composite was used to immobilize ATCC 201253 cells by forming a biofilm [75]. This study appeared to use a pH of 2.0 or 5.0 to ensure adsorption of the cells onto the plastic composite [75]. The cells adsorbed onto the plastic composite formed a biofilm reactor with a working volume of 1.5 L. The biofilm reactor was utilized to synthesize pullulan for a period of 168 h at 30 °C (Table 1). It was noted that when the initial culture medium pH was 2.0, the biofilm reactor produced less polysaccharide after 168 h than was observed if the cells were adsorbed onto the plastic composite at pH 5.0 [75]. A 1.3-fold higher level of polysaccharide was produced at pH 5.0 than was produced at pH 2.0. The polysaccharide produced in the biofilm reactor was characterized as authentic pullulan [75]. The growth conditions in biofilm reactors have been optimized for pullulan production [76,77].

4. Pullulan Production Using Cell Immobilization Using Entrapment

4.1. Polyurethane Foam and Polyvinyl Alcohol as Agents to Entrap Fungal Cells for Pullulan Production

As shown in Table 2, *A. pullulans* cells can be immobilized in a variety of agents that can be utilized to entrap fungal cells [66,78–84]. It has also been shown that pullulan production varies according to how the fungal cells are entrapped. ATCC 42023 cells (grown in culture medium pH 5.5 at 28 °C for 16 h) were immobilized by entrapment in a hydrophilic polyisocyanate solution with water to form a foam [66]. The cells entrapped in polyurethane were utilized for semi-continuous production of pullulan for four cycles of 96 h [66]. It was found that the immobilized cells synthesized increasingly higher levels

of pullulan during successive cycles of production [66]. The electron micrographs of the polyurethane foam showed that the fungal cells were not only entrapped in the foam, but they had also coated the foam. This finding likely indicates why pullulan production by the polyurethane-immobilized cells increased during each successive cycle of use [66]. Polyvinyl alcohol has also been employed as an agent to entrap *A. pullulans* cells in a gel [78]. Cells of *A. pullulans* strain Y-4137 were mixed with an 11% polyvinyl solution, stored at −80 °C for 24 h, and then thawed to yield cryogel granules of immobilized cells [78]. The immobilized cell granules were grown in a glucose-containing medium for 45 h at 26 °C for up to 16 production cycles of use with little loss of pullulan production during the analysis [78]. It appeared that the immobilized cryogels produced from the polyvinyl alcohol treatment would be very effective in the semi-continuous production of pullulan.

Table 2. Growth conditions and maximum pullulan production by *Aureobasidium pullulans* strains immobilized by entrapment.

Entrapment Agent	Strain	Carbon Source	Growth Conditions	Cycles of Use	Pullulan (g/L) First Use	Pullulan (g/L) Final Use	Reference
Polyurethane foam	ATCC 42023	Sucrose	96 h, 28 °C	4	12.0	18.0	[66]
Polyvinyl alcohol	Y-4137	Glucose	45 h, 26 °C	16	10.0	9.0	[78]
2% Calcium alginate	P56	Sucrose	120 h, 28 °C	7	21.0	10.0	[79]
5% Calcium alginate	ATCC 42023	Glucose	168 h, 30 °C	2	7.6	4.0	[80]
5% Calcium alginate	ATCC 42023	Sucrose	168 h, 30 °C	2	10.3	4.0	[80]
5% Calcium alginate	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	4.3	4.8	[81]
4% Agar	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	4.4	6.8	[81]
5% Calcium alginate	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	3.4	5.6	[82]
4% Agar	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	3.9	4.0	[82]
4% Agarose	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	4.4	5.6	[84]
3% Carrageenan	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	4.7	4.8	[84]
1% Chitosan	ATCC 201253	Maltose corn syrup	168 h, 30 °C	2	6.7	5.4	[85]

4.2. Calcium Alginate and Agar as Cell Entrapment Agents for Pullulan Production

A number of investigations have studied the immobilization of *A. pullulans* cells using calcium alginate beads (Table 2). The popularity of using calcium alginate beads is probably related to the ease of preparation, as well as to their ability to maintain fungal cell viability [79]. An initial study utilized *A. pullulans* strain P56 cells immobilized in 2% calcium alginate beads [79]. The immobilized cells were used batchwise to produce pullulan for seven cycles of use in a sucrose-containing culture medium buffered to pH 6.5 to 8.5 [79]. The polysaccharide production by the immobilized cells was maximal during the initial cycle and decreased with each subsequent cycle. Polysaccharide production during the final cycle decreased by more than 50% compared to the initial cycle of use. [79]. It was demonstrated that the calcium alginate beads were not subject to deformity during repeated use [79]. It was also noted that the polysaccharide synthesized by the immobilized cells was primarily pullulan [79]. In another study exploring pullulan production by *A. pullulans* cells immobilized in calcium alginate beads, the concentration of alginate to produce the beads was increased to 5% [80]. The cells of ATCC 42023 were grown on the carbon source glucose or sucrose in a medium containing ammonium sulfate prior to being immobilized. The glucose-grown ATCC 42023 cells immobilized in the calcium alginate beads were tested for their ability to synthesize polysaccharide for two batch production cycles of 168 h at 30 °C [80]. It was observed that polysaccharide production was higher during the initial production cycle, with the second production cycle showing a nearly 50% drop in polysaccharide production [80]. Both a decrease in productivity and yield

of polysaccharide production by the calcium alginate-immobilized glucose-grown cells was observed during the second cycle compared to the first cycle [80]. Interestingly, the pullulan content of the polysaccharide being elaborated by the immobilized cells increased during their second cycle of use compared to their first cycle of use. The sucrose-grown ATCC 42023 cells immobilized in the calcium alginate beads in batch cultures produced a higher level of polysaccharide than did the glucose-grown immobilized cells after 168 h at 30 °C [80]. During the second cycle of use, the sucrose-grown ATCC 42023 cells immobilized in the calcium alginate beads produced less polysaccharide than they did during the initial cycle of use. It should be mentioned that the polysaccharide levels produced by the glucose- or sucrose-grown immobilized cells were the same during their second cycles of use [80]. In addition, a diminution in productivity and yield of polysaccharide production by the calcium alginate-immobilized sucrose-grown ATCC 42023 cells was seen during the second cycle compared to the first cycle [80]. The pullulan content of the polysaccharide elaborated by the calcium alginate-immobilized sucrose-grown ATCC 42023 cells rose during the second production cycle compared to the pullulan content of the polysaccharide synthesized during the initial cycle [80]. The reduced pigmentation mutant strain ATCC 201253 cells grown on maltose corn syrup as a carbon source were also immobilized in 5% calcium alginate beads [81]. It was observed that polysaccharide production and productivity in batch cultures by the calcium alginate-immobilized cells increased slightly during their second cycle of use for 168 h compared to their initial cycle of use for 168 h [81]. Unfortunately, the pullulan content of the polysaccharide synthesized by the immobilized cells remained low during both production cycles [81]. The maltose corn syrup-grown ATCC 201253 cells immobilized in the 5% calcium alginate beads were also studied at 25 °C for their performance in a column bioreactor aerated at 500 mL/min [82]. Similar to what was observed when the calcium alginate beads were used in batch cultures, the beads synthesized a higher polysaccharide level during the second cycle of use for 168 h than for their initial cycle of use for 168 h [82]. The pullulan content of the polysaccharide elaborated by the calcium alginate beads in the bioreactor during either production cycle was less than 50% authentic pullulan [82]. Another agent used to entrap *A. pullulans* cells was agar (Table 2). Maltose corn syrup-grown cells of ATCC 201253 were immobilized in 4% agar cubes and investigated for their ability to synthesize polysaccharide for two cycles of 168 h in batch cultures. The agar-immobilized cells exhibited an ability to synthesize a higher level of polysaccharide during their second cycle of use than the polysaccharide level synthesized during their initial cycle of use. In addition, the productivity of the agar cubes was found to increase in their second cycle of use relative to their first cycle of use [81]. The pullulan content of the polysaccharide synthesized by the agar cubes remained relatively high and constant during both production cycles [81]. In a column bioreactor, maltose corn syrup-grown ATCC 201253 cells immobilized in the 4% agar cubes were investigated for their ability to produce polysaccharide for two cycles for 168 h at 25 °C. The aerated (500 mL/min) column bioreactor containing the immobilized cells within the agar cubes produced about the same levels of polysaccharide during each cycle of use after 168 h. The pullulan content of the polysaccharide produced by the agar-immobilized cells in the column bioreactor increased during the second cycle of use relative to their first cycle of use [82]. In another report, an agar-microporous membrane was used to immobilize cells of the fungus *A. pullulans* CNCM 1726.88 [83]. The immobilized cells were placed in 250 mL bioreactors containing glucose as a carbon source for 168 h at 28 °C, but the structure of the agar-microporous membrane became deformed due to stress from internal biomass production and their production of pullulan could not be effectively determined [83].

4.3. Agarose, Carrageenan and Chitosan as Cell Entrapment Agents for Pullulan Production

Another entrapment agent that was used to immobilize cells capable of pullulan production was agarose (Table 2). Maltose corn syrup-grown cells of *A. pullulans* ATCC 201253 were entrapped in cubes of 4% agarose [84]. The ATCC 201253 cells immobilized in agarose were examined for two batch production cycles of 168 h at 30 °C [84]. It was shown that

the immobilized cells produced a 1.3-fold higher level of polysaccharide during the second cycle compared to the polysaccharide level synthesized during the initial cycle. Similarly, the polysaccharide productivity was 1.6-fold higher during the second production cycle compared to the first production cycle [84]. The pullulan content of the polysaccharide synthesized by the agarose-immobilized cells was relatively high during both production cycles [84]. The entrapment agent carrageenan was also utilized to immobilize maltose corn syrup-grown cells of *A. pullulans* ATCC 201253 (Table 2). The immobilized ATCC 201253 cells were studied for their ability to produce polysaccharide in batch cultures for two production cycles of 168 h at 30 °C [84]. The results indicated the carrageenan-immobilized cells generated roughly equivalent levels of polysaccharide yield during both production cycles [84]. The productivity of the carrageenan-immobilized cells was 1.4-fold higher during the first production cycle relative to the second production cycle [84]. The pullulan content of the polysaccharide synthesized by the immobilized ATCC 201253 cells was low for the initial cycle of production but did improve for the polysaccharide elaborated during the second cycle [84]. Chitosan beads, formed by mixing maltose corn syrup-grown ATCC 201253 cells in a chitosan acetate solution and forming beads in a pentasodium tripolyphosphate solution, were evaluated (Table 2) for their ability to synthesize polysaccharide [85]. Polysaccharide synthesis by the chitosan-immobilized ATCC 201253 cells in batch cultures was investigated for two production cycles of 168 h at 30 °C [84]. The highest polysaccharide level synthesized by the immobilized cells was observed after the initial production cycle, with a 20% decrease in the polysaccharide level being noted after the second production cycle [85]. The pullulan content of the polysaccharide synthesized by the immobilized ATCC 201253 cells during the initial production cycle was high, while the pullulan content of the polysaccharide elaborated during the second production cycle was low [85]. There was no apparent reason for the drop in pullulan content of the polysaccharide synthesized by the chitosan-immobilized cells during the second production cycle. It may indicate that a change occurred in the entrapped cells to stimulate the synthesis of a non-pullulan polysaccharide.

5. Conclusions

Overall, it is evident that cells of *A. pullulans* can be immobilized either by adsorption onto carrier-binding supports or entrapment in gel matrices. It was observed that adsorption of the pullulan-producing fungal cells onto solid supports sustained polysaccharide production by *A. pullulans* cells grown on a variety of carbon sources. The problem associated with fungal cells adsorbed onto the solid supports for polysaccharide production is that it is difficult to differentiate between the polysaccharide synthesized by the immobilized cells and that synthesized by free cells leaked from the solid supports. The most effective solid support of those screened was found to be polyurethane foam since it could be used for 18 production cycles without any decrease in pullulan production. Higher concentrations of polysaccharide were synthesized for several cycles when the pullulan-producing fungal cells were entrapped in gel matrices compared to their being adsorbed onto the solid supports. A difficulty associated with the entrapped fungal cells is the quality of the polysaccharide synthesized. The pullulan content of the polysaccharide synthesized by the entrapped fungal cells tended to be low. This may indicate that polysaccharide synthesis in the entrapped *A. pullulans* cells is significantly altered, causing the immobilized fungal cells to elaborate non-pullulan-type polysaccharides.

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