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Review

Impact of *Aureobasidium* Species Strain Improvement on the Production of the Polysaccharide Pullulan

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Abstract: This review explores the production of the fungal polysaccharide pullulan by mutants and natural isolates of *Aureobasidium* species using strain improvement. Pullulan is a neutral polysaccharide gum whose structure is a maltotriose-containing glucan. This polysaccharide gum has applications in the fields of food, pharmaceuticals, biomedical and wastewater treatment. The strain improvement of *Aureobasidium* species has focused on the pullulan production process, including the isolation of strains exhibiting reduced pigmentation, polysaccharide overproduction, the production of pullulan with variable molecular weight, and increased osmotolerant strains promoting pullulan production at high carbon source concentrations and pullulan production on hemicellulosic substrates. The majority of studies have emphasized the isolation of reduced pigmentation and pullulan hyperproducer strains since the goal of large-scale commercial pullulan production is to synthesize non-pigmented polysaccharides. A promising area of strain improvement is the isolation of strains that synthesize authentic pullulan from hemicellulosic substrates. If strain improvement in this area is successful, the goal of commercially producing pullulan at a competitive cost will eventually be achieved.

Keywords: pullulan; polysaccharide; strain improvement; mutants; isolates; pigmentation; overproducer; *Aureobasidium pullulans*; *Aureobasidium melanogenum*

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1. Introduction

The yeast-like imperfect fungus *Aureobasidium pullulans* or *Aureobasidium melanogenum* synthesizes the complex polysaccharide pullulan extracellularly into the culture medium [1]. Pullulan is a neutral polysaccharide gum [1]. Only certain varieties of *A. pullulans* taxonomically assigned to the genus *Aureobasidium* are able to synthesize the polysaccharide pullulan [2,3]. Pullulan behaves as a biopolymer that is characterized as having nylon-like properties. The polysaccharide is water-soluble and can be precipitated by the addition of alcohol [4]. The molecular weight of the pullulan synthesized by a specific strain of *A. pullulans* has been found to vary, with the molecular weight ranging from 50,000 to 2,500,000 [5–7]. The structure of pullulan (Figure 1) has been determined to be a complex linear polysaccharide consisting of maltotriose and maltotetraose units linked through α-D-(1→6) bonds on its terminal glucose residues [5–7]. Several commercial patented applications involving pullulan have been developed. Its applications as a fungal gum include being utilized as a flocculant for wastewater purification, films for pharmaceutical coatings, an adhesive in tissue engineering, a packaging film for foods, and a gel within cosmetics and a component in nanoparticles [8–37]. Based on its properties, pullulan has proven useful for a number of commercial applications as a gum [8–37]. Commercial applications of pullulan include its use in the films in Listerine PocketPaks®, as well as the Listerine Whitening Quick Dissolving Strips® [38].

Considering the applications reported for pullulan, a number of studies have examined polysaccharide production using selected strains of *A. pullulan* [39–59]. A variety of carbon sources have been used for pullulan synthesis by *A. pullulans* [39–44]. It has

been demonstrated that the type of carbon source added to the culture medium affects the pullulan content of the polysaccharide produced by the fungal strains. A high pullulan content of the polysaccharide produced by *A. pullulans* was shown when either glucose or maltose corn syrup served as a carbon source. In contrast, it was determined that if sucrose served as the carbon source for the fungus, then a low pullulan content of the polysaccharide was synthesized by the fungus [44]. The fermentation of each carbon source by the fungus results in the pH of the culture medium becoming more acidic. As the pH of the fungal culture medium decreases, pullulan elaboration by *A. pullulans* is stimulated [45–48]. In several studies, the influence of the culture medium pH on fungal polysaccharide synthesis has been studied. From these studies, it was concluded that the optimal initial pH of the culture medium was 6.0–6.5 for fungal pullulan synthesis, with the optimal pH of the medium also being dependent on the carbon source supplemented into the medium [45–48]. Nitrogen availability is also critical to the rate of pullulan production by the fungus [49–52]. The optimal temperature for polysaccharide production by the fungus is 26 °C, independent of the carbon source tested [53]. The composition of the culture medium has also been found to influence fungal polysaccharide synthesis [54]. Yeast extract is known to be an important component in the culture medium in regard to promoting fungal pullulan synthesis [55]. In the absence of yeast extract, the optimal culture medium pH is 5.5 or below, depending on the carbon source [55]. 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, but no specific vitamin supplement could be used to replace yeast extract [55]. It does appear that hydrolyzed plant biomass may be able to be used as a substitute for yeast extract in the production medium [24,56,57]. The supplementation of the mineral salt ferric chloride or manganese chloride has been shown to increase fungal pullulan production [54,55]. Other growth factors, such as fungal cell density and oxygen concentration, are also important to pullulan synthesis [58]. The discoloration of pullulan by melanin is a significant problem during its production since the precipitated polysaccharide appears gray and requires a step involving activated carbon to remove the melanin pigment [59].

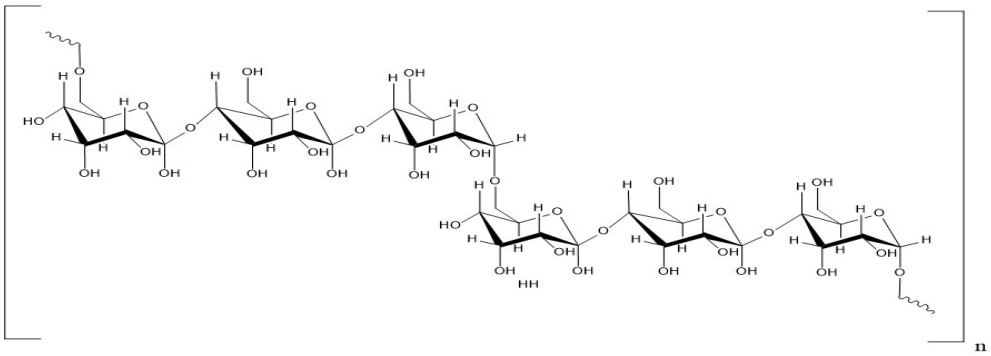

Figure 1. The structure of pullulan involving cross-linked maltotriose units.

The biochemistry of pullulan synthesis in species of *Aureobasidium* involves the glycolytic enzymes hexokinase, glucose isomerase, and phosphofructokinase to provide the substrates for pullulan synthesis [37,60,61]. Other enzymes critical to pullulan biosynthesis include the enzymes α-phophoglucomutase, uridine diphosphoglucose pyrophosphorylase, glycosyltransferase, and a pullulan synthetase contained within an α-glucan synthetase enzyme complex [62,63]. It has been shown that the upregulation of the enzymes involved in pullulan biosynthesis by induction occurs in the pullulan-producing strains of *Aureobasidium* [37,60,61].

A possible way to make the pullulan production process more economical is *A. pullulans* strain improvement. The isolation of mutant strains of *A. pullulans* that increase polysaccharide production or eliminate a downstream processing step should decrease the

cost of pullulan production. It should be noted that a number of studies have reported the isolation of mutant strains that could reduce the costs associated with the commercial production of pullulan [1,2,9]. This review examines prior investigations that have reported the isolation of *A. pullulans* and *A. melanogenum* mutants or natural isolates that could impact the large-scale production of pullulan. This would include studies reporting strains exhibiting reduced pigmentation, pullulan overproduction, the production of pullulan with variable molecular weight, increased sugar osmotolerance allowing elevated pullulan production, and hemicellulose utilization for pullulan production. In this review, the mutants or natural isolates that have been reported in prior studies are perused from a strain improvement perspective for possible impacts on the commercial production of pullulan.

2. Reduced Pigmentation Strains

In the commercial process of pullulan production using normally pigmented strains, an additional polysaccharide purification step of removing the melanin from the polysaccharide by treatment with activated charcoal is necessary. The elimination of this step in the purification of pullulan by using reduced pigmentation strains should help reduce the cost of synthesizing pullulan. Another advantage of using the reduced pigmentation strains is that it has been shown that they synthesize authentic pullulan similarly to the pigmented *A. pullulans* strains.

It appears that the tyrosinase gene cluster in *A. pullulans* is responsible for melanin pigment synthesis [64]. It was found that disruption of the pullulan synthetase in *A. pullulans* strain NP1221 also caused a disruption in the tyrosinase cluster. This resulted in the reduced pigmentation of the strain NP1221's sucrose-grown cells. The cells of strain NP1221 were unable to produce pullulan but did produce β-glucan [64]. It is likely that *A. pullulans* mutant strains exhibiting reduced melanin synthesis involve a disruption in the tyrosinase gene cluster. This was confirmed in another investigation where the pullulan synthetase gene (*pul*) was inactivated [65]. It was observed that both pullulan and melanin syntheses were halted when the pullulan synthetase in the strain was inactivated. It was thought that the enzyme polyketide synthase, critical to melanin synthesis, was affected by the inactivation of the pullulan synthase [65]. The addition of melanin to *A. pullulans* cells increased pullulan yield by 31% [66]. It is clear that there is a relationship between the production of melanin and the synthesis of pullulan [59].

A number of prior investigations have investigated the isolation of *Aureobasidium* strains that were either mutants or natural isolates. In a prior study, a reduced pigmentation mutant strain AP41 of *A. pullulans* was selected from its parent strain AP11. Initially, the mutant strain AP24 was identified from strain AP11 using ethidium bromide resistance [66]. The polysaccharide produced by strain AP24 was found to have a 13-fold higher viscosity than the polysaccharide synthesized by strain AP11 when both strains utilized sucrose as a carbon source [66]. Subsequently, the reduced pigmentation mutant strain AP41 was identified following ultraviolet mutagenesis [66]. Strain AP41 was still capable of pullulan production when using corn syrup or sucrose as a carbon source, similar to what was observed when its parent strain was grown on corn syrup or sucrose as a carbon source [66].

Another study reported the isolation of a reduced pigmentation mutant strain of *A. pullulans* ATCC 42023 using nitrous acid mutagenesis [67]. ATCC 42023 cells were subjected to nitrous acid mutagenesis, and the mutant strain ATCC 201253 was selected [67]. As indicated in Figure 2, a 15-fold reduction in melanin pigmentation was noted for ATCC 201253 cells compared to ATCC 42023 cells when the strains were grown on 2.5% (*w*/*v*) sucrose for 168 h at 30 °C [67]. Similarly, an 11-fold reduction in melanin pigmentation was noted for ATCC 201253 cells compared to ATCC 42023 cells when each strain was grown on 2.5% (*w*/*v*) glucose [67]. The pullulan and biomass levels produced by ATCC 42023 and ATCC 201253 cells on either carbon source were similar after 168 h at 30 °C [67]. The pullulan content of the polysaccharide synthesized by ATCC 201253 cells was observed to be higher than the pullulan content of the polysaccharide produced by ATCC 42023 on

either carbon source [67]. This reduced pigmentation mutant strain of *A. pullulans* has been utilized in a number of investigations exploring batch pullulan production [51,56,57].

Figure 2. The reduced pigmentation *A. pullulans* strain RP-1/ATCC 201253 (**left**) compared to the typical melanin pigmentation of its parent strain *A. pullulans* strain ATCC 42023 (**right**).

Using the previously isolated reduced pigmentation mutant strain ATCC 201253, a melanin-deficient mutant strain, *A. pullulans* SZU 1001, was selected [68]. The mutant strain SZU 1001 was isolated by ultraviolet and gamma-ray mutagenesis. It was observed that the glucose-grown cells of the mutant strain produced a slightly higher level of pullulan than the level produced by the glucose-grown ATCC 201253 cells at 30 °C with aeration (200 rpm) after 72 h [68]. The molecular weight of the polysaccharide produced by the parent or mutant strain cells on glucose as a carbon source was determined to be about 1.8×10^6 daltons. This study concluded that the lack of melanin synthesis in the mutant strain SZU 1001 cells did not affect the ability of the cells to produce pullulan.

A melanin-deficient strain P 56 of *A. pullulans* has been used in prior reports, although its isolation has not been described [69,70]. In one study, strain P 56 was used in a shake flask, batch, or continuous fermentation. The pullulan production of P 56 was shown to be higher when the strain was grown on sucrose compared to utilizing glucose, fructose, xylose, or lactose [69]. It was noted that strain P 56 produced 1.7-fold higher pullulan levels compared to glucose or fructose as a carbon source after 168 h at 28 °C [69]. A second study that used *A. pullulans* P 56 focused on pullulan production by the strain grown on the carbon source sucrose [70]. It was determined that the batch production of pullulan was more effective than fed-batch production. The pullulan synthesized by strain P 56 was determined to be authentic pullulan, with the molecular weight of the pullulan being about 10^6 daltons [70].

Using ultraviolet light mutagenesis of *A. pullulans* ATCC 42023, the strain HP-2001 was selected that exhibited reduced pigmentation of its cells [71,72]. In an initial study [71], HP-2001 was grown in a glucose-containing medium in batch cultures as well as in a 7-L or 100-L bioreactor, where it produced similar pullulan levels determined for its parent strain after 72 h at 30 °C [71]. The second investigation using HP-2001 [72] demonstrated that the strain could utilize glucose, sucrose, or dextrin as a carbon source to support pullulan synthesis. The production of pullulan by strain HP-2001 using either glucose or sucrose (100 g/L) in a 7-L bioreactor operating for 72 h at 30 °C was compared. The strain produced 74.9 g/L pullulan in the sucrose-containing medium compared to 38.1 g/L pullulan in the glucose-containing medium [72]. Using continuous culture conditions at 30 °C for 72 h for strain HP-2001 in a medium containing 100 g/L, 150 g/L, or 200 g/L sucrose, it was found that 69.39 g/L, 86.3 g/L, or 113.5 g/L pullulan was produced [72]. It was concluded that sucrose as a carbon source was more suitable for pullulan production by HP-2001 than the other carbon sources tested.

A white mutant of *A. pullulans* strain A.p.-3 was isolated using ethyleneimine, and ultraviolet light irradiation [73,74]. The isolated mutant strain B-1 was selected and identified as a reduced pigmentation mutant [73]. The cellular melanin content in the mutant strain B-1 cells was reduced by 13-fold compared to the cellular melanin in the parent strain A.p.-3 cells when grown in a medium containing 60 g/L sucrose after 96 h at 28 °C with aeration at 150 rpm [73]. The polysaccharide produced by the mutant strain was not contaminated by melanin pigments. The pullulan levels produced by the parent strain and the mutant

strain were nearly equivalent after 96 h 28 °C. It was demonstrated that authentic pullulan was produced by A.p.-3 and B-1 when the strains were grown on sucrose [73]. A later study also used the reduced pigmentation mutant strain B-1 in a 3-L bioreactor fermenter [74]. The fermenter was operated for 96 h at 28 °C, with aeration at 150 rpm. The mutant strain synthesized about 26 g/L pullulan in the fermenter [74].

Natural isolates of *A. pullulans* and *A. melanogenum* that exhibit reduced pigmentation have been isolated [75,76]. A natural isolate of *A. pullulans* was selected from strawberry fruit that produced pigment-free pullulans [75]. The swollen cells of the strain NG synthesized pullulan with little melanin contamination, which was characterized as authentic pullulan. It was shown that the strain NG cells produced pullulan in a glucose-containing medium [75]. In *A. melangogenum*, a natural isolate from mangrove, identified as P5, exhibited reduced melanin pigmentation as well as increased thermotolerance and halotolerance [76].

By virtue of the number of reduced pigmentation strains from *A. pullulans*, it is clear that the use of these strains in the large-scale production of pullulan is economically advantageous. The primary advantage of using these strains in the pullulan production process is that the polysaccharide being synthesized will not be highly pigmented, and the production level in most cases is equivalent to its pigmented parent strain.

3. Hyperproducer Strains

A number of strains of *A. pullulans* or *A. melanogenum* that exhibit the hyperproduction of pullulans have been identified (Table 1). The hyperproducing pullulan strains were either mutants or natural isolates from various sources [77–93]. In one report, the mutant strain RG-5 of *A. pullulans* was isolated from a thermotolerant parent strain using methylmethane sulfonate and the fungal growth inhibitors nystatin and 2-deoxyglucose [77]. The strain RG-5 was shown to produce higher pullulan levels on the carbon sources (4%, *w*/*v*) sucrose or glucose (4%, *w*/*v*) compared to fructose, lactose, or xylose [77]. When glucose served as a carbon source, the mutant strain produced 25.5 g/L pullulan compared to the 23 g/L pullulan synthesized by its parent strain after 96 h at 42 °C [77]. The biomass production of strain RG-5 and the parent strain were similar after 96 h at 42 °C [77].

Table 1. Growth conditions and maximum pullulan production by pullulan-hyperproducer strains of *Aureobasidium* species.

Species	Strain	Carbon Source (%, *w*/*v*)	Growth Conditions	Maximum Pullulan (g/L)	Reference
A. pullulans	RG-5	Glucose (4%)	96 h, 42 °C	25.5	[77]
A. pullulans	ICCF-68	Glucose (8%)	48 h, 28 °C	50.2	[78]
A. pullulans	NYS-1	Sucrose (2.5%)	168 h, 30 °C	14.9	[79]
A. pullulans	DG-1	Sucrose (2.5%)	168 h, 30 °C	17.7	[79]
A. pullulans	NYSRP-1	Sucrose (5%)	168 h, 30 °C	20.0	[80]
A. pullulans	M-uncol	Sucrose (25%)	96 h, 28 °C	67.0	[81]
A. pullulans	F3-2	Hydrolyzed Starch (5%)	72 h, 28 °C	20.7	[82]
A. pullulans	MTCC 6994	Sucrose (5%,)	168 h, 30 °C	23.1	[83–86]
A. pullulans	RYLF-10	Sucrose (5%)	168 h, 28 °C	45.2	[87,88]
A. pullulans	BL06ΔPMA	Sucrose (6%)	120 h, 28 °C	140.0	[89]
A. melanogenum	P16	Sucrose (12%)	120 h, 28 °C	67.4	[90]
A. melanogenum	AMY-PKS-11	Sucrose (14%)	96 h, 28 °C	103.5	[91]
A. melanogenum	A4	Glucose (16.3%) & Maltose (38.7%)	120 h, 30 °C	122.3	[92]
A. melanogenum	ZH27	Sucrose (15%)	132 h, 28 °C	115.4	[93]

Using *A. pullulans* strain YB-4515, a hyperproducer mutant strain ICCF-68 was isolated [78]. The mutant strain was grown in a 12-L fermenter with a working volume of 8-L of an 8% (*w*/*v*) glucose-containing medium. Strain ICCF-68 synthesized 50.2 g/L pullulan after 48 h at 28 °C. It was found that both pullulan and biomass production by the mutant strain increased during the 48-hour fermentation period [78].

Another study utilized the known pullulan-producing strain *A. pullulans* ATCC 42023 to isolate strains exhibiting pullulan hyperproduction [79]. Initially, ATCC 42023 cells were subjected to 1% methylmethane sulfonate for 10 min, with the mutagenized cells being allowed a period of outgrowth. The mutagenized cells were spread on a potato dextrose solid medium containing 258 units/mL nystatin or on a solid minimal containing 0.1% glucose and 0.1% deoxyglucose. After 10–14 days of incubating the plates at 30 °C, the mutant strain NYS-1 or strain DG-1 was identified as pullulan hyperproducers [79]. When strain NYS-1 was grown on 2.5% corn syrup as a carbon source, the mutant strain synthesized a 1.5-fold higher level of pullulan than did the parent strain ATCC 42023 after 168 h at 30 °C [79]. Using 2.5% sucrose as a carbon source, strain NYS-1 produced a 1.4-fold higher level of pullulan than its parent strain after 168 h at 30 °C [79]. Strain DG-1 grown on 2.5% corn syrup synthesized a 1.3-fold higher pullulan concentration than the polysaccharide concentration produced by ATCC 42023 after 168 h at 30 °C [79]. If 2.5% sucrose served as the carbon source, strain DG-1 was noted to synthesize only a slightly higher pullulan level than did the parent strain after 168 h at 30 °C [79]. The pullulan content of the polysaccharide produced by the mutants NYS-1 and DG-1 was very similar to the pullulan content of the parent strain ATCC 42023, regardless of whether the strains were grown on 2.5% corn syrup or sucrose as a carbon source [67]. A later investigation isolated a reduced pigmentation spontaneous mutant derived from strain NYS-1 that was designated as strain NYSRP-1 [80]. The cellular pigmentation of strain NYSRP-1 was reduced by at least 32-fold compared to its parent strain NYS-1 [80]. The lack of cellular pigmentation of strain NYSRP-1 had little effect on the production of polysaccharides, independent of whether it was grown on corn syrup or sucrose as a carbon source after 168 h at 30 °C [80]. It was determined that the growth of strain NYSRP-1 produced the highest levels of pullulan on either 5% (*w*/*v*) corn syrup or sucrose as a carbon source after 168 h at 30 °C [80]. The pullulan content of the polysaccharide synthesized by strain NYSRP-1 on either corn syrup or sucrose was measured and found to be high [80].

A wild-type strain of *A. pullulans* had its cells subjected to ultraviolet mutagenesis for 15 min. Subsequently, a mutant strain, M-uncol, which overproduced pullulan after 72 at 28 °C in a sucrose-based culture medium, was identified [81]. Using this mutant under optimized growth conditions in a 25% sucrose-containing medium, the strain was capable of synthesizing 67 g/L pullulan after 96 h at 28 °C [81]. It was not clear whether the mutant strain isolated was synthesizing authentic pullulan.

Using *A. pullulans* N3.387 as a parent strain, genome shuffling was performed using ethylmethane sulfonate or ultraviolet light mutagenesis at 28 °C [82]. The mutagenized cells were spread on sucrose-containing solid medium Trypan blue. Colonies on the solid medium that appeared blue were screened as possible hyperproducers [82]. Following the screening process, two mutant strains, EMS-1 and UV-1, were identified. This improved the raw material utilization ratio of the recombinant strains that were created using protoplast fusion. Using three cycles of protoplast fusion, a strain F3-2 was isolated that exhibited a 1.8-fold increase in pullulan production compared to pullulan production by its parent strain N3.387 [82].

A natural isolate of *A. pullulans* was identified following its selection from the plant *Ficus benjaming* and characterized as a pullulan hyperproducer [83]. The isolate was first designated as *A. pullulans* FB-1 and now has an accession number of MTCC 6994. Strain MTCC 6994 and another pullulan-producing strain NCIM 976 were grown in a 5% (*w*/*v*) sucrose-containing medium pH 6.5 with aeration (150 rpm) at 30 °C for 168 h [83]. It was observed that strain MTCC 6994 produced a 1.9-fold higher pullulan level than strain NCIM 976 when grown under the same conditions. It was confirmed by spectral analysis

that the polysaccharide being synthesized by strain MTCC 6994 was pullulan [83]. In a second study using strain MTCC 6994, the downstream processing of the polysaccharide synthesized on the 5% (*w*/*v*) sucrose-containing medium was characterized and found to be 94.3% authentic pullulan compared to a commercial sample of pullulan hydrolyzed by pullulanase for 360 min [84]. A third study involving MTCC 6994 was conducted to determine the optimum medium composition to synthesize pullulan [85]. After the strain was grown on the optimized sucrose-containing medium for 168 h at 30 °C, the fungus produced 44.2 g/L pullulan and 10 g/L cellular biomass and utilized 96.9% of the sucrose in the medium [85]. A final study explored the production of pullulan by strain MTCC 6994 in a 1.5-L stirred tank bioreactor with a working volume of 1 L that was agitated at 300 rpm for up to 216 h at 30 °C [86]. A 5% (*w*/*v*) sucrose-containing medium was utilized in the fermenter. The maximum pullulan and biomass levels were noted to be 48 g/L pullulan and 9.9 g/L cellular biomass after 144 h at 30 °C with sugar utilization being 97% [86]. An X-ray diffraction of the polysaccharide produced by the strain confirmed that the structure of pullulan [86].

The *A. pullulans* strain RYLF-10 was another natural isolate. The strain was characterized as a hyperproducer that produced a high level of polysaccharide on 5% (*w*/*v*) sucrose as a carbon source. It was observed that strain RYLF-10 synthesized 45.2 g/L polysaccharide after 168 h at 28 °C, with the shake flasks being aerated at 150 rpm [87]. A fungal isolate from a leaf sample was identified as a pullulan producer. The strain was shown to be *A. pullulans* and was designated as MG271838. Strain MG271838 was found to grow on 5% (*w*/*v*) glucose, sucrose, fructose, mannose, and xylose as carbon sources and yeast extract or ammonium sulfate as nitrogen sources at 28 °C for 168 h with aeration (200 rpm) in a medium buffered at pH 6.5 for 168 h [88]. When the strain was grown in a 6% (*w*/*v*) sucrose-containing medium at 28 °C, the pullulan concentration produced by the strain was determined to be 38.57 g/L [88]. In sucrose-grown MG271838 cells, it was found that the gene expression of the glucosyltransferase, phosphoglucomutase, and uridine diphosphate pyrophosphorylase were elevated [88]. This result would be expected for a pullulan hyperproducer, considering that these enzymes are associated with pullulan biosynthesis. Another strain isolated from leaf samples was *A. pullulans* strain BL06 [89]. Strain BL06 was grown in 14% (*w*/*v*) sucrose-containing phosphate-buffered medium (0.3% yeast extract) with aeration (200 rpm) at 28 °C for 168 h with 37 g/L pullulan being synthesized [89]. The pullulan content of the polysaccharide was 97%. The molecular weight of pullulan synthesized by strain BL06 was 3.3×10^6 Da, which was the highest molecular weight of pullulan reported [89]. The gene for polymalic acid synthase was deleted from strain BL06 and it was found that the resultant knockout strain produced 64.4 g/L pullulan after 120 h in shake flasks and up to 140 g/L pullulan after 120 h in a 5-L fermenter [89]. This knockout strain produced a 1.7-fold higher pullulan level than did the parent strain BL06 grown under the same culture conditions. The molecular weight of the pullulan produced by the strain lacking polymalic synthase was substantially lower than previously reported pullulan molecular weights [89]. The gene for melanin synthesis in strain BL06 was deleted to learn if pullulan production was affected by no melanin synthesis. It was observed that strain BL06 lacking melanin synthesis actually produced a slightly higher level of pullulan (42 g/L) than did the parent strain [89].

A number of pullulan hyperproducer strains of *A. melanogenum* have been isolated from natural sources. The *A. melanogenum* strain P16 was identified as a pullulan hyperproducer [90]. Strain P16 was shown to produce pullulan on sucrose, glucose, or fructose with sucrose being the optimal carbon source for pullulan production [90]. When strain P16 was grown batchwise for 72 h at 28 °C with aeration (180 rpm) in a 12% (*w*/*v*) sucrose-containing medium, it produced 65.3 g/L pullulan as well as 18.7 g/L cellular biomass [90]. A yield of 0.81 g/g of sucrose and a productivity of 0.54 g/L/h was observed at the flask level [90]. When strain P16 was grown in a 10-L fermenter in a 12% (*w*/*v*) sucrose-containing medium for 120 h at 28 °C with aeration (300 rpm), it produced 67.4 g/L polysaccharide and 23 g/L cellular biomass with a yield of 0.62 g/g of sucrose and productivity of 0.56 g/L/h [90]. The

molecular weight of the polysaccharides was 6.699 × 10^4 Da [90]. Using spectral analysis, the polysaccharide synthesized by strain P16 was characterized as pullulan [90]. Another strain of *A. melanogenum* was isolated from natural honey and was designated as strain TN3-1. The genes for α-amylase and melanin synthesis were deleted from strain TN3-1, giving rise to mutant strain AMY-PKS-11 [91]. This mutant strain produced 103.5 g/L non-pigmented pullulan from 14% (*w*/*v*) sucrose-containing medium when grown batchwise in cultures grown at 180 rpm for 96 h at 28 °C [91]. Mutant strain AMY-PKS-11 was noted to be a hyperproducer like its parent strain TN3-1 since both strains synthesized high levels of pullulan. The pullulan hyperproducer strain *A. melanogenum* A4 was isolated from soil [92]. Strain A4 was able to synthesize pullulan from glucose, fructose, sucrose, and maltose, with maltose being able to support the highest level of pullulan production after 48 h at 30 °C [92]. The strain could tolerate high concentrations of sucrose to synthesize pullulan. Following the optimization of medium components, strain A4 was grown at 30 °C in a 5-L fermenter with a 3-L working volume containing a 16.3% (*w*/*v*) glucose and 38.7% (*w*/*v*) maltose-containing medium for 120 h [92]. Strain A4 produced 122.34 g/L pullulan and 82.32 g/L cellular biomass with a pullulan productivity of 1.0195 g/L/h [92]. Another strain of *A. melanogenum* that was isolated from a natural source was strain ZH27 [93]. A number of *A. melanogenum* isolates were screened for their ability to synthesize pullulan following growth in a 12% (*w*/*v*) sucrose-containing medium (pH 6.5) at 28 °C with aeration (180 rpm) for 72 h [93]. Of those isolates screened, strain ZH24 was identified as a pullulan overproducer [93]. Subsequently, the ability of strain ZH27 to synthesize pullulan was tested in a 15% (*w*/*v*) sucrose-containing medium at 28 °C using batch fermentation for 132 h [93]. Using these growth conditions, strain ZH27 produced 115.4 g/L pullulan with a productivity of 0.87 g/L/h [93]. It was concluded that strain ZH27 was osmotolerant with respect to high sucrose concentrations [93]. Spectral analysis of the polysaccharide synthesized by strain ZH27 indicated that it was pullulan [93]. Transcriptional analysis of the genes associated with glycolysis as well as pullulan synthesis, along with genes encoding hydrolases associated with pullulan degradation, was performed in strain ZH27. It was observed that both types of genes were upregulated in strain ZH24 [93]. The studies involving hyperproducer strains indicate that such strains from *Aureobasidium* species can be identified by mutant selection or by isolation from natural sources. The hyperproducer strains may be of significant value to the commercial production of pullulan.

4. Osmotolerant Pullulan-Producing Strains

As shown in Table 2, some *Aureobasidium* strains have been reported as being osmotolerant [94–97]. The wild-type strain *A. pullulans* NCPS2016 was subjected to atmospheric and room temperature plasma mutagenesis [94]. It has been shown that mutant strains can be isolated using this type of mutagenesis, which exhibits a high level of genetic stability relative to the stability of strains isolated with conventional mutagenesis procedures. In addition to atmospheric and room temperature plasma mutagenesis, adaptive evolution was employed to isolate a highly osmotolerant mutant strain of *A. pullulans* NCPS2016 [94]. The resultant mutant strain M233-20 was isolated by 20 cycles of adaptive evolution. This mutant strain was able to grow on an 80% (*w*/*v*) glucose-containing agar plate. In contrast, the parent strain NCPS2016 did grow on 70% (*w*/*v*) glucose-containing agar plates but failed to grow on 80%-glucose-containing agar plates. It is clear that the adaptive evolution of parent strain NCPS2016 successfully resulted in the construction of strain M233-20, which was more osmotolerant relative to glucose transport [94]. The increased ability of strain M233-20 cells to transport glucose more rapidly than their parent strain resulted in a higher level of pullulan being produced by the mutant strain [94]. The mutant strain M233-20 produced 162.3 g/L pullulan after 144 h at 28 °C in a 30-L fermenter [94]. The pullulan yield and productivity of strain M233-20 reached 0.82 g/g glucose, while the pullulan productivity of the mutant strain was 1.13 g/L/h [94]. The activities of uridine diphosphate glucosyltransferase, uridine diphosphate-glucose pyrophosphorylase, and glycosyltransferase were elevated in the mutant strain compared to the wild-type strain [94].

The genes for the above enzymes involved in pullulan biosynthesis were all upregulated in expression compared to the wild-type strain [94]. The osmotolerant mutant strain produced a polysaccharide with a molecular weight of 2.890×10^5 Da, which was lower than the molecular weight of the parent strain polysaccharide which had a molecular weight of 5.936×10^5 Da [94]. It was found that the molecular weight of the osmotolerant mutant strain polysaccharide continued to decrease as the fermentation time increased due to the synthesis of enzymes hydrolyzing pullulan, such as amylases and glucoamylases. It was also determined that the molecular weight of the polysaccharide produced by the mutant strain in shake flasks was higher than the molecular weight of the polysaccharide synthesized in a 30-L fermenter [94].

Table 2. Growth conditions and maximum pullulan production by osmotolerant strains of *Aureobasidium* species.

Species	Strain	Carbon Source (%, *w*/*v*)	Growth Conditions	Maximum Pullulan (g/L)	Reference
A. pullulans	M233-20	Glucose (20%)	144 h, 28 °C	162.3	[94]
A. pullulans	*Mal31*	Glucose (5%)	72 h, 30 °C	27.0	[95]
A. pullulans	RBF-4A3	Sucrose (15%)	96 h, 30 °C	66.79	[96]
A. melanogenum	TN3-1	Glucose (14%)	120 h, 28 °C	110.3	[97]

Using the *A. pullulans* strain M2012259, a mutant strain in which a sugar transporter *mal31* was overexpressed was constructed. The *mal31* gene is essential to the transport of precursors for pullulan synthesis [95]. An increase in the transcriptional level of genes associated with pullulan synthesis and glucose transport was observed. Using a fermentation medium containing 50 g/L glucose, the mutant strain was grown at 30 °C for 72 h in a 3-L fermenter (400 pm, 1 *v*/*v*). It was found that the mutant strain *Mal31* produced a 15.9% higher pullulan level than the parent strain grown under the same conditions [95]. If the *mal31* gene is deleted in *A. pullulans* strain M2012259, pullulan synthesis decreases by 69.1% compared to the pullulan synthesis by the parent strain [95]. The deletion of the *mal31* gene in *A. pullulans* strain M2012259 caused an increase in uridine diphosphate-glucose supply for glycogen synthesis [95]. It was not indicated whether the molecular weight of the polysaccharides by the mutant and parent strains was affected [95].

A natural isolate was isolated from flowers of *Caesulia axillaris*, and it was genotypically characterized as *A. pullulans* [96]. The isolate was designated as RBF-4A3 [96]. It was observed that strain RBF-4A3 was capable of producing high pullulan levels at high sugar concentrations [96]. The strain produced 66.79 g/L in 15% (*w*/*v*) glucose, with a productivity of 16.69 g/L/d after 96 h at 30 °C.

An isolate from natural honey, designated as *A. melanogenum* TN3-1, was identified as being osmotolerant and was capable of elevated pullulan production [97]. This likely spontaneous mutant strain produced 110.3 g/L pullulan when grown in a medium containing 140.0 g/L of glucose at 28 °C and 180 rpm for 120 h in a 10-L fermenter with a working volume of 6.5 L [97]. The pullulan yield of strain TN3-1 was 0.79 g/g glucose while the pullulan productivity of the strain was 0.84 g/L/h. On a 140.0 g/L sucrose-containing medium, the strain TN3-1 produced 105.5 g/L pullulan at 28 °C and 180 rpm for 120 h in the 10-L fermenter [97]. It was seen that the gene for the primary glucose repressor in *A. melanogenum* TN3-1 was downregulated, making the strain more osmotolerant for glucose or sucrose [97]. The polysaccharide of the pullulan was characterized as authentic [97]. The osmotolerant pullulan producers appear to be valuable to the large-scale production of pullulan since many of the strains are pullulan hyperproducers.

5. Strains Synthesizing Variable Molecular Weight Pullulans

A mutant strain of *A. pullulans* and a natural isolate of *A. melanogenum* have been shown to synthesize pullulans of varying molecular weights [98]. Using *A. pullulans* CGMCC3.933,

ultraviolet mutagenesis resulted in the isolation of strain UVMU6-1 [99]. The mutant strain UVNU6-1 was grown at 30 °C for 114 h in 2 L of medium containing 40 g/L glucose in a 5-L fermenter (400 rpm) with an aeration rate of 1.5 L/min and produced 109 g/L polysaccharide [98]. The pullulan yields of the mutant strain and parent strains were not compared [98]. The polysaccharide produced by the mutant strain was characterized as authentic pullulan [98]. Interestingly, the molecular weight of the polysaccharide produced by strain UVMU6-1 was determined to be 6.7×10^4 daltons [98]. The molecular weight of the polysaccharide produced by strain UVMU6-1 is significantly lower than the molecular weight of its parent strain (10^7 daltons). It was thought that strain UVM6-1 could be used to produce a low molecular weight pullulan at an industrial scale.

In strain *A. melanogenum* strain P16, the genes for α-amylase, glucoamylase, and isopullulanase were deleted creating a new strain DT15 [99]. Strain DT15 produced 46.2 g/L pullulan in a medium containing 120 g/L glucose after 120 h at 28 °C with aeration (180 rpm) with the pullulan having a molecular weight of 3.02×10^6. Under the same growth conditions, pullulan synthesis by the parent strain P16 was determined to be 65.5 g/L, with the molecular weight of the pullulan determined to be 0.35×10^6 daltons [99]. It was thought that the presence of α-amylase, glucoamylase, and isopullulanase activities in strain P16 caused the reduction in the molecular weight of the pullulan witnessed in the parent strain compared to the mutant strain [99]. It has also been reported that glucoamylase activity was present in *A. pullulans* ATCC 42023, which promoted the degradation of pullulans [100]. Strains producing pullulans of variable molecular weights may be valuable to certain polysaccharide applications.

6. Strains Synthesizing Pullulan from Hemicellulose

Recent investigations have begun examining how hemicellulose hydrolysates could be utilized [101–103]. Table 3 shows a list of strains that can utilize hemicellulosic substrate hydrolysates. As part of these investigations, strains that are more effective in utilizing hemicellulose to produce pullulan have been isolated. In an early report, *A. pullulans* AY82 was an ultraviolet-light-induced mutant isolated from *A. pullulans* CGMCC.0837 [104]. A sugarcane bagasse hemicellulose hydrolysate contained 70% xylose, 12% glucose, and 7% arabinose [104]. The fermentation of the hydrolysate using strain AY82 in shake flasks was done for 168 h at 28 °C with aeration (200 rpm). It was shown that 14.32 g/L pullulan and 14.85 g/L cellular biomass were produced [104]. In a 5-L fermenter using a 3-L working volume with an agitation rate of 400 rpm, strain AY82 synthesized 17.63 g/L pullulan and 14.85 g/L cellular biomass after 168 h at 28 °C [104]. Another investigation was conducted using a bubble column reactor to produce pullulan using *A. pullulans* ATCC 42023 from sugarcane bagasse hemicellulosic hydrolysate [105]. In a second investigation, the mutant strain ARH-1 was isolated from *A. pullulans* CCTCC M 2012259 following 20 cycles of adaptive evolution [106]. An acid-treated, decolorized rice hull hydrolysate was used as the substrate for pullulan production by ARH-1 [106]. Strain ARH-1 produced 23.2 g/L pullulan on the hydrolysate after 48 h at 30 °C [106]. In a 5-L fermenter with a working volume of 3-L operated with aeration (400 rpm) at 28 °C, strain ARH-1 synthesized 22.2 g/L pullulan after 48 h [106]. An evolved strain of *A. pullulans* CCTCC M 2012259 was isolated after 10 rounds of subculturing to increase the tolerance to acid hydrolysates of corncob or corn straw hydrolysates [107]. The resultant strain EV6 was shown to produce 21.75 g/L and 20.25 g/L pullulan, respectively, on the acid hydrolysates of corncob or corn straw after 72 h at 30 °C [107]. In strain EV6, the enzymes involved in pullulan biosynthesis or degradation were elevated [107]. A natural isolate of *A. melanogenum* was identified from honeycomb and could utilize a wheat straw hydrolysate to synthesize pullulan [108]. The strain TN2-1-2 was shown to be able to metabolize xylose and glucose to produce pullulan and could tolerate high salt concentrations [108]. The wheat straw hydrolysate contained 110 g/L reducing sugars and could be utilized to synthesize 55.1 g/L pullulan with a yield of 0.50 g/g hydrolysate [108].

Table 3. Growth conditions and maximum pullulan production by strains of *Aureobasidium* species capable of hemicellulose utilization.

Strain	Hemicellulosic Hydrolysate	Growth Conditions	Maximum Pullulan (g/L)	Reference
AY82	Sugarcane bagasse	168 h, 28 °C	17.63	[104]
ATCC 42023	Sugarcane Bagasse	120 h, 28 °C	28.62	[105]
ARH-1	Rice hull	48 h, 30 °C	22.20	[106]
EV6	Corncob	72 h, 30 °C	21.75	[107]
EV6	Corn straw	72 h, 30 °C	20.25	[107]
TN2-1-2	Wheat straw	120 h, 28 °C	55.10	[108]

The polysaccharide produced by the strain on the hydrolysate was shown to be authentic pullulan with its molecular weight being 1.862×10^5 daltons [108]. The future of commercial pullulan production may be dependent on identifying additional strains that synthesize pullulan from hemicellulose. With the abundance of hemicellulosic substrates, the ability of these strains to produce pullulan from hemicellulose represents an excellent way to reduce the cost of this polysaccharide.

7. Conclusions

The strain improvement of *Aureobasidium* species for the production of the polysaccharide pullulan has been important in enhancing the economic viability of commercial pullulan synthesis. It is important to note that the *Aureobasidium* cultivation conditions used (including carbon source and yeast extract concentration) have to be considered when comparing the yields of pullulan produced by the strains isolated. The number of mutant strains or natural isolates of *Aureobasidium* species that exhibit reduced pigmentation, pullulan hyperproduction, increased osmotolerance, polysaccharide molecular weight variation, and hemicellulose utilization has been constantly growing and will allow commercial processes to choose the best strain to meet the needs of pullulan production. It should help to make the production of pullulan more economical in order to compete with the lower cost of other commercial polysaccharides. If the cost of pullulan can be lowered from $12,000/kg, the number of applications for pullulan could be dramatically increased [32]. As the strain improvement of *Aureobasidium* species continues, it is expected that the commercial production of pullulan should become more cost-effective as the current $68 million market continues to grow annually by more than 4%.

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