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THE EFFECT OF AGITATION ON PULLULAN PRODUCTION BY A WHITE MUTANT *AUREOBASIDIUM PULLULANS* B-1 IN BATCH CULTURE

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ABSTRACT

In this study we indicated a way of producing pullulan using a new strain of *Aureobasidium pullulans,* the white mutant B-1. The fungus culture was run in a stirred bioreactor. Five different impeller speeds were applied: 200 rpm, 400 rpm, 600 rpm, 800 rpm, and 1000 rpm. The kinetics of changes in air saturation of medium, the content of pullulan, cell biomass, residual sugar and pH were investigated. The maximum concentrations of pullulan (26.2 $g \times dm^{-3}$) and biomass (14.4 $g_{d.w.} \times dm^{-3}$), and the maximum saccharose consumption (>99%) were achieved in the culture stirred at 800 rpm after 96h of incubation. In this culture, air saturation (DO) has decreased to 45-50% within 36h. That DO value was maintained until the end of fermentation. The pH value has decreased from an initial 6.5 to 5.35. Agitation speeds in the range of 200-600 rpm and at 1000 rpm were not so beneficial. The respective cultures were characterized by both: lower biomass yield and lower pullulan content. The B-1 mutant culture with adjusted pH, maintained at 6.5, and agitation rate of 800 rpm was more beneficial for biomass growth (18.5 $g_{d.w.} \times dm^{-3}$) than for pullulan production (16.2 $g \times dm^{-3}$). The data demonstrated that agitation speed influenced pullulan biosynthesis by *A. pullulans* B-1. Mutant B-1 is suggested as a potential candidate for industrial pullulan production.

Key words: pullulan, *Aureobasidium pullulans*, white mutant, stirred bioreactor

INTRODUCTION

Pullulan is an α-glucan polymer of simple chains of three glucopyranosides linked by (1→ 4)-α–D-glycosidic bonds [20]. These α-trisaccharide sub-units are multiple-polymerized by (1→6)-α–D-glycosidic bonds occurring at the ends of glucoside residues. The degree of polymerization ranges from 100 to 5000 α-glucopyranoside units and the molecular mass of pullulan may range between 15 - 4 000 kDa depending on the applied strain and selected culture parameters. Parameters influencing pullulan biosynthesis are: temperature, initial pH of culture medium, type and concentration of carbon and nitrogen source in the medium, and culture saturation with oxygen [10, 12, 16, 17]. Over the last four decades, numerous studies have demonstrated the application of pullulan in the biomedical and pharmaceutical industries as well as in the production of foods and cosmetics [3]. Due to its non-toxic, non-

mutagenic and non-immunogenic properties, pullulan may be used as biomaterial in tissue engineering and as a carrier of controlled drugs release in a human body [13]. Furthermore, its excellent transparent film forming ability, its colorless, odorless, water soluble characteristics and its thickness are properties making pullulan suitable for use in the production of oral protective capsules for drug and dietary supplements (NPcaps® capsules) and rapidly-soluble oral films for use in therapeutic preparations [3]. Pullulan has also been demonstrated to be a polysaccharide poorly and slowly degradable by human digestive enzymes and, additionally, one that reduces appetite sensation in men [14, 24]. For these reasons, it may be directly added to foods in order to decrease their calorific value.

Pullulan is synthesized by *Aureobasidium pullulans*, which is an oligotrophic fungus abundant in nature and occurring over almost the entire globe [4, 26]. Most native strains simultaneously synthesize melanin pigments that impart typical coloration to cultures, from dark olive to black. The literature reports on many *A. pullulans* strains capable of producing pullulan [3]. Out of these, special significance is ascribed to novel strains producing pullulan not contaminated with melanins. These compounds pose the key problem in pullulan production owing to the necessity of their removal during the purification of a crude preparation, which in turn increases the cost of pullulan production. In recent years, some scientific studies have described strains deficient in melanin production, isolated from the natural environment, e.g. *A. pullulans* P56 that was used by Roukas [17], Youssef et al. [25], Lazaridou et al. [11]. Gőksungur et al. [9] , and a non-pigmented, osmotolerant strain RBF-4A3 isolated from flowers from India [5] or a strain isolated from biogas reactors from the UK [15]. Other authors have improved strains via induced mutagenesis and selected reduced-pigmentation mutants [8, 19, 21, 23]. Outcomes of these investigations are strains characterized by inhibited or significantly reduced synthesis of melanin pigments. This property has, however, not always been accompanied by the enhanced production of pullulan compared to parent strains.

The effect of medium air saturation and agitation on pullulan production in bioreactor has already been investigated by many authors [7, 9, 11, 12, 16]. Researchers have demonstrated a significant effect of these factors. Aeration provides oxygen that is indispensable for the genesis and growth of microbial cells, which is reflected in pullulan yield and the synthesis rate of this polysaccharide [16]. Stirring makes the content of components and cells in the culture medium uniform and is responsible for mass exchange. It also induces shearing forces that may influence both morphological changes in the analyzed culture and the formation of metabolites, but also damage to cells [11, 17].

Our previous investigations demonstrated that white mutant *A. pullulans* B-1 was characterized by a significant reduction in contents of cellular and exocellular melanins at an unchanged level of pullulan production compared to the parent strain. It seems that the production of pullulan with the use of B-1 mutant may reduce the production costs of this polysaccharide [8].

The objective of this study was, therefore, to determine the optimal agitation conditions for pullulan production by a white mutant *Aureobasidium pullulans* B-1 in a stirred tank bioreactor.

MATERIALS AND METHODS

Organism and growth conditions

The white *Aureobasidium pullulans* mutant B-1, isolated after mutagenesis of the natural black strain *A. pullulans* A.p.-3 using ethylenoimine and UV radiation, was used in this study [8]. Culture was maintained on the slant and stored at 4 °C. For preinoculum preparation, whole material on slant was transferred to the liquid media containing ($g \times dm^{-3}$): sucrose 60, K_2HPO_4 7.5, NaCl 1.5, $(NH_4)_2SO_4$ 0.72, $MgSO_4 \cdot 7\ H_2O$ 0.4, yeast extract 0.4. Medium pH was 6.5. All chemicals and reagents were of analytical grade and purchased from POCH (Gliwice, Poland). Culture was grown in 250 cm^3 conical flasks containing 50 cm^3 of sterile medium. The culture was shaken (150 rpm) for 24 h (SM-30/Control, GmbH, Hechingen, Germany) at 28 °C. A total of 1 cm^3 of preinoculum was then transferred into the fresh medium and cultivated for 24 h under the same conditions as described above. When the cultivation was completed the number of cells was equal to $\sim 1 \cdot 10^7$ cfu $\times cm^{-3}$.

Fermentation conditions

The fungus culture was run in a BioFlo 3000 bioreactor (New Brunswick Scientific, Edison, NJ, USA) with a 5 dm^3 vessel containing 3 dm^3 of a growth medium, with a composition identical to that used for inoculum preparation. Inoculum – in a concentration of 1 % (v/v) was added to a sterile medium. Agitation was provided by two centrally mounted six-bladed Rushton turbines. Aeration occurred through a perforated pipe sparger ring. The rate of culture aeration was 1 vvm (air volume per reactor working volume per minute). The culture was run for 96h, and the temperature was kept at 28 °C. The bioreactor was fitted with pH and dissolved oxygen (DO) probes (Mettler Toledo, OH, USA). The DO probe was calibrated according to instructions of bioreactor producer. Before inoculation, the DO probe was placed in a sterile medium. In order to achieve the value of 0%, a cable was disconnected from the DO probe and the value of 0% was set at a bioreactor console. Then, to make the value reach 100%, once the cable was connected to the DO probe, the culture medium was saturated with atmospheric air for 15

min at an aeration rate of 1 vvm, 1000 rpm, 28°C. Measurements of pH and DO were automatically registered in an AFS – Biocommand ver. 250 software (New Brunswick Scientific, Edison, NJ, USA). It was assumed that the values of the reading DO were expressed as percentage of the initial air saturation.

Study design

The study was divided into two stages. During the first stage, a culture medium with non-adjusted initial pH 6.5 that was changing spontaneously throughout the incubation period was used. Five different impeller speeds were applied: 200 rpm, 400 rpm, 600 rpm, 800 rpm, and 1000 rpm. During the second stage, culture medium pH was maintained at a constant level of 6.5 for 96h using 1% sulfuric acid, and the medium was stirred at 800 rpm. Three series of each culture were performed.

Analytical procedures

Samples (50 cm^3 each) were collected aseptically every 24 hrs, and then centrifuged at 18,000 × *g* for 20 min (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The supernatant was decanted and the centrifuged biomass was rinsed with distilled water, re-centrifuged and dried at 80 °C until reaching dry biomass. From the resultant supernatant, pullulan was precipitated with the use of ethanol added to a sample in a volumetric ratio of 2 : 1 (v/v). After vigorous stirring, pullulan was filtered through a pre-weighed Whatman GF/A filter, and purified according to the Roukas and Biliaderis method [18]. Next, it was dried overnight at 80 °C. The residual sugar content was measured colorimetrically in the filtrate using the phenol-sulfuric acid method [6]. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

The study was carried out with a white mutant *Aureobasidium pullulans* B-1 fungus that was selected after associated mutagenization (UV and ethylenoimine) of a wild strain *A. pullulans* A.p.-3. Compared to the native parent strain, the B-1 mutant was characterized by a higher productivity of pullulan with a simultaneous, beneficial lack of synthesis of melanin compounds contaminating crude pullulan preparation [8]. This modification enables a reduction of the costs of pullulan production linked with the necessity of removing melanin from the preparation of crude pullulan. Investigations on pullulan production by *A. pullulans* B-1 were conducted for 96h in a culture medium in a bioreactor (Fig. 1- 4).

The effect of agitation speeds on culture air oxidation and pullulan production

Changes in % of air saturation are shown on Fig.1. At a low rotational impeller speed (200 and 400 rpm), within the first 12 h culture air saturation rapidly decreased to almost 0 % and was maintained at this level until the end of incubation. A similar tendency was observed at 600 rpm, where within the first 18 h DO diminished to 10%. Already in the subsequent hours of the culture, the level of air saturation successively increased. Lazaridou et al. [11] observed a similar tendency in a melanin-deficient culture of *A. pullulans* (P56) strain agitated at a speed of 200-400 rpm, during which DO changed rapidly up to 48 h, and afterwards remained at a low level reaching 7-10 % of the initial saturation level up to 120 h. Our results are also consistent with the findings of McNeil and Kristiansen [12], who noted a rapid decrease in DO (below 10% of air saturation) in cultures of *A. pullulans* stirred at 100 and 220 rpm, with the decrease maintained up to almost 96 h.

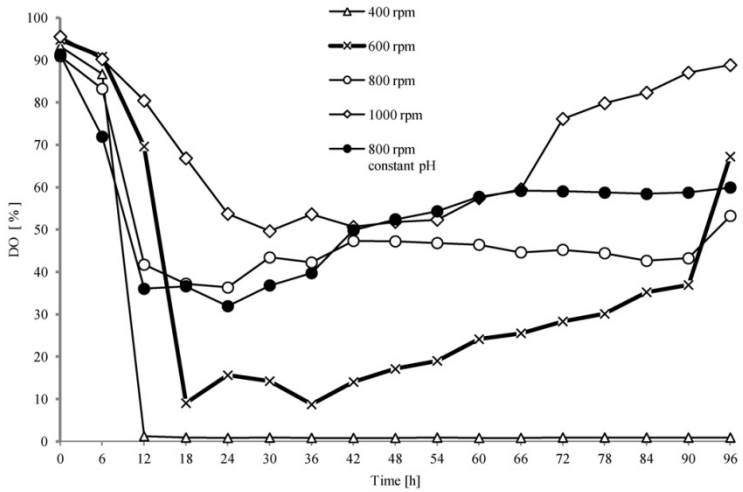

Fig. 1. DO profiles in batch cultures of *A. pullulans* B-1 at different agitation conditions. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, non-adjusted initial pH 6.5, aeration rate 1 vvm, inoculum 1% (v/v).

At a stirring speed of 800 rpm, better culture air saturation was observed. A slow decrease in relative concentration of culture oxidation proceeded up to 36 h of incubation and remained at 45-50%. The best saturated culture was that agitated at 1000 rpm, because in this culture DO reached 51 % at 42 h, and slowly increased after 72 h to reach 76 %. Our observations confirm the results reported in a study by McNeil and Kristiansen [12] who investigated the culture of *A. pullulans* agitated at 750 rpm and noted a low decrease saturation only to 70% in the first 24 h, followed by its successive increase to 100 % reached at 72 h.

The impact of agitation speed on pullulan production at a constant aeration rate reaching 1 vvm is presented on Fig. 2. At an agitation rate of 200 rpm, the production of this polysaccharide was low, at a maximum level of 8.0 g×dm^{-3}. It may be speculated that pullulan biosynthesis was correlated with the degree of medium aeration during incubation and hence the main reason for the low pullulan content could be oxygen deficit. The polysaccharide concentration increased with increased agitation speeds. After 96h, in cultures agitated at 400 and 600 rpm, the concentration of pullulan was already twofold higher and reached 15.0 and 16.4 g×dm^{-3}, respectively. These observations are in agreement with findings of other authors, who found that pullulan concentration increased with agitation speeds.

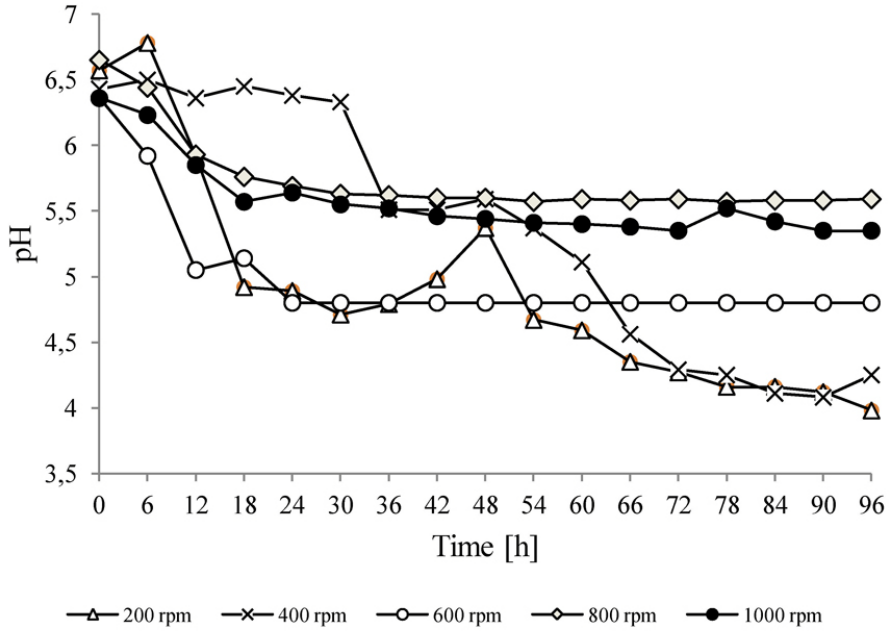

Fig. 2. The effect of agitation speeds on pullulan production by *A. pullulans* B-1. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, non-adjusted initial pH 6.5, aeration rate 1 vvm, inoculum 1% (v/v). Values are presented as mean ± SD of triple determinations.

Rho et al. [16] and McNeil and Kristiansen [12] explained this phenomenon on the basis of increasingly better oxygen mass transfer and an increase in the percentage content of unicellular forms of the fungus that are claimed to be pullulan producers. In turn, Lazaridou et al. [11] observed increasing concentrations of pullulan, synthesized by the *A. pullulans* (P56) strain, upon agitation speeds increase from 300 to 700 rpm (at a constant aeration rate of 1.4 dm^3 ×min^{-1}). In those studies, a maximum production of pullulan of 49 g×dm^{-3} was achieved at 700 rpm (from beet molasses with 100 g×dm^{-3} of saccharose). Contrasting observations were made by Gőksungur et al. [9], who applied three agitation speeds: 200, 400 and 600 rpm, at three aeration rates: 1, 2 or 3 vvm, in a culture of the same strain, i.e. *A. pullulans* (P56). The maximum concentration of pullulan was reached at a relatively slow agitation rate (400 rpm), but – in contrast – at a twofold higher rate of aeration than in our study (2 vvm). Subsequent, simultaneous increases in agitation speed (600 rpm) and aeration rate (3 vvm) were no longer so beneficial and diminished pullulan biosynthesis.

A further increase in agitation rate to 800 and 1000 rpm resulted in a significantly better aeration of culture compared to cultures agitated at 200-600 rpm (Fig. 1). The highest concentration of pullulan (26.2 g×dm^{-3}) was obtained in a culture agitated at 800 rpm after 96 h of incubation of *A. pullulans* mutant. Our results are consistent with findings of McNeil and Kristiansen [12], who also observed the maximum pullulan production during cultivation of *A. pullulans* B-1 at an agitation speed of 750 rpm and aeration rate of 1 vvm. Simultaneously, this culture was the best oxygenated. In turn, Lazaridou et al. [11] reported a decreasing pullulan concentration in a culture of P56 strain agitated at speeds over 750 rpm. The agitation speed of 1000 rpm applied in our research was no longer so beneficial, as the maximum production of pullulan by the strain reached 18.9 g×dm^{-3}. Simultaneously, it was noticed that the decrease of relative concentration of culture air saturation was the smallest and up to 30 h of incubation did not drop below 50 % (Fig. 1). Such a high aeration of the culture did not facilitate pullulan biosynthesis, which was confirmed in a study by Gibbs and Seviour [7]. These authors determined the lowest

content of pullulan in a culture in which the DO was at 100 % during the entire incubation period. Gibbs and Seviour [7] and Lazaridou et al. [11] concluded that at high agitation rates (>750 rpm) the production of this polysaccharide may be kept at a high level but only in condition that the DO was low (15 %) during the initial stage of fungus culture, i.e. for the first 16 h. Such culture conditions hinder the formation of the micellar forms of *A. pullulans* fungus, and these do not synthesize pullulan.

The effect of agitation speeds on biomass growth, residual sugar and pH value

The effect of agitation speeds on the growth of fungus cellular biomass is shown on Fig.3. The biomass increased with the increase in agitation speed from 200 to 1000 rpm. In the cultures agitated at 800 and 1000 rpm the maximum biomass concentration as compared to cultures agitated at 200, 400 and 600 rpm was observed. At those cultures run at slower agitation rates (200-400 rpm), intensive biomass growth was observed only up to 24 h, and the maximum biomass yield reached 5.0-5.5 g $_{d.w.}\times dm^{-3}$. In the same time, a rapid decrease of DO was observed (Fig. 1), which was due to a rapid increase in biomass yield. In the cultures stirred at 600, 800 and 1000 rpm, the yield of biomass increased up to 96 h, and its maximum level ranged from 8.7 to 14.4 g $_{d.w.}\times dm^{-3}$, but still the maximum biomass yield was achieved at 800 rpm (Fig. 3). The highest agitation rate (1000 rpm) did not facilitate either high concentrations of biomass or pullulan.

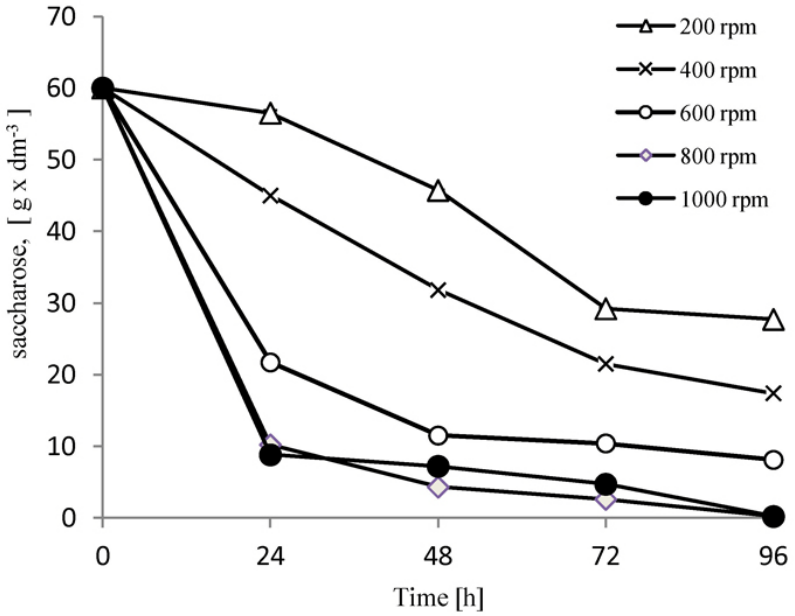

Fig. 3. The effect of agitation speeds on cell biomass of *A. pullulans* B-1. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, non-adjusted initial pH 6.5, aeration rate 1 vvm, inoculum 1% (v/v). Values are presented as mean ± SD of triple determinations.

Gibbs and Seviour [7], in their experiment with a controlled level of medium aeration above 90 % throughout the incubation of *A. pullulans*, obtained a very high biomass yield and a significantly lower concentration of pullulan than in those cultures with non-controlled aeration level. It may, thus, be speculated that the high degree of medium aeration facilitates, most of all, the growth of biomass and not the formation of secondary metabolites, including pullulan. Lazarodou et al. [11] claim that agitation has a positive impact on the growth and functioning of *A. pullulans* cells by improving oxygen supply. Better mixing of the culture medium helps maintain the gradient of concentrations of substrates and products at the internal and external side of a cell. This assures better diffusion of a carbon source and other nutrients to cells and facilitates the removal of by-products from a cell microhabitat.

Changes in the concentration of saccharose during cultivation of *A. pullulans* B-1 at different agitation speeds were presented on Fig. 4. Along with an increasing impeller speed, an increasing assimilation of saccharose was observed, which was due to the enhanced pullulan biosynthesis and fungus growth. After 96 h of incubation, at agitation rates of 200 and 400 rpm, relatively poor consumption of saccharose by fungus cells was observed, i.e. only 54 and 78 %, respectively. In the cultures agitated at 600, 800 or 1000 rpm, the consumption of the saccharide contained in the medium by the fungus was maximal (over 99.9 %). In these cultures, after the first 24 h, saccharose was 69 % (at 600 rpm) or 99 % (at 800 and 1000 rpm) consumed. Our observations are consistent with results achieved by Lazarodou et al. [11], who noted that the assimilation of sugars increased along with agitation speed, which was in agreement with increasing concentrations of biomass and pullulan.

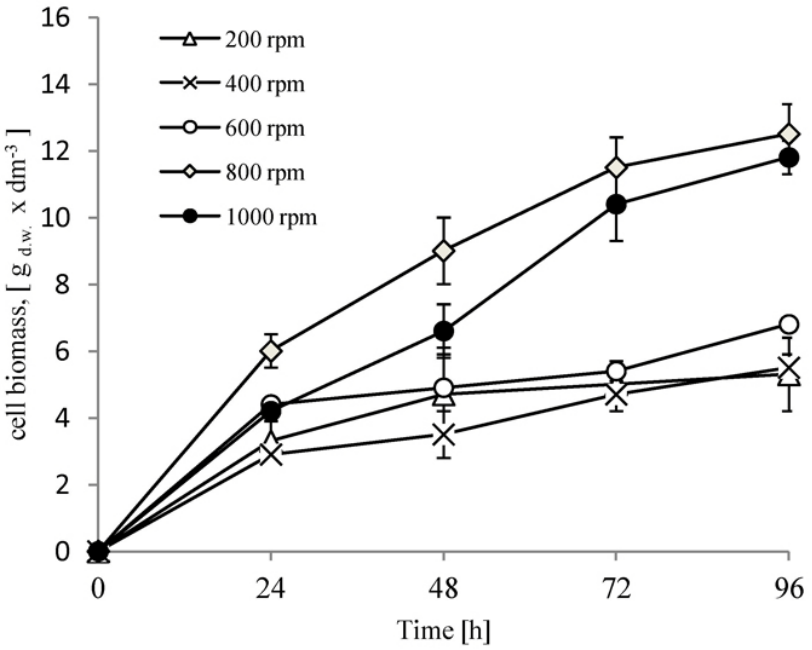

Fig. 4. The effect of agitation speeds on saccharose consumption by *A. pullulans* B-1. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, non-adjusted initial pH 6.5, aeration rate 1 vvm, inoculum 1% (v/v). Values are presented as mean ± SD of triple determinations.

Changes in pH values of the analyzed cultures are depicted on Fig. 5. The pH value was observed to decrease from an initial 6.5 to 3.98 – 5.59, depending on the agitation speed. An increase in medium acidity is ascribed to the synthesis of organic acids by *A. pullulans* strains [7]. In the cultures agitated at 200 and 400 rpm, the pH value dropped maximally to 3.98 and 4.25, respectively. A similar tendency was noted in the culture agitated at 600 rpm, where the greatest changes in pH occurred within the first 24 hours. At increasing agitation speeds, i.e. 800 and 1000 rpm, the decrease in medium pH was less and after 96 h its value was 5.35 and 5.59. When comparing changes in pH values of the culture of white strain *A. pullulans* (P56) analyzed by Lazarodou et al. [9] with those in *A. pullulans* B-1 culture, it may be noticed that they were similar only at the initial stage of strains growth, when pH decreased from an initial value of 7.0 to 5.2 – 5.7 after 24h incubation. At the final stage of (P56) strain growth, the pH values were observed to increase. This could be due to the deamination of amino acids present in molasses (used as a source of carbon) and the production of ammonia.

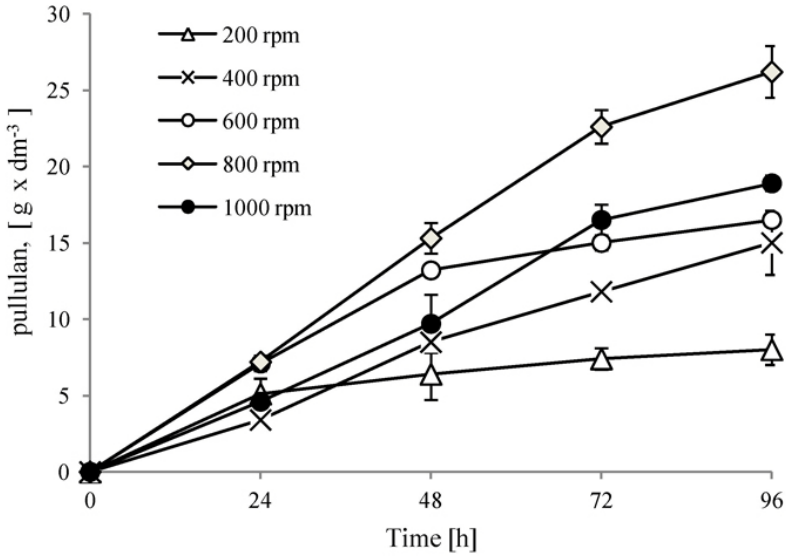

Fig. 5. The effect of agitation speeds on pH changes during the culture of *A. pullulans* B-1. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, non-adjusted initial pH 6.5, aeration rate 1 vvm, inoculum 1% (v/v).

In order to establish pH effect on pullulan production by *A. pullulans* B-1, an additional experiment was carried out in which pH was kept at a constant level of 6.5 throughout the incubation period. Results of this culture were

compared with those of the above-discussed culture where the initial pH was 6.5 and changed spontaneously within 96 h of incubation.

Changes in contents of biomass, pullulan and saccharose in cultures with adjusted and non-adjusted pH

Changes in contents of biomass, pullulan and saccharose in cultures with adjusted and non-adjusted pH are presented on Fig. 6. The cultures were agitated speed of 800 rpm, at which pullulan production was the highest. Data achieved indicate that the constant pH of 6.5 facilitated more enhanced growth of biomass than of pullulan. Within the first 24 h, biomass yield increased up to 11.4 g×dm^{-3} and increased maximally to 18.5 g $_{d.w.}$×dm^{-3} after 96 h. Under these conditions, analyses also showed the maintenance of a high air saturation of culture, with ranging between 30 and 60 %, especially after 42 h of incubation (Fig. 1).

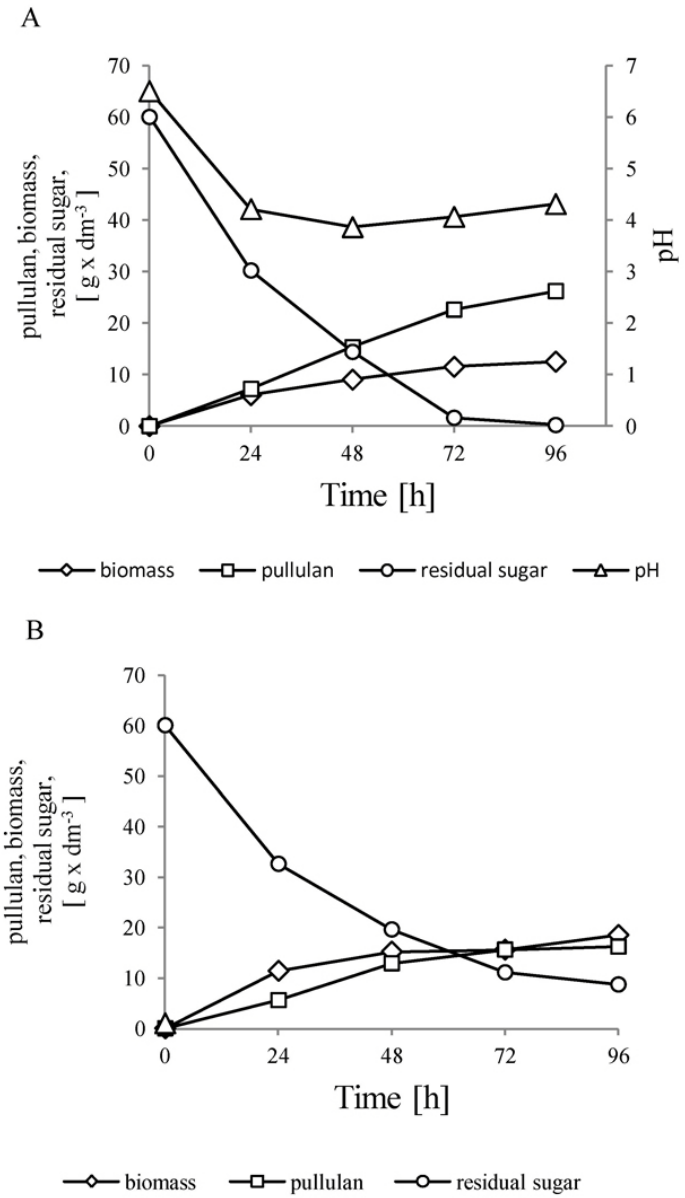

Fig. 6. Changes in contents of biomass, pullulan and saccharose in cultures with adjusted and non-adjusted pH. Saccharose 60 g×dm^{-3}, culture temperature 28 ^{0}C, aeration rate 1 vvm, agitation speed 800 rpm, inoculum 1% (v/v). A) non-adjusted pH 6.5, B) constant pH 6.5.

Such high quantities of biomass were not reported in any of the previously analyzed cultures with non-adjusted pH. In turn, the production of pullulan was lower compared to the culture where pH changed spontaneously. In the end, the content of the polysaccharide did not exceed 16.2 g×dm^{-3} and was lower by as much as 10 g×dm^{-3} when compared to the analogous culture with non-adjusted pH. After 96 h, 8.6 g×dm^{-3} of non-consumed saccharose remained in the medium, whereas in the medium with non-adjusted pH the strain utilized all the available saccharide (Fig. 6A). Some authors suggest that the conditions of environmental pH determine whether saccharose from the culture medium would be consumed for cell biomass growth or for pullulan production. Our study demonstrates that the stable pH of 6.5 did not facilitate pullulan production by the strain and that saccharose was utilized mainly for

biomass growth. According to West [22], a drop in pH stimulates pullulan synthesis during fermentation, which is consistent with our observations. In addition, it is common knowledge that pullulan is produced already at the late stationary phase of *A. pullulans* growth. It is likely that in the culture medium with a stable pH of 6.5 the population of *A. pullulans* B-1 reached the stationary phase later and, thus, not consumed saccharose remaining in the culture medium could be used for pullulan production already at a significant elongation of the incubation time.

Recent works report on results of experiments with the use of stable pH during the culture of *A. pullulans* in a bioreactor. Cheng et al. [1] demonstrated that in a culture medium containing 75 $g \times dm^{-3}$ of saccharose and 3 $g \times dm^{-3}$ of a yeast extract, at 30ºC and stable pH of 5.0, the *A. pullulans* ATCC 201253 strain finally produced 19.3 $g \times dm^{-3}$ of pullulan. In further studies of these authors, the production of pullulan was increased by using a PCS biofilm reactor [2]. Results of these studies indicate that a stable pH value maintained at 5.0 throughout the culture period was enhancing pullulan production by the analyzed strain. The maximum concentration of pullulan after 7-day culture reached 32.9 $g \times dm^{-3}$ and was 1.8-fold higher compared to the culture with uncontrolled pH.

CONCLUSION

This study The effect of different agitation speeds, in a wide range from 200 to 1000 rpm, on the pullulan production by a white mutant *A. pullulans* B-1 in a stirred bioreactor was investigated in this study. It demonstrated that the agitation speed had a significant impact on pullulan production by *A. pullulans* B-1. The maximum concentration of pullulan and biomass were achieved at 800 rpm after 96h of incubation. These studies provide new information and encourage the use of *A. pullulans* B-1 mutant as present and future biotechnology applications. Further research should focus on the production of pullulan on a larger scale.

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REFERENCES

1. Cheng K.C., Demirci A., Catchmark J.M., 2009. Effect of temperature, carbon source, yeast extract, and pH on pullulan production by *Aureobasidium pullulans*. ASABE Paper No. 096033. American Society of Agricultural Engineers. St. Joseph, M1, 14 pp.
2. Cheng K.C., Demirci A., Catchmark J.M., 2010. Effect of plastic composite support and pH profiles on pullulan production in a biofilm reactor. Appl. Microbiol. Biotechnol. 86, 853-861.
3. Cheng K.C., Demirci A., Catchmark J.M., 2011. Pullulan: biosynthesis, production, and applications. Appl. Microbiol. Biotechnol. 92, 29–44.
4. Chi Z., Wang F., Chi Z., Yue L., Liu G., Zhang T., 2009. Bioproducts from *Aureobasidium pullulans*, a biotechnologically important yeast. Appl. Microbiol. Biotechnol. 82, 793–804.
5. Choudhury A.R., Sharma N., Prasad G.S., 2012. Deoiledjatropha seed cake is a useful nutrient for pullulan production. Microb. Cell Factor. 11, 39-47.
6. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F., 1956. Colorimetric methods of determination of sugar and related substances. Anal. Chem. 28, 350-356.
7. Gibbs P.A., Seviour R.J., 1996. Does the agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aureobasidium pullulans*. Appl. Microbiol. Biotechnol. 46, 503-510.
8. Gniewosz M., Duszkiewicz-Reinhard W., 2008. Comparative studies on pullulan synthesis, melanin synthesis and morphology of white mutant *Aureobasidium pullulans* B-1 and parent strain A.p.-3. Carbohydr. Polym. 72, 431-438.
9. Gőksungur Y., Dağbağli S., Uçan A., Güvenç U., 2005. Optimization of pullulan production from synthetic medium by *Aureobasidium pullulans* in a stirred tank reactor by response surface methodology. J. Chem. Technol. Biotechnol. 80, 819-827.
10. Kim J.H., Kim M.R., Lee J.H., Lee J.W., Kim S.K., 2000. Production of high molecural weight pullulan by *Aureobasidium pullulans* using glucosamine. Biotechnol. Lett. 22, 987-990.
11. Lazaridou A., Roukas T., Biliaderis C.G., Vaikousi H., 2002. Characterization of pullulan produced beet molasses by *Aureobasidium pullulans* in stirred reactor under varying agitation. Enzyme Microb. Technol. 31, 122-132.
12. McNeil B., Kristiansen B., 1987. Influence of impeller speed upon the pullulan fermentation. Biotechnol. Lett. 9, 101-104.
13. Mishra B.M., Vuppu S., Rath K., 2011. The role of microbial pullulan, a biopolymer in pharmaceutical approaches: A review. J. Appl. Pharm. Sci. 1, 45-50.
14. Peters H.P.F., Ravestein P., van der Hijden H.T.W.M., Boers H.M., Mela D.J., 2011. Effect of carbohydrate digestibility on appetite and its relationship to postprandial blood glucose and insulin levels. Eur. J. Clin. Nutr. 65, 47–54.
15. Ravella S.R., Quiňones T.S., Retter A., Heiermann M., Amon T., Hobbs P.J., 2010. Extracellular polysaccharides (EPS) production by a novel strain of yeast-like fungus *Aureobasidium pullulans*. Carbohydr. Polym. 82, 728-732.
16. Rho D., Mulchandali A., Luong J.H.T., Leduy A., 1988. Oxygen requirement in pullulan fermentation. Appl. Microbiol. Biotechnol. 28, 361 -366.
17. Roukas T., 1999. Pullulan production from brewery wastes by *Aureobasidium pullulans*. World J. Microbiol. Biotechnol. 15, 447-450.

18. Roukas T., Biliaderis C.G., 1995. Evaluation of carob pod as a substrate for pullulan production by *Aureobasidium pullulans*. Appl. Biochem. Biotechnol. 55, 27-44.
19. Seo H.P., Son C.W., Chung C.H., Jung D.I., Kim S.K., Gross R.A., Kaplan D.L., Lee J.W., 2004. Production of high molecular weight pullulan by *Aureobasidium pullulans* HP-2001 with soybean pomace as a nitrogen source. Biores. Technol. 95, 293-299.
20. Taguchi R., Kikuchi Y., Sakano Y., Kobayashi T., 1973. Structural uniformity of pullulan produced by several strains of *Pullularia pullulans*. Agricultur. Biol. Chem. 37, 1583-1588.
21. Tarabasz-Szymańska L., Galas E., 1993. Two-step mutagenesis of *Pullularia pullulans* leading to clones producing pure pullulan with high yield. Enzyme Microb. Technol. 15, 317-320.
22. West T.P., 2003. Exopolysaccharide production by entrapped cells of the fungus *Aureobasidium pullulans* ATCC 201253. J. Basic Microbiol. 40, 397-401.
23. West T.P., Reed-Hamer B., 1993. Polysaccharide production by a reduced pigmentation mutant of the fungus *Aureobasidium pullulans*. FEMS Microbiol. Lett. 113, 345-350.
24. Wolf B.W., Garleb K.A., Choe Y.S., Humphrey P.M., Maki K.C., 2003. Pullulan is a slowly digested carbohydrate in humans. J. Nutr. 133, 1051-1055.
25. Youssef F., Roukas T., Biliaderis C.G., 1999. Pullulan production by non-pigmented strain of *Aureobasidium pullulans* using batch and fed-batch culture. Proc. Biochem. 34, 355-366.
26. Zalar P., Gostinčar C., de Hoog G.S., Uršič V., Sudhadham M., Gunde-Cimerman N., 2008. Redefinition of *Aureobasidium pullulans* and its varieties. Stud. Mycol. 61, 21–38.

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