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Full Length Research Paper

Optimization of physico-chemical and nutritional parameters for pullulan production by a mutant of thermotolerant *Aureobasidium pullulans* in fed batch fermentation process

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A mutant of thermotolerant Aureobasidium pullulans was isolated using growth inhibitors viz. nystatin and deoxyglucose in the growth medium. An improved mutant strain, RG-5, was developed using methyl methane sulfonate (MMS) (0.5%) for 5 minutes, having killing rate of 70% level, produced 6 g l-1 higher pullulan as compared to the wild type without loosing thermotolerant and non-melanin producing ability. The mutant required 15 rpm higher agitation as compared to the wild type and consumed 1% and 0.1% less sucrose and ammonium sulphate, respectively than the wild strain with lesser incubation period (12h), and with slight increase of pH optima (6.5) in a fed-batch fermentation process. This mutant is very stable and producing significantly higher pullulan with lesser carbon and nitrogen as well as pH tolerance capacity along with lesser incubation period. Therefore, the mutant can be used for industrial production in order to reduce the cost of production.

Key words: *Aureobasidium pullulans*, pullulan, mutant, methyl methane sulfonate (MMS).

INTRODUCTION

Pullulan is the generic name given to water-soluble homopolysaccharide that is produced extracellularly by a polymorphic fungus, *Aureobasidium pullulans* (De Bary) Arnaud. It is a linear α-D-glucan connected with α-1, 4 glycosidic bond mainly of maltotriose repeating units interconnected by α-1,6 linkages (Leathers, 2002). The regular alternation of α-1,4 and α-1,6 bond results in two distinctive properties, the structural flexibility and enhanced solubility (Leathers, 1993). This polysaccharide is of great economic importance with increased application in food, pharmaceutical, agricultural and chemical industries (Seviour et al., 1992; Leathers, 2003). Pullulan produces a high viscosity solution at a relatively low concentration and can be used for oxygen-impermeable films and fibers, thickening or extending agent or adhesives or encapsulating agents (McNeil and Kristiansen, 1990; Leathers, 2002, 2003; Shingel, 2004). Pullulan is also being used for the production of biodegradable plastics in Japan and U.S.A., because it resembles polyethylene with properties like tensile strength and ability to form thin transparent oxygen impermeable films. *A. pullulans,* a polymorphic fungus, has been exploited for pullulan production world-wide. This fungus has been frequently isolated from different environments, mainly tropical and subtropical zones. Much work has been done on isolation and optimization for pullulan production at different physico-chemical levels at laboratory scale. Morphological forms have also been studied during fermentation for pullulan production and found that yeast like phase is responsible for pullulan production favorably in submerged fermentation (Catley, 1980; Heald and Kristiansen, 1985; Campbell, 2004).

Most of the mutants of *A. pullulans* have been developed either for the reduced or no melanin production during pullulan fermentation, while some mutants could produce higher pullulan when compared to wild strain (West and Reed-Hamer, 1993; West and Reed-Hamer, 1994; West

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and Strohfus, 2001). Few mutants have shown both reduced melanin as well as enhanced pullulan yields (Bock et al., 1991; Tarabasz-Szymanska and Galas, 1993). Some mutants initiated higher percentage of yeast like cells during fermentation resulting in higher yield, because yeast phase of growth is generally responsible for pullulan production (Kelly and Catley, 1977; Pollock et al., 1992). Till date, all the studies have been employed for the development of mutant by mesophilic wild strains of this fungus having temperature range of 24 - 32°C (Lee et al., 2001, Lazaridou et al., 2002; Chi and Zhao, 2003; Thirmavalavan et al., 2008; Cheng et al., 2009). The fate of mutant of a thermotolerant strain is not yet done, as thermotolerant strain may be better than mesophilic strain at commercial level, because large scale production increases the temperature of the fermentor, hence cooling devices are required to lower the temperature of fermen-tor, thereby increasing the cost of production, and therefore, cost of production of pullulan can be effectively reduced. Moreover, melanin is produced by most of the *A. pullulans* strains during fermentation. This will make it more difficult to purify polysaccharide after fermentation. Therefore, non melanin producing thermotolerant strain is always a better choice for industrial production of pullulan. Keeping these facts, an attempt was made to isolate a temperature tolerant non melanin producing strain of *A. pullulans* from the natural environment, and further optimized at different physico-chemical and nutritional levels, then subjected to chemical mutagenesis for strain improvement which is resistant to fungal growth inhibitors without losing the thermotolerant character for achieving higher yield of pullulan.

MATERIALS AND METHODS

Microorganism

Wild type thermotolerant strain of *A. pullulans* was isolated from hibiscus flowers using medium containing: Glucose 2%; ammonium sulphate 0.06%; dipotassium hydrogen orthophosphate 0.5%; sodium chloride 0.1%; magnesium sulphate 0.04% and yeast extract 0.04% with pH 5.0 and incubated at 42°C.The wild type was maintained on slants of the same medium and preserved at 4°C, and sub-cultured monthly. The wild type was optimized for pullulan production at different physico-chemical and nutritional levels and then subjected to mutagenesis for the improvement of strain.

Mutant development

Wild type of thermotolerant *A. pullulans* was subjected to chemical mutagenesis using methyl methane sulfonate (MMS) (Sigma Chemicals, USA) by the method of West and Strohfus (2001). An actively growing cells (24 h) containing approximately 5 x 107 CFU/ml was suspended in 80 mM potassium phosphate buffer (pH 8.0) containing 2% glucose and 0.5% methyl methane sulfonate for 5 min at 30°C. The mutagenesis was halted by inclusion of 6% sodium thiosulphate (10 ml) to the mixture. The cell pellet was collected by low speed centrifugation, and washed again with sodium thiosulphate. The cells were then washed with 0.85% NaCl (10 ml) and re-suspended in the broth medium, and grown for 48h at 30°C. Approximately 2 x 102 CFU/ml survived cells (approximately 30% cells) were spread onto basal agar medium plate containing 258 units nystatin ml^{-1} and on the solid minimal medium containing 0.1% glucose and 0.1% deoxyglucose (pH 6.0). After 10 -14 days of incubation at 42°C, colonies were observed on the medium and were then collected for further analysis. The mutant colonies appeared light or pale pink in colour on the respective medium. All the possible mutants were screened for pullulan production at different temperature (data not shown). The selected mutant was designated as RG-5 and optimized at different fermentative parameters for the pullulan production.

Inoculum preparation

Cell suspension was prepared by inoculating 1 ml of 48 h grown culture in 200 ml basal nutrient broth was used for inoculation, and then incubated at 42°C for 24 h to achieve active exponential phase of the culture for transfer into the fermentor.

Optimization of fermentation conditions

The various process parameters influencing pullulan production by fermentation were optimized individually and independently of the others, therefore, the optimized conditions were subsequently used in all the experiments in sequential order. For optimization, the basal fermentation medium contained glucose 2.0%; ammonium sulphate 0.06%; di-potassium hydrogen orthophosphate 0.5%; sodium chloride 0.1%; magnesium sulphate 0.04% and yeast extract 0.04% with pH -5.0 was used for inoculation with 0.5 % of A. pullulans having 50x107 CFU/ml and then incubated for different periods viz. 12, 24, 36, 48, 60, 72, 84 and 96h at different temperature viz. 25, 30, 35, 40, 42, 44 and 46oC. For pullulan production stirred fed-batch fermentation process was followed. Initial pH also plays an important role in pullulan production, so pH of medium was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 using either 1N HCL or 1 N NaOH. For the optimal production of pullulan, the strain may require additional carbon and nitrogen sources with varying concentrations in its growth media. Therefore, the growth medium was supplemented with the carbon sources viz. glucose, fructose, sucrose, lactose and xylose (at the level of 4%) along with nitrogen sources viz. ammonium sulphate, yeast extract, sodium nitrate, sodium nitrite and histidin (at the level of 0.5%) in the fermentor (YORCO Y555, INDIA, 6 liters capacity with a working volume of 4.5 liters). The fermentation medium was sterilized at 121oC for 15 minutes and incubation was done at 42oC with all the other conditions at the optimal levels determined previously. The 1.5 liters of initial volume of the medium was further fed with 1.5 liters in two successive batches at every 5 hours interval into the fermentor. The sterile air was supplied only upto 10 h at the rate of 0.5 vvm (at different incubation periods).

Extraction and estimation of pullulan

After fermentation, the culture medium was heated at 100oC in water bath for 15 minutes, and then cooled to room temperature. The heated culture was centrifuged at 12,000 rpm at 4oC for 8 minutes to remove the cells and other precipitates. Three milliliters (3 ml) of the supernatant were transferred into a test tube, and then 6 ml of cold ethanol (absolute ethanol or 95% ethanol) was added to the test tube and mixed thoroughly, and held at 4°C for 12 h to precipitate the extracellular polysaccharide. Further, after removal of the residual ethanol, the precipitate was dissolved in 3 ml of deionized water at 80°C and the solution was dialyzed against deionized water for 48 h to remove small molecules in the solution.

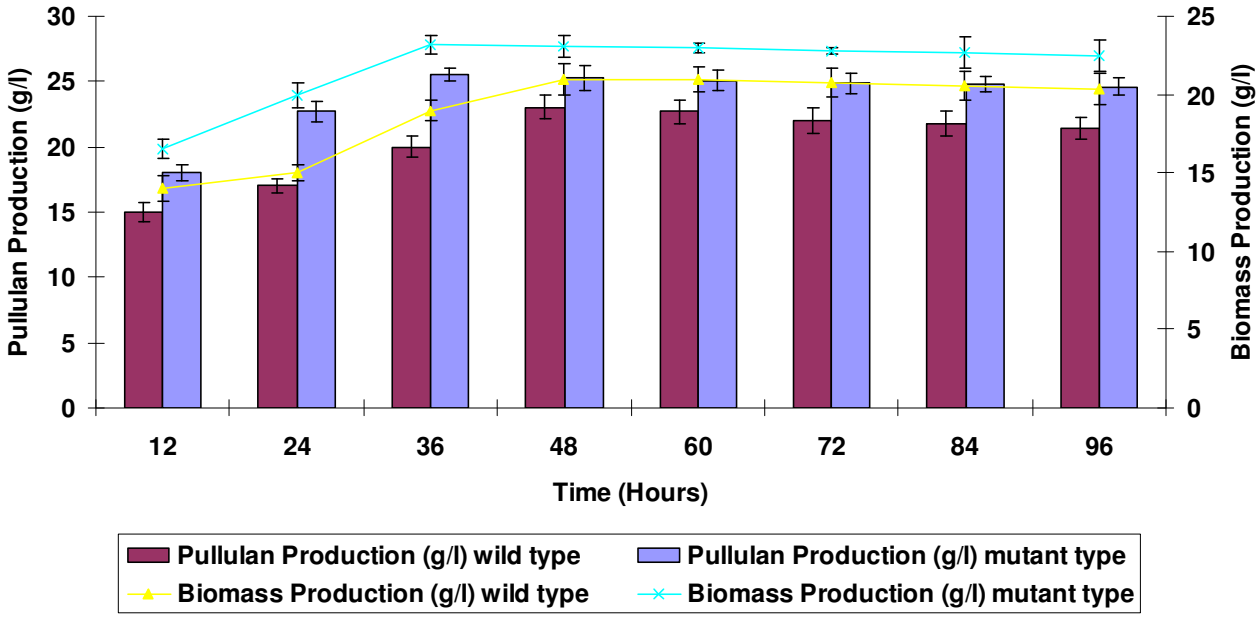

Figure 1. Effect of incubation period on pullulan and biomass production.

After that, exopolysaccharide was precipitated again by using 6 ml of the cold ethanol and after that the residual ethanol was removed and filtered using gelman filter paper (England). Finally, the precipitate was dried at 80°C to a constant weight (Badr-Eldin et al., 1994). Pullulan dry weight was measured using electronic balance (Sartorius, USA) and expressed in gram/liter.

Hydrolysis of the purified extracellular polysaccharide and assay of reducing sugar

To assay the component of the extracellular polysaccharide, the purified precipitate was vacuum desiccated to no alcohol by using a vacuum pump, then dissolved in 3 ml deionized water at 80°C in water bath. The dissolved substrate was hydrolyzed by incubating the mixture of 0.5 ml of the substrate, 0.4 ml of 0.2 M Na_2HPO_4 / 0.1 M citric acid buffer (pH 5.0) and 0.1 ml pullulanase (Sigma Chemicals, USA) for 2 h at 40°C (Su, 1986). The released reducing sugar was determined by using modified dinitrosalicylic acid (DNS) method (Lee et al., 1999) for the confirmation of pullulan.

Statistical analysis

All the experiments were done in triplicate and mean values were calculated using standard deviation.

RESULTS

A mutant of thermotolerant *A. pullulans* was developed through chemical mutagenesis using MMS at 0.5% concentration for 5 min and designated as RG-5 which produced higher amount of pullulan at a temperature (42°C) just like wild type (Figure 2). Therefore, no change in temperature optimum for wild type as well as mutant could be recorded. Furthermore, the mutant utilized 1% lesser amount of sucrose and produced 6 g l-1 higher pullulan as compared to wild type (Figure 5). The thermotolerant wild strain was non-melanin producing, and the mutant also did not produced melanin.

Time course of pullulan production and biomass yield during fermentation

Incubation period for pullulan production differ from strain to strain, therefore, incubation period has also been evaluated for pullulan and biomass production by the mutant. Results in Figure 1 have clearly indicated that maximum pullulan production (23.0±0.9 g l-1) was at 48h of incubation in case of the wild strain, while mutant produced maximum pullulan (25.5±0.5 g l-1) in 36h. Mutant could produce higher pullulan in lesser time period as compared to wild strain. Beyond this time period, the production of pullulan became stable in the case of both, wild and the mutant strain. Similarly, maximum biomass production (21.0±1.0 g l-1) was recorded in 48h in the case of wild type; while in case of mutant maximum biomass (23.2±0.6 g l-1) was obtained at 36h of incubation.

Effect of temperature on pullulan production

Fermentation temperature is one of the important factors for pullulan production, therefore, pullulan production by the wild and mutant types were evaluated at different temperature range (25oC-46oC). It is clearly indicated in Figure 2 that 42oC was found suitable for higher pullulan.

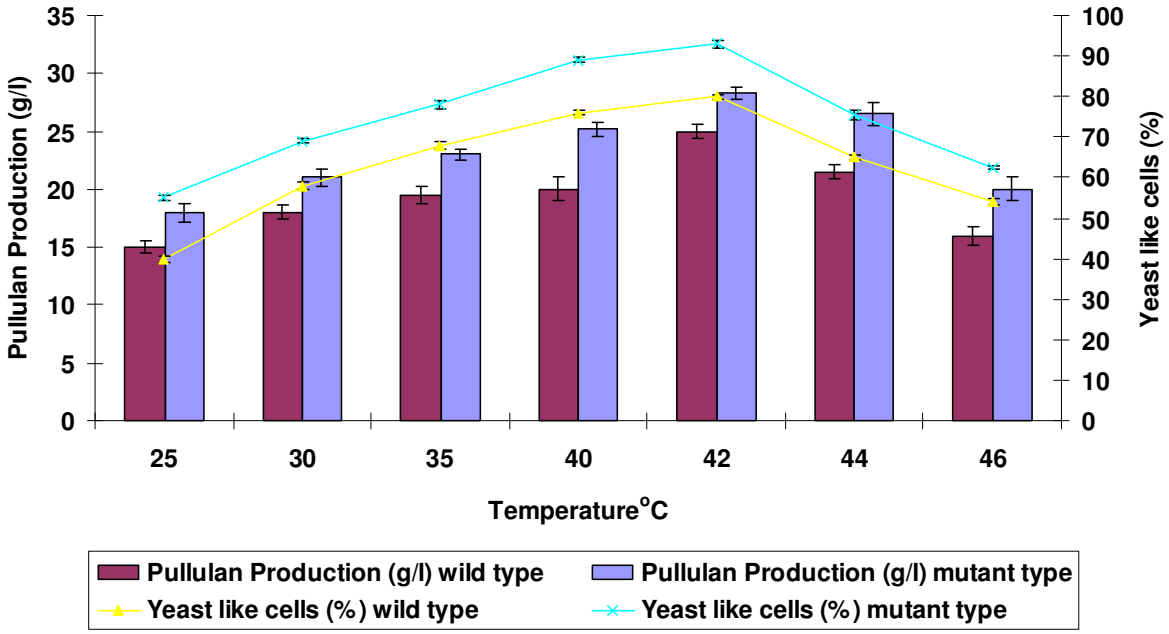

Figure 2. Effect of temperature on production of pullulan and percentage of Yeast like cells.

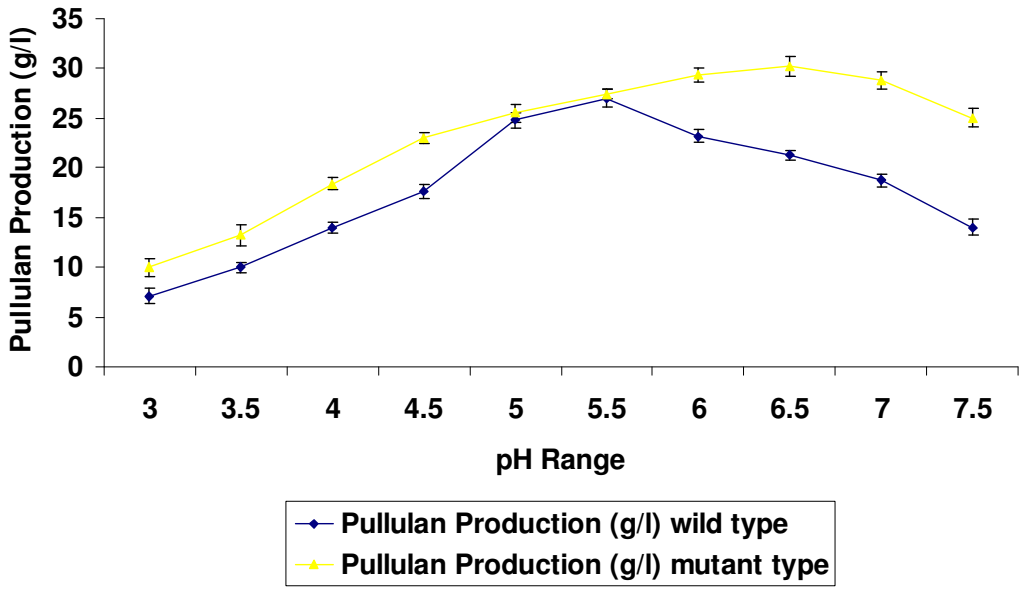

Figure 3. Effect of different pH range on pullulan production.

production and for yeast cells number by the mutant as well as wild types

Effect of initial pH on pullulan production

It has been reported that pH has profound effect on both, the rate of production and the synthesis of pullulan. Therefore, wild type temperature tolerant strain of A. pullulans was compared with its mutant for pullulan production at different pH range (Figure 3). The results clearly indicated that the optimal pH for the pullulan production by mutant was 6.5, at which maximum pullulan production (30.2±1 g l-1) was reported, while in case of the wild type, the optimal pH for higher pullulan production (27.0±0.9 g l-1) was 5.5. Further, increase or decrease of medium pH reduced the pullulan production sharply with the wild type but mutant showed slight reduction in pH. Therefore, mutant is more stable with the increasing pH.

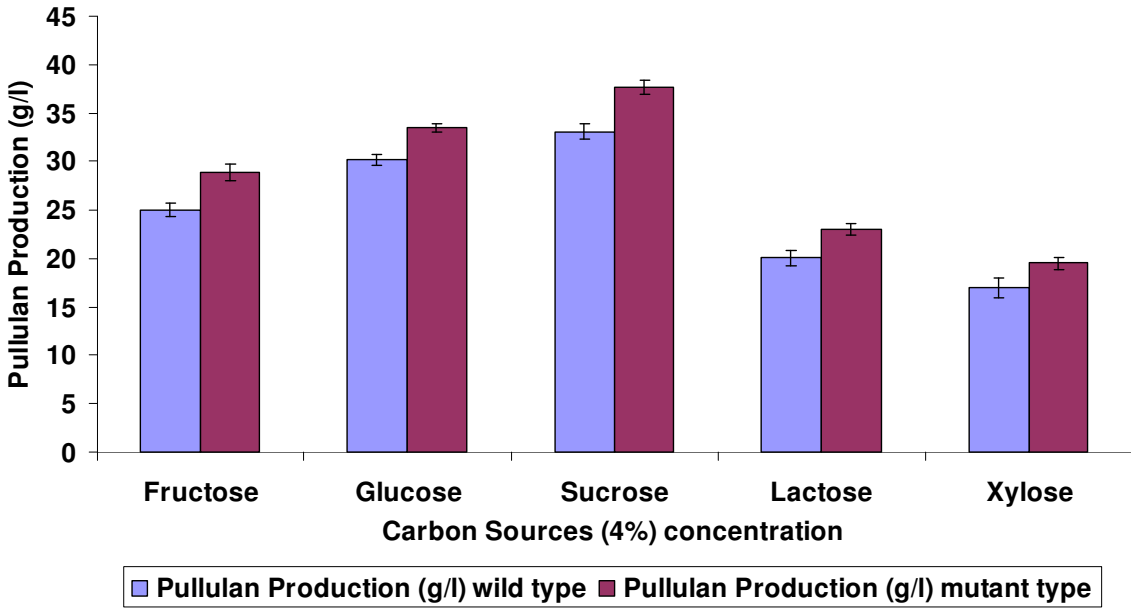

Figure 4. Effect of different carbon sources on pullulan production.

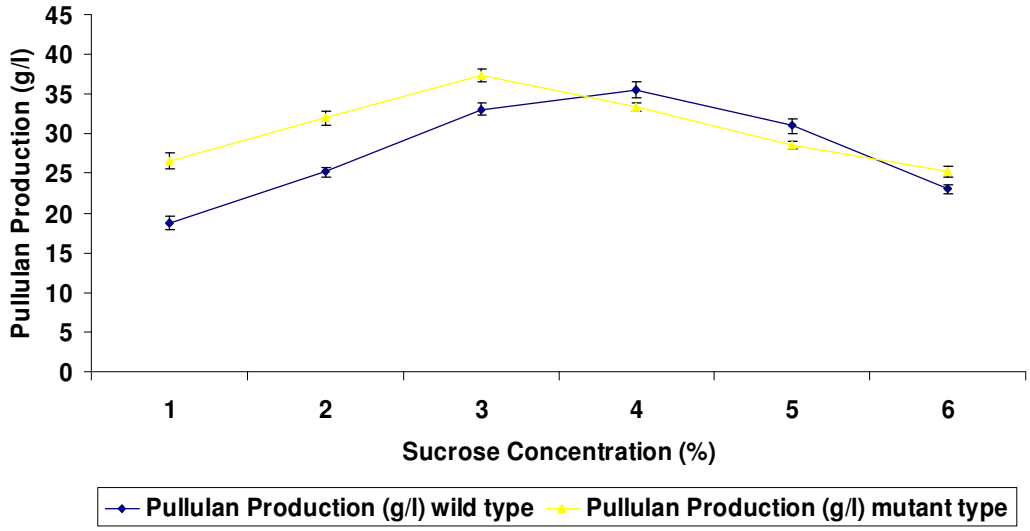

Figure 5. Effect of different sucrose concentration on pullulan production.

Effect of different carbon sources and concentrations on pullulan production

Carbon sources and its concentration play a vital role in the production of pullulan. Among different carbon sources used, highest pullulan production was reported in sucrose by the wild type and the mutant (Figure 4). Further, when different concentration of sucrose was used the mutant strain showed maximum production of pullulan (37.4±0.8 g l-1) at (3%) sucrose concentration, while the wild type showed maximum production of pullulan (35.5±1 g l-1) at (4%) sucrose concentration (Figure 5). Therefore, mutant strain showed better results than wild type as it utilized lesser amount of sugar than the wild type and produced more pullulan. Hence, the mutant strain is more economical than wild type of A. pullulans for pullulan production.

Effect of different nitrogen sources on pullulan production

Different strains of A. pullulans require different concentration of inorganic or organic nitrogen sources or specific

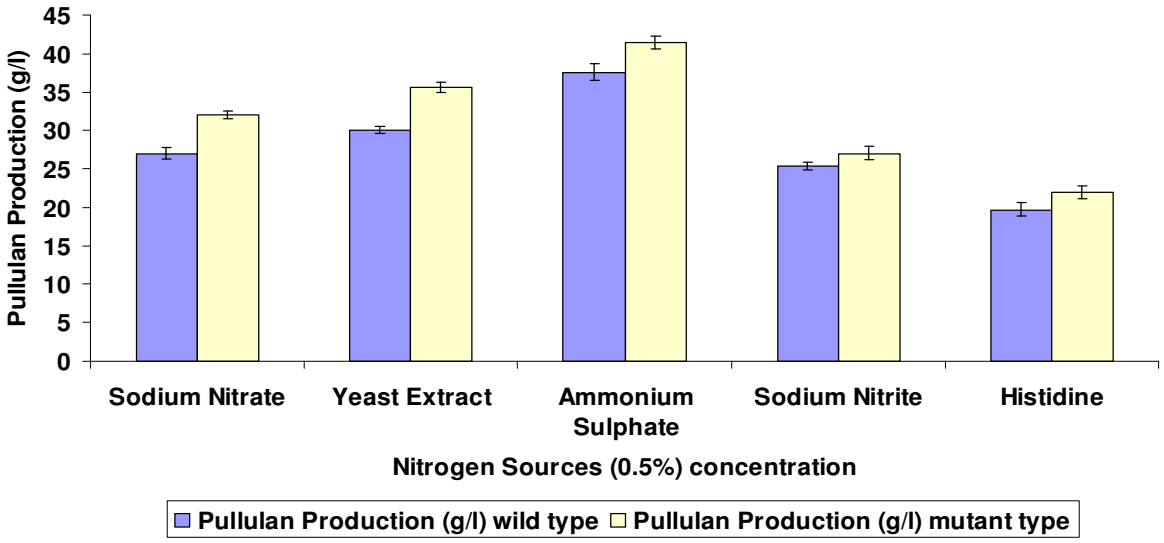

Figure 6. Effect of different nitrogen sources on pullulan production.

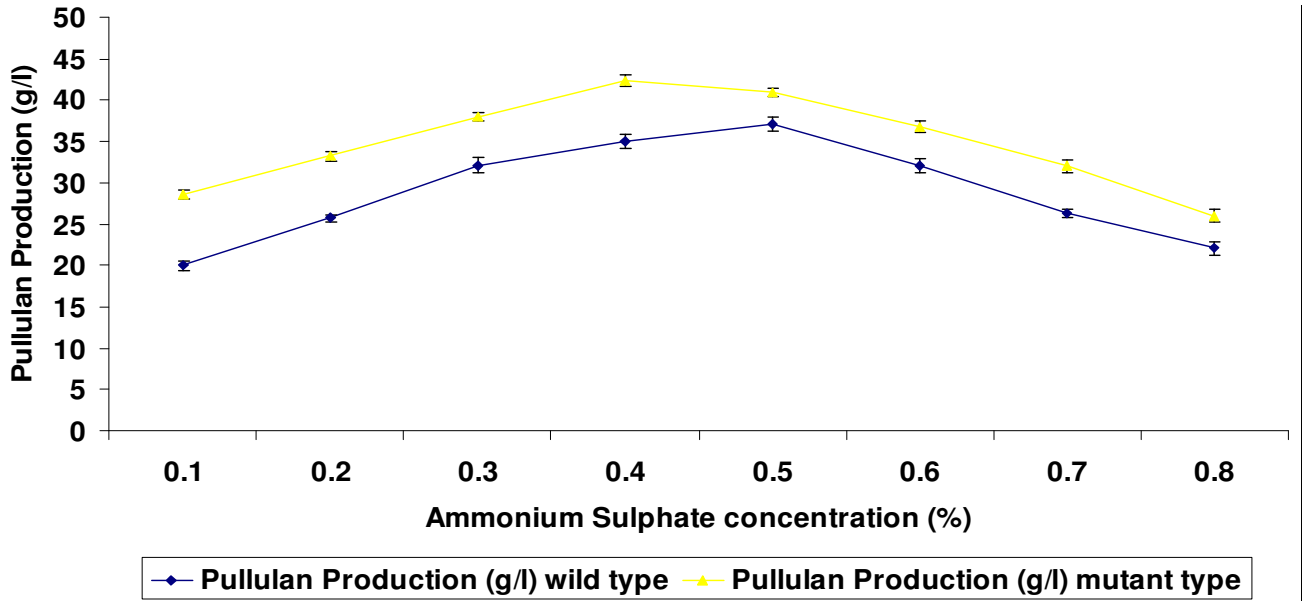

Figure 7. Effect of different ammonium sulphate concentration on pullulan production.

specific combination of both for better pullulan production and support to yeast phase of growth of A. pullulans during fermentation. Among different nitrogen sources taken at 0.5% concentration, the highest pullulan production was reported in ammonium sulphate in case of both, the wild type and the mutant (Figure 6). In view of this context, effect of different concentrations of ammonium sulphate for pullulan production by the mutant and the wild type has been evaluated. The highest pullulan production (42.4±0.7 g l-1) was reported at a concentration of 0.4% by the mutant which was optimal for the production of pullulan, while in case of the wild strain, the highest pullulan production (37.1±0.8 g l-1) was observed at the same concentration of ammonium sulphate taken previously (Figure 7).

Effect of stirred and non stirred fermentation process on pullulan production

Agitation rate is one of the important factors which influence pullulan production during fermentation because oxygen requirement by A. pullulans vary from strain to strain. In the present investigation, different agitation

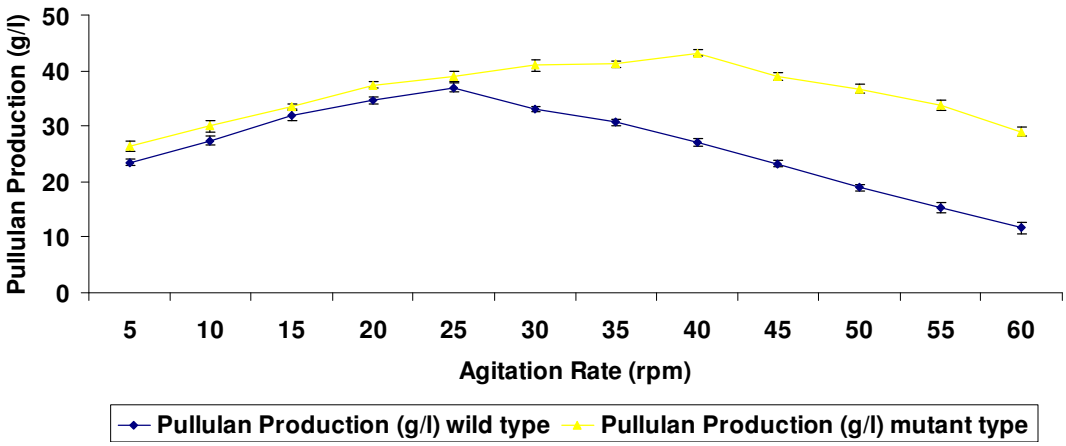

Figure 8. Effect of agitation rate on pullulan production.

speeds were assed for pullulan production by the wild as well as the mutant strain (Figure 8). Maximum pullulan production (37.0±0.8 g l-1) was reported at 25 rpm for wild type and maximum pullulan production (43.2±0.6 g l-1) at 40 rpm for mutant type.

DISCUSSION

The mutant - RG-5 produced higher amount of pullulan as compared to wild type with lesser expense of carbon, nitrogen and incubation period at the same temperature (42°C) at which wild type could produce higher production. The parent strain was non-melanin producing, and the mutant also did not produce melanin. This is an important finding since the mutant did not loose the temperature tolerance capacity and produced non-melanin pullulan which is the requirement of the industry to reduce the cost of pullulan production. Incubation period is an important factors for pullulan production during fermentation and it various from strain to strain (Chi and Zhao, 2003). Aureobasidium pullulans has mainly four different growth phases: yeast-like cells; young blastospores; swollen blastospores and chlymydospores in its life cycle (Guterman and Shabtai, 1996). Yeast like cells are mainly responsible for pullulan production (Cately, 1980, Heald and Kristiansen, 1985, Campbell, 2004). The authors' findings also show similar type of effects with the wild type as well as mutant regarding the pullulan production by yeast-like cells. Mutant produced higher pullulan than the wild type in lesser time interval which is required for industry in order to get higher productivity. Hence, the mutant strain is better than the wild strain. Thus reduction of incubation period by the mutant is also a biotechnological approach for selection of microbial strain. The early production of pullulan is due to change in the physiological status by the mutant. This may be due to the limitation of nutrition, fast metabolism and higher uptake of oxygen at initial level. Similar trend of pullulan production was also observed by Chi and Zhao (2003), even though nutrient was remaining in the med-ium. Similarly, maximum biomass was also produced by mutant strains and in lesser time interval than wild strain, since formation of biomass directly depends on pullulan formation which indirectly depends on some of the other factors like sucrose concentration and other physicochemical parameters. Maximum pullulan production was achieved when the cells reached their stationary phase which was at 48 and 36 h in the case of wild and mutant strains, respectively.

Fermentation temperature is one of the important factors which alter the morphological forms of *A. pullulans* from yeast like cells to blastospores or filamentous, as change in the morphology of *A. pullulans* at elevated temperature adversely affect pullulan production. Yeast phase of growth in submerged system in the presence of suitable carbohydrates and its concentration have shown maxi-mum pullulan production by this organism (McNeil and Kristiansen, 1990). Previous study have shown that in submerged system having sucrose as carbon source in medium supported 90 - 95% yeast like cells resulting in higher pullulan production (Cately, 1980; Heald and Kristiansen, 1985; Campbell, 2004). It was observed that 42°C was found suitable for higher pullulan and biomass production by both wild and mutant strain, respectively. Hence, we can conclude that the optimum temperature for the pullulan production was same for both the wild as well as the mutant strain, although the production of pullulan was more in case of mutant strain than the wild type. This is an important finding since the mutant strain did not loose its thermotolerant nature as that of wild strain. On the other hand, we can also conclude that the highest yeast like cells could also grow at 42°C. Till date, most of the pullulan production by *A. pullulans* has been reported at a temperature range of 24 -32°C by wild types and mutant strains (Kelly and Cately, 1977; Pollock et al., 1992; West and Reed-Hamer, 1993; West and Reed-Hamer, 1994; West and Strohfus, 2001). Thus, it may be

suggested that the optimal temperature for pullulan production and cell morphological forms also depend on the variation of yeast strains and its genetic diversity because *A. pullulans* have been isolated from a very wide range of climatic conditions.

Pullulan production by *A. pullulans* at different pH has shown different trend of pullulan production, and this aspect has been discussed in the light of inorganic nitrogen sources and its concentration in the medium. Production of pullulan was studied at different range of pH (3.0 - 7.0) by various workers and have reported that the change of the medium pH is due to presence of inorganic and organic sources of nitrogen because different rate of ammonium and hydrogen ions may be released in the medium resulting in increase or decrease of pH of the medium affecting the rate of production of microbial metabolites (Lee et al., 2001; Chi and Zhao, 2003; Cheng et al., 2009). The optimal pH for the pullulan production by the mutant was 6.5, while in the case of wild type, the optimal pH for higher pullulan production was 5.5 and further increase or decrease in pH reduced the production drastically in wild type while in case of mutant the reduction of production was not so drastic and was much stable as compared to wild type. Hence mutant strain is stable as compared to wild type which is required for industrial production of pullulan. The mutant strain was not able to produce pullulan at the same pH range as that of wild strain because of either change in the physiology of the cell or in the membrane permeability of the cell releasing hydrogen ion concentration in the medium. This implies that the optimal pH value for pullulan production depends on yeast strain, composition of fermentation medium, growth conditions, the physiology of the cell as well as the membrane modification resulting in the change of transport system of the organism.

Carbon and nitrogen sources are also important parameters which affect pullulan production in a fermentor. Their different concentrations play a vital role in pullulan production. Maximum pullulan production was observed in sucrose as carbon source and ammonium sulphate as nitrogen source in this investigation, while West and Reed-Hamer (1993) have reported that pullulan production was more in glucose grown cells than in sucrose. Another study reported that the pullulan content elaborated by Aureobasidium was lower in sucrose grown cells than in corn starch grown cells (Leathers et al., 1988). Further, West and Strohfus (2001) have reported that mutant produced more pullulan on sucrose than on the corn syrup. In our finding, optimal sucrose concentration for pullulan production was (3%) in the case of mutant type, while in case of wild type optimal concentration was observed at (4%). Mutant strain showed better results than wild type, hence, the mutant is more economical than wild type of A. *pullulans* for pullulan production. Similar carbon source (sucrose) was also found best for pullulan production (Cheng et al., 2009). Therefore, sugar utilization also selectively differs from strain to strain. This is perhaps because of the metabolic and biochemical activities which regulate higher production of pullulan at lesser energy expanses by the cell at their optimal temperature and pH value. Level of sucrose uptake by the cell also varies from strain to strain, and some time, uptake of carbon sources and polysaccharide production depend on the modification of the membrane for less/higher release of microbial metabolite due to increase/decrease in the permeability of cell membrane (Hankin and Anagnostakis, 1975). Some strains of *A. pullulans* has the ability to hydrolyse lactose for utilization of pentose sugars for pullulan synthesis, while some strains have ability to produce such enzymes which hydrolyzes disa-ccharide and oligosaccharide to produce glucose, fructose and other associated monosaccharide mainly hexose attached with glucose (Catley, 1971).

Among nitrogen sources, (0.5%) ammonium sulphate concentration was optimal for pullulan production in case of wild type while in the case of mutant the optimal concentration of ammonium sulphate was (0.4%). Hence, mutant strain is more economical than wild type as it could utilize lesser amount of nitrogen source and produce more pullulan. Below and above this concentration, the production was reduced. Polysaccharide production commenced on reaching nitrogen limiting condition, and the yield of pullulan got down when excess ammonium ions were present, even under conditions which other-wise supported its synthesis. Similar results were shown by Seviour and Kristiansen (1983), Auer and Seviour (1990), Badr-Eldin et al. (1994) and Lee et al. (2001). This is perhaps because ammonium ions are responsible for more synthesis of pullulan or the production of higher biomass resulting in higher pullulan production. Agitation rate has profound effect on pullulan production. Maximum pullulan production (37.0 ± 0.8 g l^{-1}) was observed at 25 rpm in the case of wild type while the mutant required 40 rpm for maximum pullulan (43.2 ± 0.6 g l^{-1}). Some workers have reported an increase in the rate of pullulan production with increase in agitation rate (McNeil and Kristiansen, 1987), while others have reported a decrease production of pullulan with increase in the agitation rate (Gibbs and Seviour, 1996; Wecker and Onken, 1991). It is evident from our previous finding that this thermotolerant isolate of Aureobasidium produced higher pullulan (37.1 ± 1.0 g l^{-1}) in non-stirred condition (Gaur et al., unpublished data) while stirred condition (25 rpm) did not effect the production significantly. The mutant of this fungus, RG-5, required slight higher agitation (40 rpm) for higher pullulan production. Therefore, it seems that mutant requires higher oxygen for their cell growth and this could have been due to modification in physiological conditions of the mutant. There may be several other factors like increase permeability of membrane or cell bound polysaccharide could be released into the medium due to nature of more binding capacity of mutant to the poly-

saccharide. Fed-batch volume of initial 1.5 liters of nutrient solution fed into the fermentor at 5 h intervals in 2 successive stages had been evaluated for pullulan production. Simple batch fermentation did not produce much pullulan as compared to fed batch fermentation process. The data of simple batch fermentation is not presented in the text. Therefore, fed batch fermentation was used for optimization of growth and production parameters for mutant strain.

Conclusion

A mutant of thermotolerant *A. pullulans,* RG-5, was developed which produced higher amount of pullulan by utilizing lesser amount of sugar as compared to the wild type without loosing the thermotolerant nature. This is an important finding because in industries the temperature goes up during fermentation and cooling devices are required to lower the temperature involving higher cost. Therefore, this strain can effectively be used for pullulan production by the industry to cut down the cost of pullulan. Temperature also decreases the viscosity and facilitates oxygen in the medium resulting in better growth and production. Further, the mutant strain can be optimized for continuous production of pullulan by immobilized cells of *A. pullulans* on suitable support matrix by manipulating other fermentation conditions. Therefore, the mutant is also being optimized for continuous production using immobilized cells in our laboratory.

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