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Research Article

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Production of Pullulan from a high yielding strain of *Aureobasidium pullulans* isolated from Jabalpur Region of Madhya Pradesh in Central India

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ABSTRACT

Aureobasidium pullulans, popularly known as black yeast, is one of the most widespread saprophytic fungus associated with wide range of terrestrial and aquatic habitats. The fungus has widely been employed in production of an economically important polysaccharide pullulan. Total 50 isolates of Aureobasidium pullulans were isolated from different flowers and leaves samples, out of which 10 thermotolerant strains produced pullulan. One thermotolerant non-melanin pullulan producing strain, designated as RS-4, produced highest pullulan (6.5±0.02 g/100ml) at 37°C, pH 5.0 in 72h of incubation with 5% sucrose and 0.5% yeast extract in a non-stirred flask fermentation system with working volume 100ml.

Key words: *Aureobasidium pullulans*, Pullulan, Production, Black Yeast.

INTRODUCTION

Aureobasidium pullulans (De Bary) Arnaud is cosmopolitan yeast like fungus that occurs in diverse habitats, including the phylloshere of many plants and also on various tropical fruits. The fungus is industrially important because of its capability to produce the polysaccharide pullulan [5, 11]. It is a linear α-D-glucan, made mainly of maltotriose repeating units interconnected by α-1, 6 linkages. The regular alternation of α-1, 4 and α-1, 6 bonds results in two distinctive properties that is structural flexibility and enhanced solubility [8]. This polysaccharide is of great economic importance with increased applications in food, pharmaceutical, agricultural, blood plasma substitute and chemical industries [4-5; 11]. Pullulan produces a high viscosity solution at a relatively low concentration and can be used for oxygen-impermeable films and fibers, thickening or extending or adhesives or encapsulating agents [5, 11].

Yeast cells are mainly responsible for pullulan production (2). Till date most of the studies have been reported on mesophilic strain of this fungus which produces pullulan up to level of 30-36 g/l in flask shake experiments or in fermentor experiments at a temperature range of 24-32°C at different physico-chemical conditions [3- 4, 9, 11-12, 15]. However, a high yielding strain of *A. pullulans* has not been reported so far which can also sustain a high temperature above 35°C.

Non-stirred fermentation system is beneficial over stirred fermentation system as the later one cuts down the cost and energy. Therefore, strains capable of producing high amount of pullulan in non-stirred fermentation system may

be beneficial for commercial production of pullulan. A thermo tolerant strain is better than mesophilic strain at a commercial level, because it can sustain high temperature and also cut down the price involved in cooling devices during fermentation. Furthermore, melanin is produced by most of the *A. pullulans* during fermentation. This will make it more difficult to purify polysaccharide after fermentation. Therefore, non-melanin producing strain is another important choice during screening and selection of strain for pullulan production.

In view of the importance of this polysaccharide, an attempt is made to isolate a high yielding thermotolerant, non-melanin producing strain of *A. pullulans* from various plant parts from St. Aloysius College Campus, Jabalpur, Madhya Pradesh, India, and optimized at various physico-chemical as well as nutritional parameters for higher pullulan production.

EXPERIMENTAL SECTION

Isolation and Maintenance of Micro-Organism

A. pullulans was isolated from the flowers and leaves samples collected within the college campus. Isolation was done by selective enrichment method. Flower and leaf samples were soaked in sterile water for 3 days at 35°C, and then 0.1 ml was transferred to 10 ml of basal fungal medium (pH 5.0). After 2 days, the turbid cultures were allowed to sit undisturbed for 20 minutes to settle the filaments and aggregates to the bottom. About 10µl of upper partially clarified culture was spread onto base agar medium plates containing 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. (Qualigens Chemicals, Mumbai, Maharashtra, India). Isolates were maintained on the same medium at 4°C in slants and sub-cultured monthly. The isolates were identified on the basis of morphological and cultural characteristics using standard identification manuals of fungi [6-7] on its respective medium.

Inoculum Preparation

Cell suspension was prepared by inoculating 1 ml of 48h grown culture in 200 ml nutrient in broth of the same medium and incubated at 42°C for 48h to achieve active exponential phase of the culture.

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%; dipotassium 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 *A. pullulans* having $50x10^7$ CFU/ml and then incubated for different periods *viz.* 24, 48, 72, 96 and 120h at different temperature *viz.*30, 37, 43, 50 and 60°C. For pullulan production non-stirred flask fermentation process was followed. For evaluation of pH, the basic medium pH was adjusted to 2.0, 3.0, 5.0, 7.0 and 9.0 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, sucrose and maltose (at the level of 2%) along with nitrogen sources *viz.* ammonium sulphate, yeast extract, sodium nitrate (at the level of 0.5%). Erlenmeyer flasks were used for fermentation having total volume of 250 ml and working volume 100 ml. The fermentation medium was sterilized at 121°C for 15 minutes and incubation was done at 37°C with all the other conditions at the optimal levels determined previously. The sterile air was supplied only up-to 10h at rate of 0.5 vvm (at different incubation periods).

Extraction and Estimation of Pullulan

After fermentation, the culture medium was heated at 100°C in water bath for 15 minutes, cooled to room temperature and centrifuged at 12,000 rpm at 4°C for 10 minutes to remove cells and other precipitates. Three milliliters of the supernatant were transferred into a test tube, and then 6ml of cold ethanol (absolute or 95% ethanol) was added to the test tube and mixed thoroughly and held at 4°C for 12h to precipitate the extracellular polysaccharide. 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 48h to remove small molecules in the solution. The exopolysaccharide was precipitated again by using 6ml of the cold ethanol and the residual ethanol was removed, the precipitate was dried at 80°C to a constant weight [1]. Pullulan weight was measured by using electronic balance (Sartorius, U.S.A.) and expressed in g/100 ml.

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 3ml 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 Na2HPO4 (0.2M), citric

acid buffer 0.1M (pH 5.0), and 0.1 ml pullulanase (Sigma Chemicals, U.S.A.) for 21 hour at 40°C, (Su, 1986).The released reducing sugar was determined by using the modified D.N.S. method (10) for the confirmation of pullulan.

Statistical Analysis

Karl Pearson method (Variability) was followed for statistical analysis. All the experiments were done in triplicate and mean values were calculated using standard deviation.

RESULTS AND DISCUSSION

A total of 50 strains of *A. pullulans* were isolated. Out of these, 10 isolates of *A. pullulans* were screened which produced significant amount of pullulan at 37^{o}C within 72 hours of incubation. Only one non-melanin pullulan producing strain, designated as RS-4, produced highest amount of pullulan at 37^{o}C in 72h of incubation (Data not shown). Thus, this strain was selected for optimization of physico-chemical parameters for higher pullulan yield.

During preliminary screening, stirred and non-stirred fermentation conditions were evaluated for pullulan production at all range of temperature from 30^{o}C to 60^{o}C under aeration. Stirred and non-stirred process produced almost same amount of pullulan in 72h of incubation at 0.5 vvm of sterile air supplied for 10h. Thus in this text only non-stirred result is presented and discussed in the light of different range of temperature, pH, carbon and nitrogen sources at various concentrations for pullulan production in non-stirred flask fermentation process. Non-stirred fermentation process is always beneficial over stirred fermentation process as it saves energy and requires less amount of cost. This strain of *A. pullulans* is capable of producing satisfactorily high amount of pullulan in non-stirred condition, therefore, can be exploited for commercial production of pullulan.

Furthermore, it is an interesting finding that our isolate of *A. pullulans* strain RS-4 could tolerate 37°C mainly for higher yeast count which is mainly responsible for pullulan production. *A. pullulans* is a ubiquitous fungus of polymorphic characteristic and frequently isolated from different ecosystems namely terrestrial to aquatic. The various morphological forms protect the fungi for long term existence in all sort of environment. This fungus has been reported to produce yeast-like cells at 25^{o}C to 35^{o}C mainly responsible for pullulan production. Besides this, our isolate has produced a very high amount of pullulan which has not been reported so far. Therefore this isolate can be used successfully in industries where temperature goes very high and cooling devices are required to lower the temperature. This strain has both the quality of high production as well as high tolerance of temperature. India is a temperate as well as subtropical country, and thus *A. pullulans* diversity in different climatic conditions may be a reason of this finding. Till date a starin has been not isolated which produced such a high amount of pullulan.

Time Course of Pullulan Production and Biomass Yield during Fermentation

Pullulan production is directly related to yeast phase of growth. Yeast-like cells are mainly responsible for pullulan production (2). Incubation period for pullulan production differs from strain to strain; therefore incubation period has also been evaluated for pullulan production and biomass accumulation. Maximum pullulan production (5.6±0.05 g/100ml) was recorded at 72h of incubation. Further, after 72h of incubation the production of pullulan became stable. This was mainly because the cells reached their stationary phase (Figure 1).

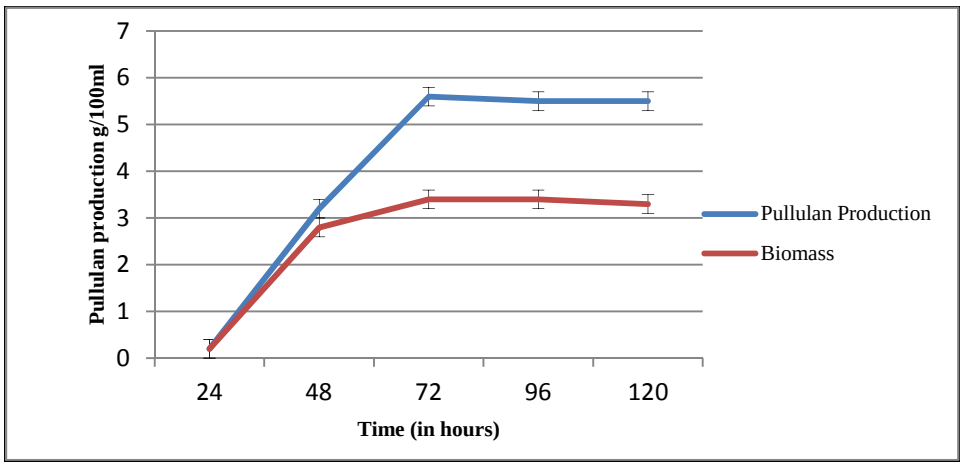

Figure 1: Time Course of Pullulan Production and Biomass Yield during Fermentation

Effect of Temperature on Morphology and Pullulan Production

Fermentation temperature is one of the most important factors for pullulan production affecting yeasts phase of *A. pullulans* growth because change in the morphology adversely affects pullulan production. In this fungus, yeast form of growth is mainly responsible for pullulan production in flask fermentation system. It is clearly indicated in Figure 2 that strain has able to produce high amount of pullulan (3.1±0.03g/100ml) at 37°C. Hence, we can conclude that the highest yeast like cells survived and produced pullulan at 37°C. The optimal temperatures for pullulan production by *A. pullulans* vary from strain to strain.

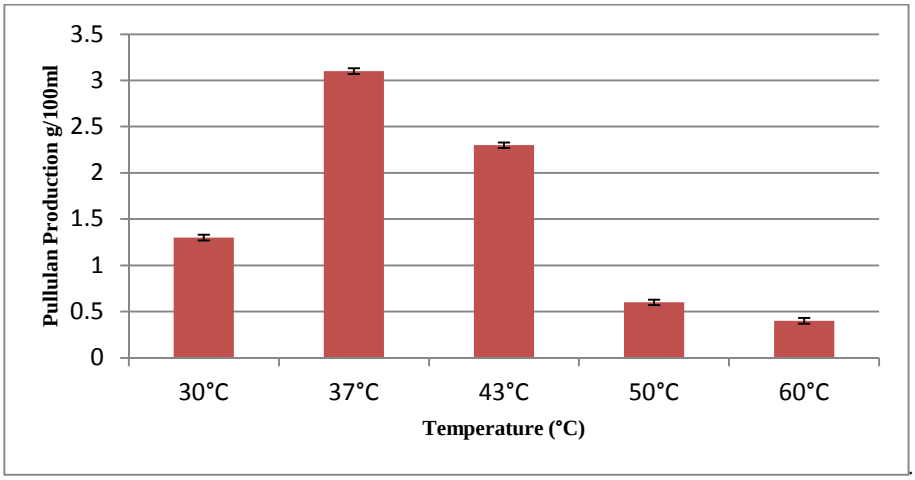

Figure 2: Effect of different temperature on pullulan production

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. Different workers have reported pullulan production at different pH range of 2.0-9.0 in the medium (3, 4, 9, 12). The maximum production (3.1±0.01g/l) of pullulan in the flask fermentation conditions was recorded at an initial pH 5.0 (Figure 3). This implies that the optimal initial pH values for pullulan production depend on different yeast strains, composition of the fermentation medium and growth conditions. Therefore, the physiological function of *A. pullulans* varies from strain to strain in case of pH also. This is perhaps due to either special structure of the membrane and cell wall or transport system of the organism along with the change of cytosole pH due to medium constituents affecting the critical level at specific pka value of medium and ultimately affecting more or less hydrogen ion concentration which in turn affected cell growth or pullulan synthesis. The pH value reached much lower due to organic acid production by yeast cells after growth of *A. pullulans*, which affects negatively the extracellular polysaccharide production.

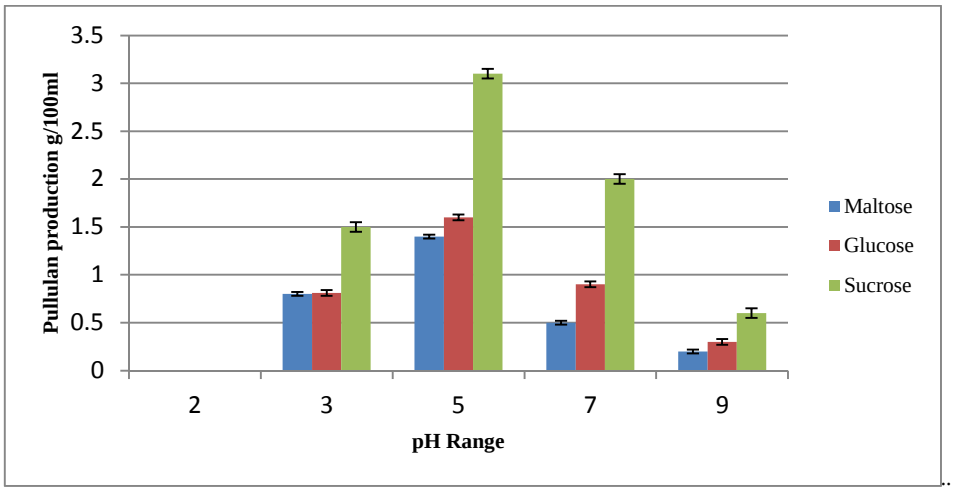

Figure 3: Effect of pH on pullulan production

Effect of Different Carbon Sources and Concentrations on Pullulan Production

Carbon sources play a vital role in the production of pullulan (4). Sucrose showed best result (2.5±0.02 g/100ml) among all the carbon sources used at level 2% (Figure 4). The effect of different concentration of sucrose on

pullulan production by this strain was investigated at 37°C in the production medium. The optimal concentration of sucrose for pullulan production by this strain was 5% (w/v), resulted in pullulan yield of 2.8±0.02 g/100ml (Figure 5). Similar carbon source (*i.e.* sucrose) was also found best for pullulan production as reported by other workers [3]. The pullulan production increased with the increase in initial sugar concentration level from 2% to 5% (w/v). Further increase of sugar concentration resulted in reduction of pullulan yield. The decline in polysaccharide production encountered with high sugar concentrations in the medium is probably due to osmotic effects, such as lower level of water activity as well as plasmolysis events.

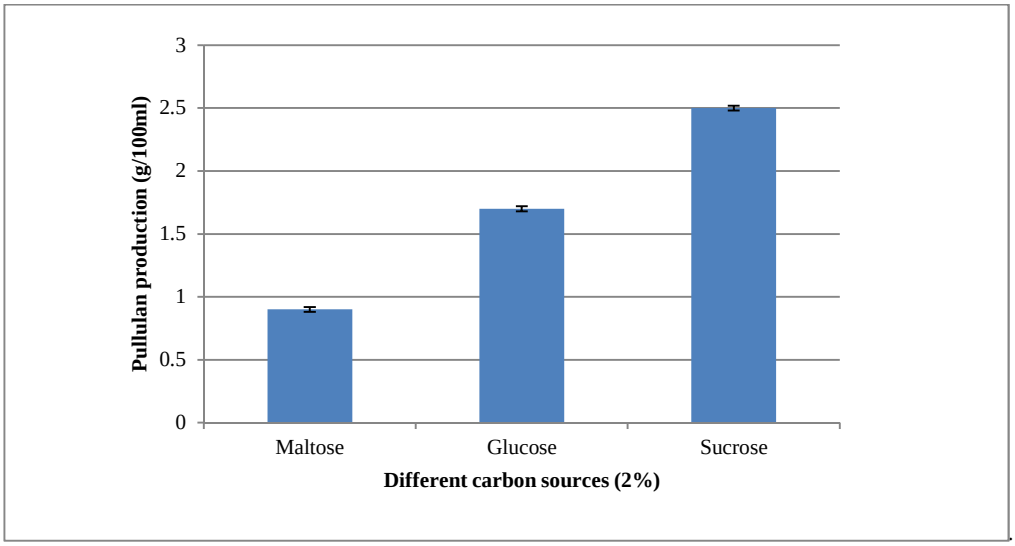

Figure 4: Effect of different carbon sources on pullulan production

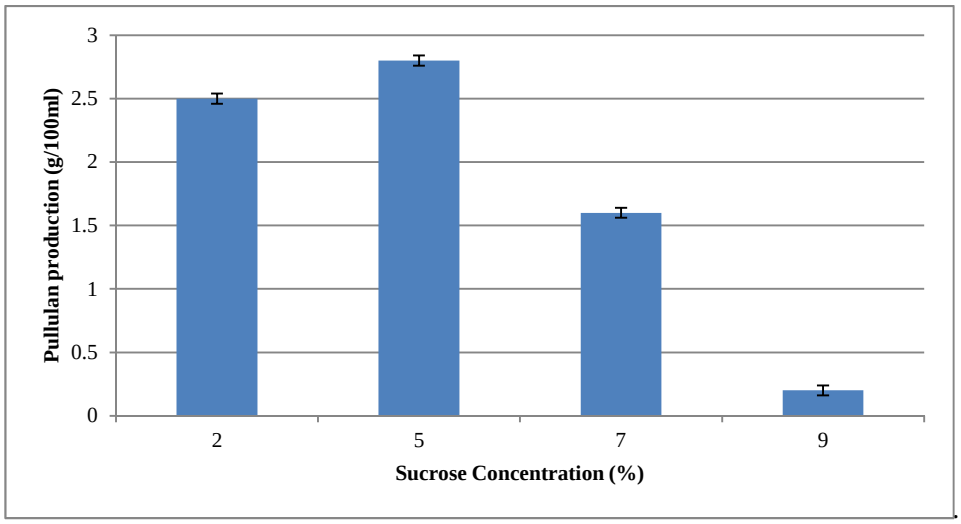

Figure 5: Effect of different sucrose concentration on pullulan production

Effect of Different Nitrogen Sources on Pullulan Production

Different nitrogen sources were also tested to optimize pullulan production in non-stirred flask fermentation by this fungus. Among different nitrogen sources (organic and inorganic), the highest pullulan production (6.2±0.04g/100ml) was reported with yeast extract at the level of 0.5% (Figure 6). In view of this context, different concentrations of yeast extract were further studied. Highest pullulan production (6.5±0.02g/) was reported at the same concentration of yeast extract. Below and above this concentration (0.5%) pullulan production decreased (Figure 7). Polysaccharide production commended on reaching nitrogen limiting condition, and the yield of pullulan fell when excess yeast extract was present, even though other conditions were favourable for pullulan production. Various nitrogen sources were optimized for pullulan production by different workers (3, 4).

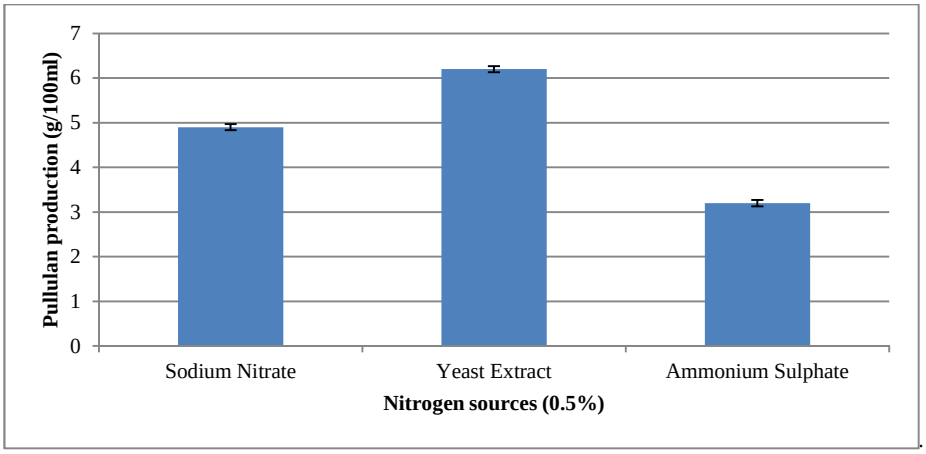

Figure 6: Effect of different nitrogen sources on pullulan production

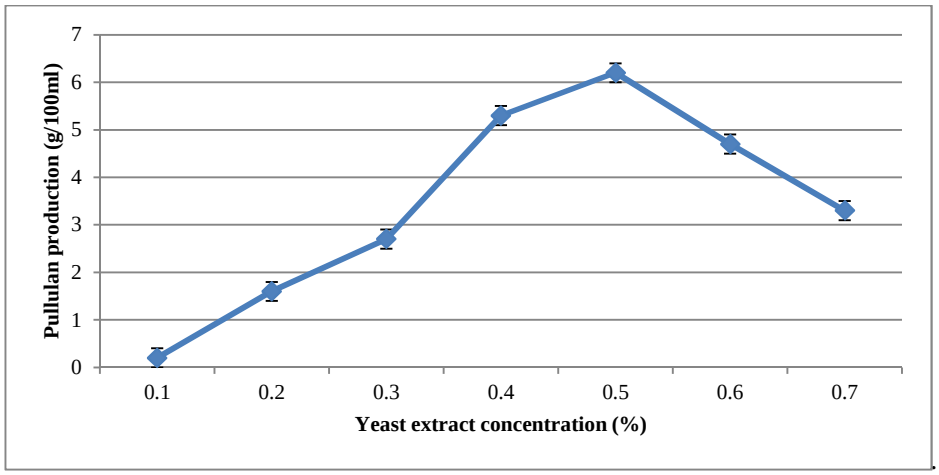

Figure 7: Effect of different Yeast Extract Concentration on pullulan production

CONCLUSION

From the ongoing study it can be concluded that the isolated strain of *Aureobasidium pullulans* was able to produce higher amount of pullulan by utilizing lesser amount of sugar (5%) at 37°C. This is the first report of a high yielding strain of this fungus from the world. The strain can sustain high temperature and is of great use in industry where temperature reaches very high and cooling devices are needed.

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