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Short Communication

Short term atmospheric pressure cold plasma treatment: A novel strategy for enhancing the substrate utilization in a thermophile, *Geobacillus* sp. strain WSUCF1

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ABSTRACT

The aim of this study was to investigate the effect of atmospheric pressure cold plasma on the microbial substrate utilization and biomass yield in a thermophilic strain. *Geobacillus* sp. strain WSUCF1, a thermophile capable of producing cellulolytic enzymes with higher activity was used for this investigation. Treatment with cold plasma for 4 min increased the rates of glucose utilization by 74% and biomass yield by 60% when compared with the control. WSUCF1 treated with plasma also displayed enhanced biofilm formation. This study for the first time, reports the use of cold plasma for enhancing the substrate utilization and biofilm formation in a thermophile.

1. Introduction

Biomanufacturing technologies have shown rapid growth in recent years, with special promise for production of renewable chemicals, biofuels, enzyme, and high value, low volume products (Erickson et al., 2012; McFadden and Smyth, 2018). Microorganisms are attractive options for producing nutraceuticals, cosmetics, and therapeutic molecules (Rathinam et al., 2015). Microbial processes have significant advantages over conventional chemical processes due to their ability to produce stereospecific molecules, their environmentally benign nature, and their ease of scale up (Rathinam et al., 2017). According to *Microbial Products: Technologies, Applications and Global Markets Report,* the global market for microbial products is expected to escalate to a value of $302.4 billion in 2023 from a value of $186.3 billion in 2018, and this compound annual growth rate (CAGR) of 10.2% is expected to translate into a demand for industrial microbial technologies reaching $11.45 billion by 2026 (BCC Research, 2018).

Different bioprocess/biochemical engineering strategies such as process optimization, and reactor design were reported to increase the yield of microbial processes (Schmidt, 2005; Singh et al., 2017). Mutagenesis and recombinant DNA technology significantly helped in strain development to enhance the yield of microbial products both in terms of quantity and quality (Adrio and Demain, 2006). Recent advancements in synthetic biology, system biology and metabolic engineering strategies helped to precisely tailor their molecular systems for production of a wide range of commodity chemicals in microbial hosts (Chubukov et al., 2016). However, these strategies suffer from several limitations as they are laborious, time consuming, and could not significantly increase the yields to meet the industrial needs.

Thermophilic bioprocessing is an attractive option for increasing the yield of the products as well as cutting down the costs. Many thermophiles are shown to be resistant not only to elevated temperatures but also to extreme environments such as high toxic environments and extreme pH (Rathinam et al., 2018; Rathinam and Sani, 2018). Herein, an attempt is made to investigate the effect of cold plasma on the metabolic activity and biofilm formation in a selected thermophilic strain, *Geobacillus* sp. strain WSUCF1. Atmospheric cold plasma is a fourth state of matter and has several biological applications. Cold plasma is shown to influence biological systems in different ways. Biocidal activity of cold plasma is well explored and is shown to inactivate a wide range of bacteria and some bacteriophages. Singlet oxygen is shown to mediate the inactivation of viruses by damaging the nucleic acids and proteins (Rajan et al., 2013a,b). Cold plasma is shown to be promising for sterilization applications in the food processing and

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packaging industry (Rajan et al., 2013a). In addition to singlet oxygen, cold plasma also contains multiple chemically reactive species, including reactive oxygen species such as ozone, atomic oxygen, superoxide, peroxide, and hydroxyl radicals; and reactive nitrogen species which plays a key role in inactivating the microorganisms (Laroussi et al., 2004). Detailed reports on the effects of plasma treatment on the changes in conformation and function of proteins are also documented (Tolouie et al., 2017). Reports are also documented in the literature on the plasma mediated cell disruption and genetic changes in the microbial cells leading to phenotypic and genotypic changes. This in turn could contribute towards enhanced mass transfer of the solutes across the cell membrane as well as changes in the metabolic activity leading to enhanced catalytic activity (Han et al., 2016; Xu et al., 2015).

WSUCF1 strain used in this investigation is a cellulose degrading thermophilic strain isolated from a compost facility at Washington State University (WSU), Pullman, WA that can grow at an optimal temperature of 60 °C. WSUCF1 was shown to retain 89% of its initial CMCase activity after incubation at 70 °C for 1 day (Rastogi et al., 2010). Cellulolytic activity of this strain is evident from its genome sequence encoding several genes for glycoside hydrolases (Bhalla et al., 2015). This strain was shown to produce highly thermostable β-xylosidase (when compared with other thermophiles reported in the literature) with high specific activity (133 U/mg when incubated with p-nitrophenyl xylopyranoside) (Bhalla et al., 2014). Developing novel strategies for improving the performance of these thermophilic strains will aid in further enhancing the rates of degradation of lignocellulosic feedstocks and developing extremophilic processes for industrial applications.

To the best of our knowledge, this is the first work reporting the use of cold plasma treated thermophilic cells for enhancing the biomass yields. Thermophilic bioprocess being robust are promising for industrial application. This strategy will help in improving the effect of cold plasma treatment on substrate utilization, biomass yield, and biofilm formation in a thermophilic strain that can grow at a temperature of 60 °C.

2. Materials and methods

2.1. Growth of microorganisms

Geobacillus sp. strain WSUCF1, a thermophilic bacterial strain isolated from the compost sites was used for this investigation. The details on isolation of WSUCF1 was described elsewhere (Rastogi et al., 2010). The cultures of WSUCF1 were inoculated in LB broth and incubated at 60 °C for 24 h. After incubation, WSUCF1 cell pellet was obtained by centrifuging the broth containing the cells at 10,000 rpm for 10 min. The cell pellet was washed thoroughly using sodium phosphate buffer (0.1 M, pH 7). This removes unwanted cell debris and broth constitutes from the cell pellet. The washed cells were dispersed in sodium phosphate buffer, stored at 4 °C and used throughout the experiment.

2.2. Cold plasma treatment

3 ml of phosphate buffer containing the cells was placed in petri plates (60 mm diameter) and exposed to cold plasma. Nanosecond-pulsed dielectric barrier discharge (DBD) having a cylindrical internal brass electrode covered by a glass test tube with a semispherical tip as dielectric (1 mm thick) was used as the source for cold plasma. Petri plates containing the WSUCF1 cells were kept at 1.5 cm distance from the tip of plasma source (Rajan et al., 2013a). Experiments were conducted to investigate the effect of cold plasma treatment for different time intervals such as 2, 4, and 8 min. Petri plate containing the same concentration of WSUCF1 cells without exposing to cold plasma was used as a control.

2.3. Substrate utilization and biomass yield

WSUCF1 cells treated with cold plasma and control were inoculated in 0.1 g nitrilotriacetic acid, 1-ml $FeCl_3$ solution (0.03%), 0.05 g $CaCl_2{\cdot}2H_2O$, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g NH_4Cl, 1.8 g of 85% H_3PO_4, 0.005 g methionine, 0.05 g yeast extract, 0.01 g casamino acids, and 1-ml of Nitsch's trace element solution (per liter). 1 mM of glucose was used as the sole source of carbon. The incubation temperature was maintained at 60 °C for 5 days. Substrate utilization kinetics of cold plasma treated cells in minimal media was analyzed by measuring the glucose and chemical oxygen demand levels for every 24 h (Miller, 1958). The dinitrosalicylic acid method was used for quantifying the levels of glucose. A Merck Spectroquant was used for quantifying the chemical oxygen demand (COD) levels. The broth inoculated with control and cold plasma treated cells was centrifuged at 10,000 rpm for 10 min. Wet weight of the cell pellet was noted for 4 different sets to analyze the effect of plasma treatment on biomass yield.

2.4. Biofilm formation

Electrogenic activity of WSUCF1 was harnessed for analyzing the biofilm formation kinetics. Minimal media described in Section 1.3 was used as electrolyte and inoculated with WSUCF1. Carbon felt (procured from Fuel Cell Earth, LLC) (of dimension 1.0 cm^2) was used as the electrode material for adhesion of microbial cells. The carbon felt electrodes were fabricated by making electrical contact with a brass rod. The fabricated carbon felt electrodes were used as the working electrode and silver wire was used as the pseudo reference electrode. The potential difference between the working electrode and the reference electrode was used as the tool for monitoring the biofilm formation (Bhuvaneswari et al., 2013).

3. Results and discussions

3.1. Substrate utilization and biomass yield

Investigations on the effect the cold plasma treatment for different times (0, 2, 4, and 8 min) on glucose utilization in *Geobacillus* sp. strain WSUCF1 showed that the glucose oxidation rates increase with cold plasma treatment for 2 min and 4 min. Among the three different exposure time intervals tested, the set treated for 4 min displayed the maximum glucose utilization. The treatment of *Geobacillus* sp strain. WSUCF1 with cold plasma for 2 min and 4 min increased the glucose utilization by 66% and 74% respectively when compared with the control. This is a very significant increase which, in turn, could be expected to positively influence the growth rates of the cells. However, exposing the cells for 8 min decreased the substrate utilization rates by 36% when compared with the set treated for 4 min. Investigation of COD removal with the control and cells treated with plasma for different time intervals, showed that treatment of WSUCF1 at 2 min, 4 min, and 8 min increased the COD removal rates by 13%, 45%, and 25% respectively. Evidently, increasing the treatment time to 8 min showed a decrease in COD removal rate by 11% when compared with the cells treated for 4 min. Whereas the results of the COD utilization well corroborate our findings on the effect of cold plasma treatment on glucose utilization in WSUCF1, one difference between the glucose and COD utilization to be highlighted is the higher COD levels compared with the glucose levels. The higher levels of COD in all the cases, relative to the glucose levels, is due to the presence of metabolites produced by the cells.

The results of glucose utilization and COD utilization also corroborate the biomass yields. Among the three different sets treated with cold plasma for 2, 4 and 8 min, the one treated for 4 min had the highest yield. Treatment of WSUCF1 with cold plasma for 2, 4 and 8 min increased the yield by 33.56%, 59.8%, and 44.5% respectively. Evidently

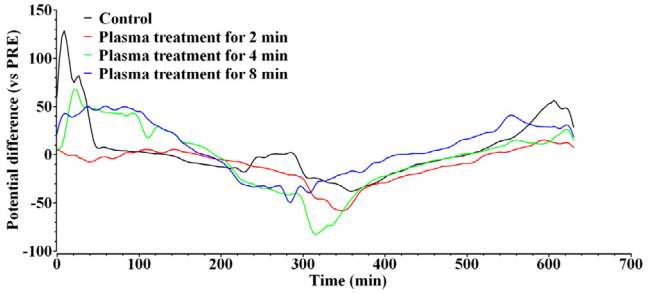

Fig. 1. Effect of cold plasma treatment on biofilm formation in *Geobacillus* sp. strain WSUCF1.

the biomass yield of WSUCF1 cells treated for 8 min with cold plasma was 9.5% lower when compared with the cells treated for 4 min. These findings clearly show that the *Geobacillus* sp strain WSUCF1 could survive and metabolize (utilize sugar and produce metabolites) even on exposing the cells with cold plasma for 8 min. This survival time could vary depending on the inherent property of microorganisms and, more importantly, the membrane characteristics of the microorganisms and their resistance to survive in extreme conditions, such as a higher intensity cold plasma. Lee et al. (2015) reported the decimal reduction time for *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* using atmospheric-pressure cold plasma treatment as 18 s, 19 s, and 115 s respectively. In comparison to mesophiles, thermophiles have increased hydrophobicity of the transmembrane helices, increase in hydrogen bonds and salt bridges, shorter length of surface loops, reduced number of kinks in the transmembrane helices, and reduced length of transmembrane helices (Meruelo et al., 2012). These features of the thermophiles offer stability to the *Geobacillus* spp., which may explain why the observed results qualify the majority of previous reports where cold plasma is demonstrated as a potential biocidal strategy for inactivating or killing microorganisms.

These findings open a new door for using the cold plasma technology for increasing the catalytic activity of the microorganisms. Several reports in the literature document the effect of cold plasma treatment on disruption of the cell membrane in microorganisms, whereas WSUCF1 has been demonstrated to survive in extreme environments such as exposure to cold plasma for unusually long times.

Furthermore, exposing the microbial cells with cold plasma for a relatively short time improved substrate utilization and cell biomass yield which may be due to partial disruption of cell membrane, or changes at the genetic level. However, treating the cells for a longer time, leads to inactivation of cells and decreased metabolic activity. Comparative studies on the glucose utilization, COD removal and biomass yield in the control and cold-plasma treated cells showed that the optimal plasma treatment time for enhancing the biomass was about 4 mins. Harnessing this optimized time will aid in developing a facile, inexpensive, and green strategy for increasing the product yield to meet industrial demand.

Previously, a report was made on modifying the rheology of the broth to avoid the cells forming flocs. Flocs settle faster when compared with the individual cells, and modifying the rheology of broth helped to overcome the limitations in diffusion of solutes to cells within the flocs (Rathinam et al., 2018). The present study, on the other hand, addresses the diffusion limitations at the cellular level. Surface display technology is another approach which is often used to overcome mass transfer limitations, by engineering the microbial cells to express the enzymes on the cell surface. However, the protein expressed using this strategy suffers from poor folding leading to decreased catalytic activity (Park et al., 2006).

3.2. Biofilm formation

This study also provides a preliminary study of biofilm formation kinetics of WSUCF1 cells treated with cold plasma for 2, 4, and 8 min. The potential difference tends to change with the adhesion of microbial cells onto the electrodes (Veerubhotla et al., 2017). This change in potential difference is used as a tool to monitor the changes in biofilm formation kinetics in WSUCF1 cells treated with cold plasma for 2, 4, and 8 min, as shown in the Fig. 1. WSUCF1 cells without treatment with cold plasma reached the maximum negative potential of −40 mV (vs PRE) after 358 min. WSUCF1 cells treated with cold plasma for 2 min and 4 min reached the maximum voltage of −65 mV (vs PRE) at 345 min and of −86 mV (vs PRE) at 310 min, respectively. These results clearly indicate that the cells treated with cold plasma for 4 min form a much more stable biofilm at a shorter time, compared with the control cells and the cells treated for 2 min. Cells treated with plasma for 8 min formed the biofilm at a faster time of 273 min, but the biofilm appears to be less stable when compared with the others.

4. Conclusion

This investigation revealed the potential of using short term cold plasma treatment for increasing the mass transfer of solutes leading to enhanced substrate utilization and biomass yields. This strategy is promising for enhancing the performance of a wide range of bioprocesses, ranging from bioremediation of wastes to synthesis of therapeutic molecules. Further investigations on optimizing the operating conditions will throw light on developing this technology for practical applications.

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