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A study on cell surface hydrophobicity, growth and metabolism of *Zymomonas mobilis* influenced by PEG as pretreatment agent

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Abstract

This study investigates the effects of polyethylene glycol (PEG) 4000, a non-ionic surfactant, on the cell surface hydrophobicity (CSH) of *Zymomonas mobilis*, as well as its growth and metabolism. Different concentrations of PEG were used in fermentation medium to determine the toxic concentration of PEG on *Z. mobilis*. PEG in 1% (w/v) did not influence growth, but increased glucose consumption and bioethanol production yield by 20 and 30%, respectively. PEG in 3% diminished growth profile slightly and had a negligible effect on glucose consumption, while bioethanol production yield was increased. However, 5% PEG inhibited growth considerably and glucose consumption was decreased. An increased concentration of PEG caused increased CSH values of cells cultivated for 12h under anaerobic conditions. Results indicate the hormetic effect of PEG, which stimulates bioethanol production at low concentrations and impedes growth and metabolism in a 5% concentration by altering the hydrophobicity of the cell surface.

Keywords: *Zymomonas mobilis*; Bioethanol; Polyethylene glycol; Metabolism; Hydrophobicity

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Introduction

The exhaustion of petroleum-based fuels and environmental problems related to their consumption has created a great interest in the production of biofuels[1]. Among the currently known biofuel options, bioethanol production has been studied more frequently because of its highly desirable fuel characteristics [2]. Ethanol can be used in combination with petrol or in its pure form, but the latter requires the modification of spark-ignition engines.

In comparison, one litre of ethanol contains 66% of the energy provided by one litre of petrol but has a higher octane level; thus, its mixture with petrol improves fuel combustion performance [3]. Bioethanol can be produced from various feedstocks classified as simple sugars, polysaccharides or lignocelluloses [4]. Simple sugars and polysaccharides make up expensive feedstock and the majority of the price of bioethanol as a product regards feedstock costs; thus, production of bioethanol from the first two groups is not economical [5].

Attention has been paid to lignocelluloses as economically attractive substrates for bioethanol production[6]. Lignocelluloses require a three-step process for bioethanol production: (1) pretreatment for delignification is necessary to make cellulose and hemicellulose accessible prior to hydrolysis; (2) hydrolysis of cellulose and hemicellulose to produce fermentable sugars; (3) sugar fermentation to produce bioethanol[4].

Pretreatment is the most challenging step in the production of bioethanol from lignocelluloses. Different chemical materials are utilized as pretreatment agents in this process[7]. Recently, surfactants have been introduced as an effective agent for the pretreatment of lignocelluloses and as an additive in the hydrolysis step [8-10].

The formation of strong interactions between surfactant molecules and lignocelluloses causes the firm adsorption of surfactants to cellulose and lignin [11, 12]. It has been suggested that

surfactants containing ethylene oxide adsorb on adsorbent by hydrogen bonding with oxygen ethylene of the hydrophilic head and by hydrophobic interaction with the hydrophobic tails [11, 13]. Thus, surfactants used in the upstream processing of bioethanol production could hinder the microorganisms utilized in the fermentation step, as complete removal of these materials after washing is not possible [14]. *Zymomonas mobilis* is an alcohol-tolerant microorganism that has a high potential for the industrial production of ethanol and several other important metabolites. It is a gram negative bacterium capable of producing up to 1.9 mole of ethanol per mole of glucose fermented [15, 16]. *Z. mobilis* is one of the few facultative anaerobic bacteria that metabolize glucose and fructose through the Entner-Doudoroff (E-D) pathway, which is usually present in aerobic microorganisms [16].

Several studies have investigated the effect of various environmental conditions and shocking procedures such as freeze-drying, freezing and thawing on the viability and ultra-structure of *Z. mobilis* [17-20]. Zikmanis et al.[18] studied the effect of different amphiphilic compounds on the hydrophobicity and protein secretion of *Z. mobilis*. The effect of some surfactants, especially nonionic surfactants of the tween group on *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae* and *Mycobacterium paratuberculosis* were examined; the studies mentioned above reflected the change of growth and ethanol tolerance of these microorganisms as influenced by the tested surfactants through the changes of membrane constituents [21-24]. The effects of surfactants on the metabolism of *Z. mobilis* have to date not been studied.

In this work, the effect of PEG 4000 in 1, 3 and 5 (%w/v) concentrations as residual agent present in the structure of pretreated lignocelluloses on *Z. mobilis* cell surface hydrophobicity (CSH), growth, glucose consumption and bioethanol production as a form of metabolism were studied. The cell surface properties of bacteria are known to be vital to the survival of an

organism, since the various surface constituents organize the contact and interaction of the bacterium with its environment [20]. Thus CSH determination is crucial for understanding the PEG effect on the growth and metabolism of *Z. mobilis*.

2. Materials and methods

2.1. Chemicals

PEG 4000 was purchased from Sigma-Aldrich. All growth components were obtained from Merck; 3, 5-dinitrosalicylic acid and potassium dichromate was purchased from Merck.

2.2. Strain and culture conditions

Experiments were conducted using the microorganism *Z. mobilis,* sub species *mobilis*. Strain PTCC 1718 was obtained from the Persian Type Culture Collection. Cultures were maintained in a liquid medium with one transfer every two days and stable cultures were maintained in a medium containing 10 g/L yeast extract, 10 g/L peptone and 20 g/L glucose with 10% glycerol as cryoprotectant, and stored at -80˚C. The seed culture medium contained D-glucose 20 g, yeast extract 10 g, peptone from meat 10 g, $(NH_4)_2$ SO_4 1g; KH_2PO_4 1g Mg SO_4 0.5 and deionized water 1l, pH 5. The cultivation of seed was carried out at 30˚C and 150 rpm in aerobic conditions. The fermentation culture medium contained D-glucose 50 g, yeast extract 10 g, $(NH_4)_2$ SO_4 1g, KH_2PO_4 1g, Mg SO_4 0.5 and deionized water 1l, pH 5. Fermentation was carried out by inoculation of 5% (v/v) seed in serum bottles sealed with a butyl rubber and incubated at 30˚C in anaerobic conditions. All experiments were carried out in duplicate.

2.3. Growth and fermentative metabolism

For monitoring the growth kinetics of *Z. mobilis* in a medium containing various concentrations of PEG and without PEG, optical density (OD) was measured at regular intervals at 600 nm using a spectrophotometer (Carry 100 UV-Vis, Agilent, USA). Fermentation was monitored by taking samples at 3h time intervals. Samples were immediately stored at 4˚C and analysed within 48h.

2.4. Analytical methods

For measuring glucose concentrations, an aliquot of the sample was centrifuged at 14000 rpm for 5 min (B. Braun A15, Germany). DNS method was utilized for glucose concentration measurement[25]. For measuring ethanol concentration, 10 ml of the sample was distilled at atmospheric pressure to obtain a mixture containing only ethanol and water. Potassium dichromate method was utilized for ethanol concentration determination [26].

2.5. Cell surface hydrophobicity and E_h measurement

Anaerobically cultured *Z. mobilis* cells were centrifuged at 12000 rpm for 10 min (Vifion, VF550). Recovered cells were washed three times with a 50 mM phosphate buffer (pH 7.1) to remove surfactant and culture medium constituents, then cells were resuspended in the same buffer at a standardized concentration of cells (OD_{400}=0.5-1) to perform measurements of the cell surface hydrophobicity (CSH). Various volumes of hexadecane were added to 1.2 ml of bacterial suspension. After pre-incubation at 30˚C, the two phases were vortexed for 2 min on a mixer. After the phases were allowed to separate, the aqueous phase was carefully removed and its light absorbance measured at 400 nm. The proportion of adherent cells was determined by comparing

the drop in absorbance following the mixing procedure with the absorbance of the suspension prior to the assay [19, 27]. E_h of the samples was measured by pH/E_h meter Metrohm model 691 (Switzerland).

3. Results and discussion

3.1. Growth of *Z. mobilis*

Growth of *Z. mobilis* in a fermentation medium without PEG and containing 1% (w/v) PEG is shown in Fig. 1 (a). The growth of *Z. mobilis* was characterized by distinct exponential and early stationary phases. The addition of PEG in 1% (w/v) to the fermentation medium did not affect the growth of the bacteria. However, PEG in a 3% (w/v) concentration slightly influenced the growth profile and the final optical density decreased by 10.9% in comparison with the medium free of PEG (Fig. 1 (b)). PEG in a 5% concentration retarded the growth of *Z. mobilis* and final optical density decreased significantly (Fig. 1 (c)). The results of *Z. mobilis* growth in media containing various concentrations of PEG and without PEG can be interpreted alongside the results of cell surface hydrophobicity values. CSH percentage values of the control medium, 1%, 3% and 5% PEG containing media are 11.80±1.64, 16.08±0.92, 26.95±1.13 respectively and 41.20±2.76 and the corresponding E_h values of media are 67±1, 74.5±1.5, 78±4 and 86.5±0.5 mV, respectively. The CSH of *Z. mobilis* increased alongside increasing PEG concentrations in the culture media; additionally, the E_h of the medium increased alongside increasing PEG concentrations, which accords with the CSH results. Increasing the CSH was equivalent to the reduction of the negative surface charge of cells, denoting an increased tendency to gain electron and to be reduced.

Results yielded by the CSH confirmed that PEG changed the cell surface of *Z. mobilis* by decreasing the cell surface charge [28]. Lipopolysaccharides (LPS) form the last outer layer of the cell wall in gram negative proteobacteria [29] and are therefore affected first when environmental conditions change. Shakirova et al. [20] showed a carbohydrate content reduction in LPS fractions of more hydrophobic *Z. mobilis* cells concomitantly with a proportional decrease of ketodeoxy gulonic acid concentration in corresponding LPS samples. Therefore, it can be concluded that PEG changes the LPS of *Z. mobilis*, possibly by altering the amounts of LPS constituents.

LPS is the essential part of the cell envelope structure, rendering proteobacteria resistant to a variety of adverse environments. LPS consists of closely packed O-specific polysaccharides, a polysaccharide core and a highly ordered hydrocarbon chain region, which provides the hydrophilic and hydrophobic protective barriers, respectively [20,29]. Zikmanis et al. [18] reported an increased total amount of secreted protein in the medium as the linear function of CSH values, indicating the damaged barrier function of more hydrophobic cells. Thus, cell wall permeability increases alongside increasing CSH values. Ethanol also inhibits *Z. mobilis* using the same mechanism. It has been reported that ethanol inhibits *Z. mobilis* growth by increased leakage through the plasma membrane, allowing for the loss of cofactors and coenzymes involved in the glucose metabolism pathway [20]. An increase in cell permeability may facilitate ethanol diffusion rate outside the cell envelope, which is favourable to the cell survival; on the other hand, the cell must bear the increased loss of vital cell constituents or the elevated gain of substances normally not present or in strictly limited amounts [21]. It can therefore be concluded that at lower concentrations of PEG, an increase in cell permeability benefits cell survival or alternatively, does not have any negative effect on growth, while at a PEG concentration of 5%, the negative

effect of increased cell permeability overcomes the positive effect, which is the reason for a decline in growth at higher surfactant concentrations.

3.2. Fermentative metabolism

In addition to growth, the fermentative metabolism of *Z. mobilis* was influenced by the presence of PEG in the fermentation medium. Results of glucose concentration during anaerobic fermentation conditions over 12h in the presence of various concentrations of PEG and without PEG are shown in Fig. 2. The addition of PEG in a 1% concentration had little effect on glucose consumption, while ethanol production yield increased by 27.6% compared to the control culture, which was free of PEG. Glucose consumption in the medium containing 1% PEG was commensurable with the growth pattern, because both were completely consistent with the control culture growth and glucose consumption, respectively. Accordance between the growth and glucose consumption pattern of the PEG 1% medium with the control medium, in addition to increasing ethanol production, suggests that increased permeability allows only an elevated diffusion rate for ethanol and not for the essential building blocks of the cell. PEG at 3 and 5% influenced glucose utilization to such a degree that glucose concentration at 3, 6 and 9h of sampling in the medium containing 3% PEG was higher than in the control culture; however, the final glucose concentration was the same as the control culture. This means that glucose consumption in the medium containing 3% PEG had a delay compared to the medium containing 1% PEG and control medium. This may have been due to increased leakage of cell vital constituents such as purines and pyrimidines, which drives the cell to invest more energy in supplying them, rather than directly on growth [21]. Thus, a delay in glucose consumption resulted. Additionally, in the medium containing 5% PEG, glucose concentrations at 3, 6 and 9h of sampling were higher than the medium containing 1% of PEG and in the control culture. Glucose

concentrations in the presence of 5% PEG at 12h were significantly higher than in the control culture, confirming the inhibitory effect of PEG at 5% on *Z. mobilis*' metabolism. The glucose concentration at 12h was approximately equal for the media containing 1 and 3% of PEG and in the control culture.

Ethanol production by *Z. mobilis* in various concentrations of PEG at 3h intervals of sampling is shown in Fig. 5. Ethanol production in the medium containing 1% PEG was not similar to the pattern of ethanol production in the control medium, unlike the glucose consumption and growth profile of *Z. mobilis*. Ethanol concentration in the medium containing 1% PEG at 3 and 6 h of fermentation were nearly the same as the ethanol concentration in the control medium. However, ethanol production at 9 and 12 h in medium containing 1% PEG increased by 16.3% and 27.6%, respectively, compared to the control. Thus, PEG at 1% concentration enhanced final ethanol production.

In addition to increased cell permeability, which favours cells at lower concentrations, PEG likely weakened the inhibitory effect of ethanol produced by cells. This can occur through the formation of hydrogen bonding between hydroxyl and hydrogens of both PEG and ethanol. Final ethanol production yield at 1% of surfactant is slightly more than 100% calculated on the basis of glucose concentration, which means that PEG was utilized as a carbon source by *Z. mobilis*. However, thorough investigations that determine the movement of labelled compounds into the cell are required to clarify questions regarding the consumption of PEG as a carbon source.

Ethanol concentration at 6h was increased by increasing PEG concentration by 10.4%, 87% and 147% for PEG concentrations of 1, 3 and 5%, respectively, compared to the control. Nonetheless, the final ethanol concentration decreased slightly as a result of the presence of PEG in 3 and 5%.

The results of this study demonstrated that PEG has a hormetic effect, which is clearly observable in the results of *Z. mobilis* growth and metabolism in media with various concentrations of PEG. PEG in low concentrations stimulates bioethanol production and glucose consumption; however, at high concentrations, PEG inhibits *Z. mobilis* growth and metabolism. Thus, PEG could be utilized to increase Z. *mobilis* bioethanol production via physiological changes of the strain.

4. Conclusion

This study revealed the hormetic effect of PEG on *Z. mobilis* metabolism. The results show that addition of 1% (w/v) concentration of PEG increased bioethanol production yield of the *Z. mobilis* by 27%. The findings of the present study bring up a novel approach to enhancement of bioethanol production via induced physiological alteration in the Z. mobilis by PEG. This physiological variation of cells cultivated in medium containing PEG is the change of cell wall constituents, as the cell surface hydrophobicity of the cells cultivated anaerobically in exposure to PEG decreased, compared to the cells cultivated in medium without PEG.

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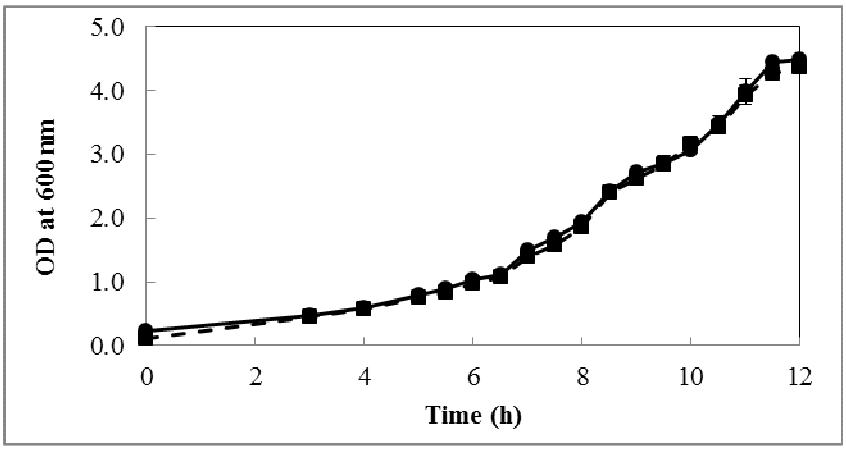
Figures captions

Fig. 1: (a) Variation of optical density during the growth of *Z. mobilis* in medium without PEG (control) and medium containing 1%(w/v) concentration of PEG; (b) control and mineral salts medium containing 3%(w/v) concentration of PEG; (c) control and mineral salts medium containing 5%(w/v) concentration of PEG.

Fig. 2: Glucose concentration during the anaerobic fermentation of *Z. mobilis* in mineral salts medium (control) and mineral salts medium containing 1, 3 and 5%(w/v) concentrations of PEG.

Fig. 3: Ethanol concentration, produced during the anaerobic fermentation of *Z. mobilis* in mineral salts medium (control) and mineral salts media containing 1, 3 and 5%(w/v) concentrations of PEG.

(a)



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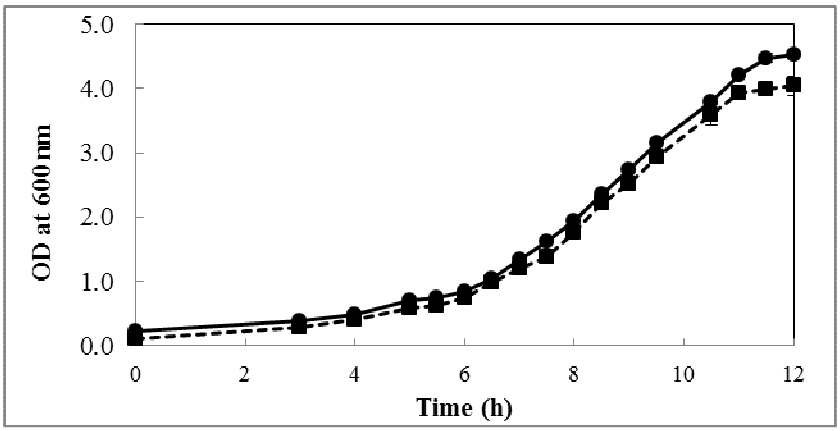

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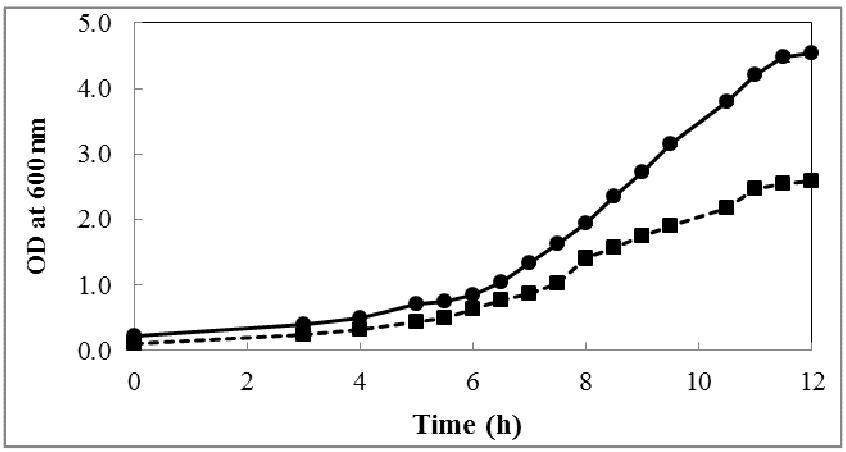

Fig. 1

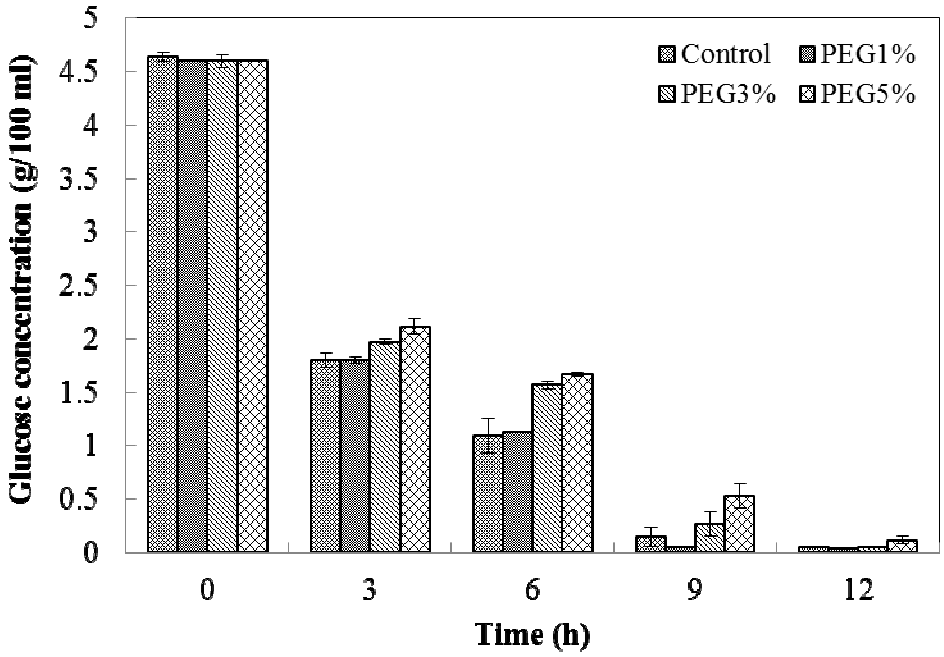

Fig. 2

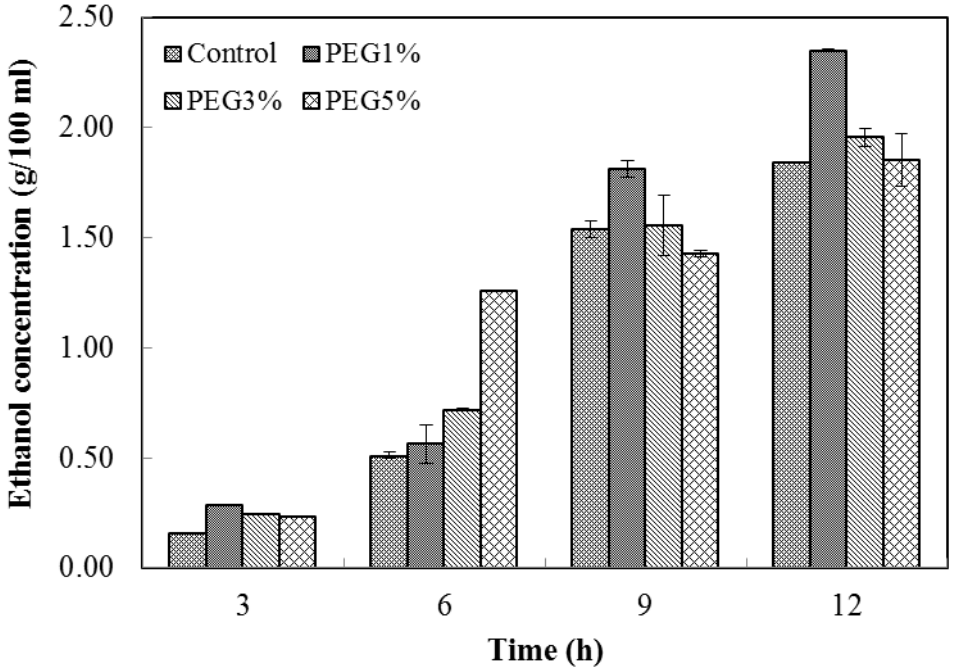

Fig. 3

PEG Influenced growth and metabolism of Z. mobilis