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ORIGINAL PAPER

Biohydrogen Production by Antarctic Psychrotolerant *Klebsiella* sp. ABZ11

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Abstract

Lower temperature biohydrogen production has always been attractive, due to the lower energy requirements. However, the slow metabolic rate of psychrotolerant biohydrogen-producing bacteria is a common problem that affects their biohydrogen yield. This study reports on the improved substrate synthesis and biohydrogen productivity by the psychrotolerant *Klebsiella* sp. strain ABZ11, isolated from Antarctic seawater sample. The isolate was screened for biohydrogen production at 30°C, under facultative anaerobic condition. The isolate is able to ferment glucose, fructose and sucrose with biohydrogen production rate and yield of 0.8 mol/l/h and 3.8 mol/g, respectively at 10 g/l glucose concentration. It also showed 74% carbohydrate uptake and 95% oxygen uptake ability, and a wide growth temperature range with optimum at 37°C. *Klebsiella* sp. ABZ11 has a short biohydrogen production lag phase, fast substrate uptake and is able to tolerate the presence of oxygen in the culture medium. Thus, the isolate has a potential to be used for lower temperature biohydrogen production process.

Key words: *Klebsiella* sp., biohydrogen, facultative psychrotolerant, oxygen uptake, carbohydrate consumption

Introduction

Hydrogen is an attractive alternative energy carrier due to the high energy density, and the cleaner by-products generated when used in automobiles (Khan et al. 2017). Biological hydrogen production is a technique of producing hydrogen through biological processes, using microorganisms as the biocatalysts. Among all the biological processes, bacterial dark fermentation is the most promising one, due to the high biohydrogen yield, and the ability to ferment different substrates to produce biohydrogen (Khan et al. 2017; Miandad et al. 2017).

Psychrophiles and psychrotolerant bacteria are abundant in the colder environment, e.g. Antarctica. Psychrophiles grow optimally at 20°C, and their fermentative processes have been considered beneficial due to the unique enzymes they possess (Corr and Murphy 2011). However, the reduced metabolic rate in psychrophiles is one key factor that affects substrate uptake and synthesis, which invariably affect the rate of substrate degradation and fermentative yield (Lettinga et al. 2001; Thauer et al. 2010; Lu et al. 2011). Psychrotolerant bacteria on the other hand, can grow above 20°C (Morita 1975; Pesciaroli et al. 2012). Thus, they are expected to be more useful for biohydrogen production at ambient temperature. Some psychrotolerant strains can thrive between 0–40°C (Pikuta et al. 2016), giving them an advantage over psychrophiles. Temperature

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contributes significantly to fermentative biohydrogen yield due to its influence on the rate of metabolism and enzyme activity (Hallenbeck et al. 2012). Antarctic seawater is subjected to a wide variation in environmental conditions which permits the survival of a wide range of bacteria, including the fermentative strains (Delille 1992). This study therefore focused on the isolation and characterization of psychrotolerant biohydrogen producing bacteria from Antarctic seawater.

More recently, a growing number of research has focused on biohydrogen production using cold-active bacteria isolated from Antarctica. For instance, Alvarez-Guzmán et al. (2016) investigated biohydrogen productivity of psychrophilic bacteria at 25°C under anaerobic condition. They reported a prolonged biohydrogen production lag phase and carbohydrate uptake after the start of fermentation. We hypothesized that utilizing facultative psychrotolerant bacteria with tolerance to mesophilic temperature may be a good option for improving substrate uptake and biohydrogen productivity of cold-adapted bacteria. Therefore, the objective of this study was to investigate the biohydrogen production ability of Antarctic facultative psychrotolerant bacteria under mesophilic temperature conditions. In addition, the influence of oxygen on the substrate uptake and biohydrogen productivity was also examined.

Experimental

Materials and Methods

Isolation of bacterial strains. Antarctic seawater was collected near Ryswyck Point, Anvers Island, Antarctic Peninsula (S64°39.189', W62°47.776') during the UTM Antarctic Research Expedition in February-March 2015. The bacteria was cultured on Marine Agar 2216 (Difco™, USA). The water samples (2 ml) was inoculated into 20 ml of the Marine Broth 2216 and incubated at 25°C for 7 days, before being transferred (0.1 µl) onto an agar plate using spread plate technique and incubated at 30°C for three days. Colonies obtained were then further sub-cultured on fresh agar plates to obtain pure isolates, before being preserved in 50% glycerol stock at –80°C for further use (Peeters et al. 2012).

Screening for biohydrogen production. The isolates were screened for biohydrogen production in 60 ml serum bottles containing 40 ml of Marine Broth 2216 as the fermentation medium. Each of the five isolates was used as an inoculum in separate serum bottles. The isolates were pre-cultured in the Marine Broth overnight, centrifuged and re-suspended in 0.1 M phosphate buffer pH 7.6. Then 4 ml of the culture was introduced into the fermentation media. Fermentation was carried out at 30°C for 24 h. Headspace gas (1 ml) was collected using airtight syringe (Agilent Technology) and analysed using a Gas Chromatography-Thermal Conductivity Detector (GC-TCD) (Agilent 7890B) equipped with five columns (two Hayesep Q80/100 SS, one Hayesep Q 80/200, and two Molsieve 5A 60/80 SS). All experiments were performed in triplicates. Among the five isolates screened for biohydrogen production, only one strain (ABZ11) which showed significant biohydrogen production was selected for further identification and characterization.

Growth profile. To investigate the growth profile of the isolate, it was first cultured in Marine Broth under agitation condition (100 × g) and incubated at 30°C for 30 h. Cells at optical density at 600 nm (OD_{600}) of 1.0 (10 ml) was introduced into 100 ml sterile Marine Broth in a conical flask and incubated at 30°C. The culture (1 ml) was then transferred into pre-weighed microcentrifuge tubes before being centrifuged at 10 000 × g for 5 min and dried at 40°C overnight. The average final dry cell weight was used to represent the growth of the isolate (Silvaa et al. 2016).

Biochemical tests and electron microscopy. ABZ11 was characterized using Gram staining, capsule staining, and blood haemolysis test. Blood haemolysis test was performed on 5% sheep blood agar and incubated at 37°C for 48 h (Shirron et al. 2008). The morphology of ABZ11 was also examined using scanning electron microscopy (SEM) (JEOL JSM-6390LV) with accelerating voltage of 10 kV (Xiong et al. 2015). Cells for SEM was prepared by centrifuging 5 ml of the overnight bacterial culture at 10 000 × g for 5 min. Cell pellet was washed three times with 1 ml of 0.1 M phosphate buffer pH 7.6. The cells were fixed for 2 h with 2.5% glutaraldehyde and dried in a critical point dryer (Leica EM CPD300).

Identification using 16S rRNA analysis. The genomic DNA of ABZ11 was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's instruction. The 16S rRNA gene was then amplified in a thermocycler (Eppendorf AG22331) using forward primer 27-F: 5'-AGAGTTTGATCCTGGCTCAG-3' and a reverse primer 1492R: 5'-CGGTTACCTTGTTACGACTT-3'. PCR was set at the following condition: 94°C for 4 min, 30 cycles at 94°C for 1 min, 48°C for 30 sec, and 68°C for 2 min, and kept at 4°C for amplification. The PCR products obtained were purified and sequenced by First Base Laboratories (Malaysia) Sdn. Bhd. The partial 16S rRNA gene sequence obtained was analysed using the BLAST tool at NCBI database, by comparing with the sequences in the Genbank database. Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Specific taxa of the organism was collapsed in less than 50% replicates from 1050 bootstrap replicates (Felsenstein 1985). The analysis was conducted using MEGA7 (Kumar et al. 2016).

Temperature range for optimum growth. Overnight culture of ABZ11 with OD_{600} of 1.0 was serially diluted to 10^{-5}, before 0.1 ml was plated by spread plate method and incubated at different temperatures (20, 30, 37, 40 and 45°C). All experiments were prepared in triplicates and colonies between 30 and 300 were considered for determination of cell viable numbers in CFU/ml (Pesciaroli et al. 2012).

Oxygen uptake capability. Oxygen uptake experiments were performed in a reconstituted medium containing 5 g/l glucose and 0.2 g/l beef extract in 0.1 M phosphate buffer pH 7.6, with trace elements (in g/l) $ZnCl_2$; 0.07, $MnCl_2 \cdot 4H_2O$; 0.1, H_3BO_3; 0.06, $CoCl_2 \cdot 6H_2O$; 0.2, $CuCl_2 \cdot 2H_2O$; 0.02, $NiCl_2 \cdot 6H_2O$; 0.025 and $NaMoO_4 \cdot 2H_2O$; 0.035 (Liu and Fang 2007). The medium (40 ml) was dispensed into 60 ml universal bottles before being inoculated with 4 ml of the overnight culture of the ABZ11. Afterwards, the bottles were tightly covered and sealed with parafilm and incubated at 25°C for 24 h. Dissolved oxygen uptake by the isolate were measured at 2, 4, 6, and 24 hours. Dissolved oxygen level was measured with a DO meter (Mettler Toledo FG4) (Santana 2008). All experiments were performed in triplicate and fermentation was carried out under static condition to avoid introducing oxygen into the medium.

Biohydrogen production using different carbon sources. Different carbon sources were tested, namely glucose, sucrose and fructose at 5, 7.5, 10 and 12.5 g/l concentrations. Beef extract and trace element solution were added as nitrogen and mineral source, as described above. The C/N ratio was fixed at 1/30. Fermentation was initiated by adding 10% (v/v) of the fresh culture (1.0 OD_{600}) of ABZ11 into 40 ml of the sterile medium in 60 ml serum bottles sealed with rubber stopper and aluminium cap (Islam et al. 2006). Experiments were prepared in triplicate and incubation was carried out at 30°C for 48 h. Headspace gas (1 ml) was collected with an airtight syringe and analysed in a GC-TCD as described above at different time intervals. Carbohydrate consumption was determined by 3,5-dinitrosalicylic acid (DNS) assay (Miller 1959). Growth profile was investigated as described above.

Kinetic analysis. Biohydrogen productivity and cell growth during the fermentation were evaluated using two equations $Y_{P/S}$ and $Y_{X/S}$ to determine whether the substrate taken up was channelled towards growth or product formation (Hahn-Hägerdal 1994; Silvaa et al. 2016). In the equations, biohydrogen yield ($Y_{P/S}$) refers to the fraction of the biohydrogen produced in (mol/l) and the substrate consumed (mg/ml).

$$Y_{P/S} = \frac{P_{max} - P_o}{S_{max} - SP_{max}}$$

Where Y = yield, P = product and S = substrate consumed (glucose, fructose and sucrose). P_{max} = biohydrogen produced at 48 hour, S_{max} = maximum carbohydrate concentration, P_o = biohydrogen produced at initial fermentation and SP_{max} = carbohydrate consumed at maximum biohydrogen production period.

While growth yield ($Y_{X/S}$) was defined as the fraction of the dry cell weight (g/ml) and the substrate consumed (mg/ml).

$$Y_{X/S} = \frac{X_{max} - X_o}{S_{max} - SX_{max}}$$

Where Y = yield, X = dry cell weight and S = carbohydrate consumed. X_{max} = maximum growth rate, X_o = initial dry cell weight, S_{max} = carbohydrate consumed at maximum growth period and SX_o = carbohydrate concentration at initial growth period.

Statistical analysis. Statistical analysis (analysis of variance, ANOVA) was carried out using SPSS software, version 24. ANOVA tests were applied at the significance level of $p < 0.05$. The data were from three independent experiments.

Results

Isolation and characterization of biohydrogen psychrotolerant bacteria. Five pure colonies were obtained from the Antarctic seawater sample, with milky mucoid color. All isolates grew on Marine Agar plates after three days incubation. These isolates were designated ABZ4, ABZ7, ABZ10, ABZ11 and ABZ12.

Out of the five isolates screened for biohydrogen production, only two isolates (Fig. 1) is able to produce biohydrogen, namely ABZ11 (0.49 mol H_2/mol glucose) and ABZ4 (0.02 mol H_2/mol glucose). Due to higher biohydrogen production, ABZ11 was selected for further characterizations.

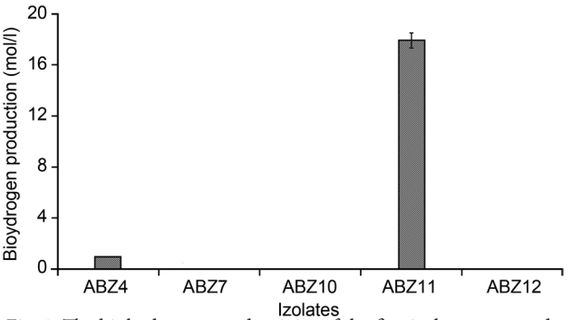

Fig. 1. The biohydrogen productivity of the five isolates screened.

ABZ11 has a short growth lag phase (1 h) (Fig. 2) but a longer stationary phase between 2 and 18 h of incubation. The cells of ABZ11 are rod-shaped, with an approximate length of 1.7 µm and diameter of 0.4 µm (Fig. 3). Capsule staining revealed no capsule around the cells as a result of the absence of visible halo zones after staining (Fig. 4).

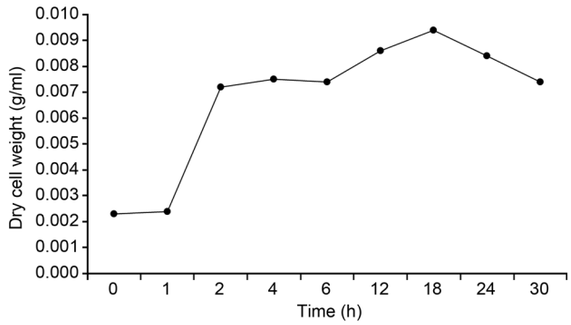

Fig. 2. Mean dry cell weight (DCW) of *Klebsiella* sp. ABZ11 during growth at 30°C.

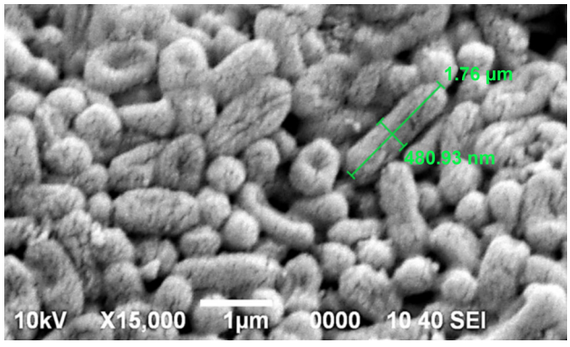

Fig. 3. Scanning electron micrograph of *Klebsiella* sp. ABZ11. Bar represents 1 µm scale.

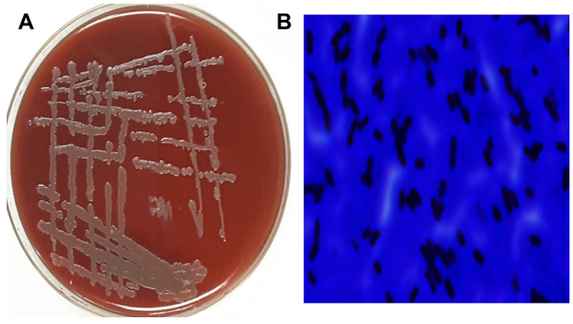

Fig. 4. A. Growth of *Klebsiella* sp. ABZ11 on blood agar plate. No blood haemolysis was observed after 48 h incubation at 30°C. B. Capsule staining of the ABZ11. No capsule is visible around the cells suggesting the non-pathogenicity of this strain.

Analysis of the partial 16S rRNA sequence showed that ABZ11 is closely related to *Klebsiella pneumoniae* (99% identity). The isolate was thus designated *Klebsiella* sp. ABZ11, with a Genbank accession number KX266892. Based on the phylogenetic relationship identified using neighbor-joining method, ABZ11 cluster with *Klebsiella pneumoniae* subsp. *rhinoscleromatis* R-70 strain on the same taxon among the 13 closely related strains that were used (Fig. 5).

Temperature tolerance of the *Klebsiella* sp. ABZ11 was tested by observing the viable cells after incubation at several different temperatures. ABZ11 is able to grow

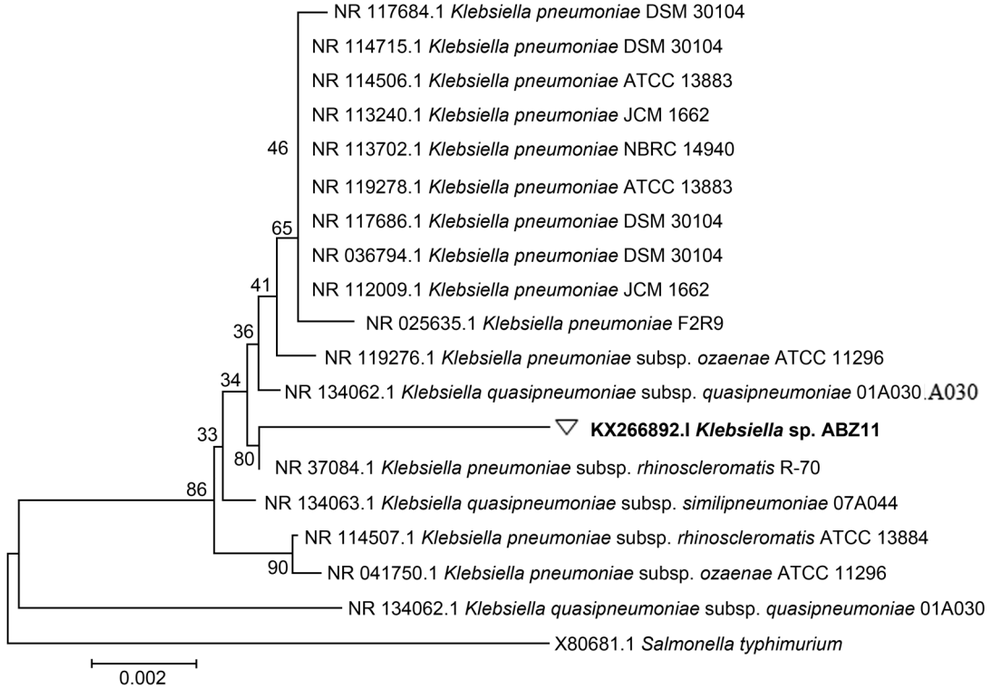

Fig. 5. Phylogenetic tree constructed using the partial 16S rRNA gene sequences by neighbor-joining method (19 nucleotides, 1303 data sets, branch length of 0.033 and all gaps removed) showing the relationship between ABZ11 and the 17 most closely related reference species. The gene sequence of *Salmonella typhimurium* X80681 was used as the outgroup. Bootstrap values (expressed as percentages of 1050 replications) are shown at the branch points. Bar represents 0.002 substitutions per nucleotide position.

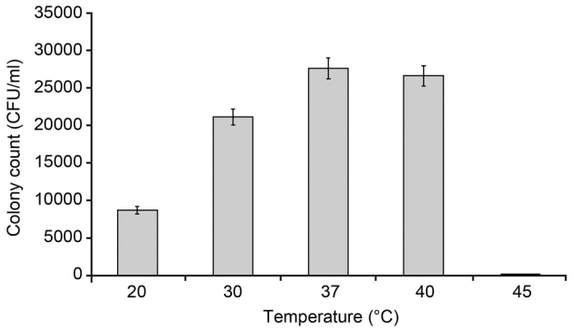

Fig. 6. Mean growth temperature range and optimum temperature of *Klebsiella* sp. ABZ11.

Biohydrogen production using different carbon sources. Biohydrogen production was observed to start after 2 hours of fermentation for all the substrate concentration except 12.5 g/l sucrose concentration that begins production at the 4th hour. Table I shows the summary of the biohydrogen production of bacterial with different concentrations of the carbohydrate sources. Meanwhile, the biohydrogen production was observed to increase with fermentation time for all the carbohydrate concentrations with maximum productivity observed at different time. Productivity was also observed to decrease with increased substrate concentration. A maximum biohydrogen of 38.55 mol/l

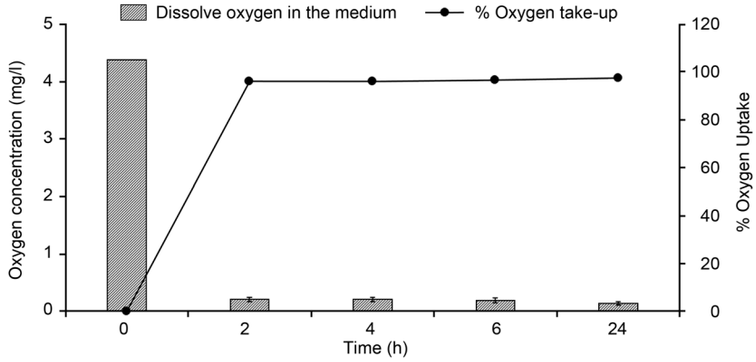

Fig. 7. Oxygen uptake by ABZ11 at different time intervals.

at 20, 30, 37, and 40°C, but no growth was observed at 45°C (Fig. 6). The optimum growth was observed at 37°C after three days of incubation.

Oxygen tolerance of ABZ11 was then investigated by measuring the level of dissolved oxygen in the culture medium. An oxygen uptake of 4.17 ± 0.03 mg/l was observed after 2 h, representing 95% of the oxygen in the medium that was scavenged by *Klebsiella* sp. ABZ11 (Fig. 7). Meanwhile, oxygen content was slightly reduced by 0.5% in 6 h and 0.02% in 24 h leading to a total of 96.8% of oxygen uptake in 24 h.

observed at 10 g/l was significantly ($p > 0.05$) different from the productivity of other substrate concentrations. Further, carbohydrate consumption started immediately with 74% of the total carbohydrate consumed after 2 h (Fig. 8). It was also observed that the carbohydrate in the medium was totally exhausted before 48 h in all tested parameters.

Biohydrogen yield in relation to the biomass during fermentation is 24.09 mol/mg while cell growth in relation to substrate utilization is 0.532 g/mg, at 10 g/l of glucose (Fig. 9). The maximum biohydrogen

Table I
Biohydrogen production and kinetic parameters of substrate utilization by *Klebsiella* sp. ABZ11.

	Carbohydrate concentrations (g/l)		5	7.5	10	12.5
Biohydrogen production at initial incubation time and at maximum production (mol/l)	Glucose	Initial	0.21 ± 0.00	0.21 ± 0.02	2.35 ± 0.03	0.77 ± 0.01
		Maximum	26.23 ± 2.18	23.80 ± 3.29*	38.55 ± 2.19*	21.11 ± 0.14*
	Fructose	Initial	0.21 ± 0.00	0.47 ± 0.05	0.65 ± 0.46	0.81 ± 0.09
		Maximum	25.47 ± 2.02	22.77 ± 2.01	19.97 ± 1.60	16.97 ± 0.12
	Sucrose	Initial	0.21 ± 0.00	0.23 ± 0.04	0.23 ± 0.01	4.52 ± 0.36
		Maximum	28.24 ± 2.96*	22.37 ± 2.19	18.21 ± 0.77	15.91 ± 1.49
Ketic analysis	$Y_{P/S}$ (mol/mg)		23.53	17.00	24.09	11.11
	$Y_{X/S}$ (g/mg)		0.170	0.424	0.532	0.205

* Maximum biohydrogen production in the group and the productivity considered for the kinetic

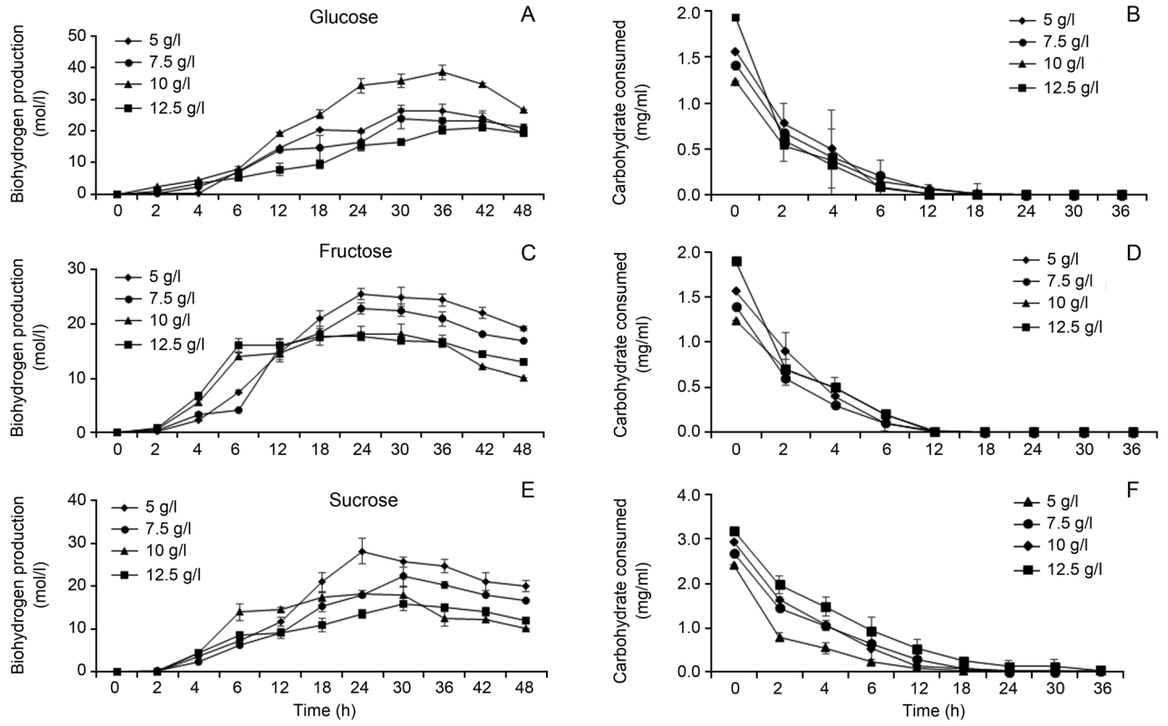

Fig. 8. Average biohydrogen productivity and carbohydrate uptake after 48 h fermentation of glucose (A, B), fructose (C, D) and sucrose (E, F) by *Klebsiella* sp. ABZ11.

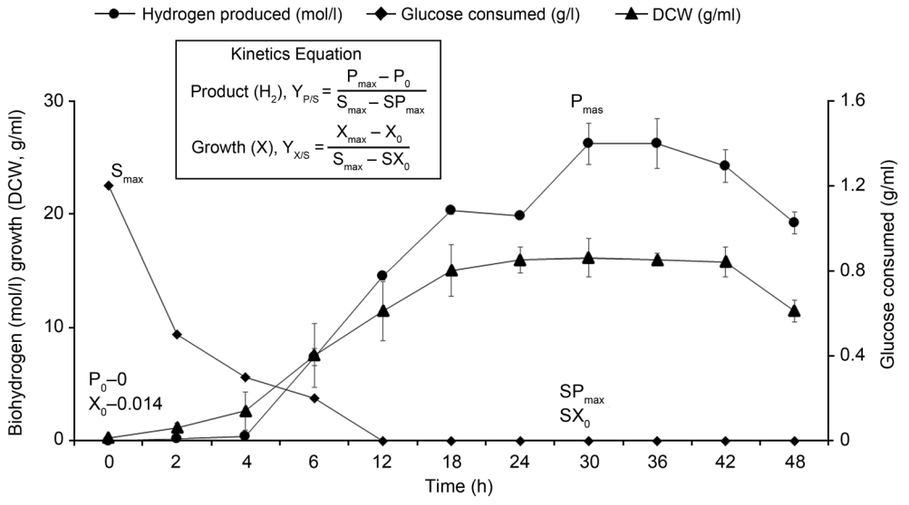

Fig. 9. Biohydrogen production kinetic of *Klebsiella* sp. ABZ11 at 5 g/l glucose concentration.

production and kinetic data for all the parameters investigated are summarized in Table I.

Discussion

Generally, there is a prolonged period between the fermentation start time and the beginning of biohydrogen production in psychrophilic bacteria, providing a challenge for application in lower temperature biohydrogen reactors. This study was thus initiated to overcome this challenge by using Antarctic psychrotolerant bacteria, with higher metabolic activity in order to improve its substrate uptake and biohydrogen production. We have successfully isolated a psychrotolerant, biohydrogen-producing *Klebsiella* sp. (closely related to *Klebsiella pneumoniae*) from Antarctic seawater. This *Klebsiella* sp. strain ABZ11 has the ability to withstand

a wide growth temperature, with optimum at 37°C, suggesting that this strain is psychrotolerant (Carrión et al. 2011; Lavin et al. 2016).

Klebsiella spp. has previously been isolated from Antarctic sea animals, samples obtained from islands, as well as other environments such as sewage sludge and hot springs (Minnan et al. 2005; Kargel et al. 2008; Brat et al. 2016). The diverse environmental conditions from which this genus was isolated suggest the unique physiological characteristics that allows their survival. *Klebsiella* sp. ABZ11 that we isolated from Antarctic seawater is devoid of capsules and does not show the ability to lyse blood cells, suggesting the non-pathogenic nature of this strain.

The carbon sources tested in the fermentation experiment were glucose, fructose and sucrose. The highest biohydrogen production of 38.55 ± 2.19 mol/l was observed at 10 g/l glucose concentration, at pH 5.6. A decrease in biohydrogen production with the increase in substrate concentration was also observed in almost all carbon sources tested, as reported previously (Ginkel et al. 2001; Kamalaskar et al. 2010) except for glucose. The optimum glucose concentration of 10 g/l observed in this study is in agreement with mesophilic *Bacillus* sp. FS2011 and *Clostridium beijerinckii* Fanp3 strains (Pan et al. 2008; Song et al. 2013) but differs with other work on hydrogen producing *Klebsiella* strains (Niu et al. 2010; Chookaew et al. 2012). Strain ABZ11 also shows high carbohydrate uptake, with 74% of glucose consumed after 2 h of fermentation due to high substrate conversion efficiency attributed to the incubation temperature and oxygen, before complete utilization around 48 h.

Biohydrogen production was detected within the exponential and stationary phase with maximum production found in the stationary phase of *Klebsiella* sp. ABZ11 growth, indicating that hydrogen production is more of a secondary metabolite in its fermentative process. This is similar to the report on biohydrogen production by mesophilic *Clostridium* sp. DMHC-10 (Kamalaskar et al. 2010). More importantly, strain ABZ11 shows a very short production lag phase, rapid carbohydrate uptake, and is able to substantially decrease culture oxygen level in a short period of time. Our findings thus suggest an improved metabolic biosynthesis for biohydrogen production compared to the psychrophiles and psychrotolerant strains (Alvarez-Guzmán et al. 2016). There is a possibility that *Klebsiella* sp. ABZ11 possesses oxygen tolerant hydrogenases, as previously reported in *K. oxytoca* HP1 (Minnan et al. 2005). The improvement in metabolic activity yielding early productivity may be attributed to the temperature condition at which the fermentation was performed. The rapid decrease in oxygen concentration in the fermentation medium could be another factor that influences the rate of the subsequent carbohydrate hydrolysis due to release of large extracellular hydrolytic enzymes and increase in their activity, which in turn increases hydrogen biosynthesis. A small amount of oxygen in the fermentation medium has been shown before to enhance the rate of substrate hydrolysis (Johansen and Bakke 2006; Ramos and Fdz-Polanco 2013).

Klebsiella sp. ABZ11 biohydrogen production kinetics show that the strain is inclined towards biohydrogen production rather than growth at the stationary phase, under the experimental conditions described here. Experiments performed under batch fermentation revealed the potential of this strain to be able to utilize different carbon sources for biohydrogen production. In the future, optimization of experimental parameters may further improve the biohydrogen-producing ability of this facultative psychrotolerant bacteria.

Conclusions

A psychrotolerant, facultative, oxygen-insensitive biohydrogen-producing bacterial strain has successfully been isolated from Antarctic seawater. The strain, identified as *Klebsiella* sp. ABZ11, has been investigated in terms of its biohydrogen productivity, temperature tolerance, and dissolved oxygen uptake. This strain shows up to 95% oxygen uptake, and can tolerate growth temperature of 20–40°C, with optimum growth observed at 37°C. Maximum biohydrogen productivity of 38.55 mol/l was observed at 30°C. This study demonstrates the feasibility of using a psychrotolerant bacterial strain for biohydrogen production at lower temperature, with high oxygen tolerance.

Acknowledgements

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