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Assessment of bio-hydrogen production from glycerol and glucose by fermentative bacteria

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Microorganisms are capable to produce hydrogen during fermentation of organic substrates and industrial waste products can be used as feedstock for hydrogen producing bacteria. One of the substrates that can be effectively used for microbial hydrogen production is glycerol, which is a by-product from the process of biodiesel production, but glucose is mainly used as a model substrate. Different bacterial isolates were tested for hydrogen gas production rates from glucose and glycerol with test-systems constructed in our laboratory. Test-systems were optimised to allow adequate substrate and bacterial strain hydrogen productivity estimation in the liquid and gaseous phases. It was concluded that several of the isolated bacterial strains are suitable for bio-hydrogen production using glycerol as a substrate. Assessment was developed to establish whether microbial conversion of glycerol is an economically and environmentally viable possibility for bio-hydrogen production. The raw material cost noticeably decreases because of large quantities of available crude glycerol after biodiesel production and the highly reduced nature of carbon in glycerol *per se*.

Key words: bio-hydrogen, fermentation, substrates, prototype bioreactor

INTRODUCTION

Biological production of hydrogen using bacteria is a promising and advantageous area, especially when hydrogen is gained from a variety of renewable resources [1, 2]. Industrial and agricultural organic waste used as feedstock for hydrogen producing bacteria is a perspective way for alternative energy production and it noticeably decreases the raw material cost. During the conversion of organic wastes, in anaerobic environment, hydrogen gas is produced as a by-product. Substantial factors like availability and cost are highly important in the selection of waste materials to be used in hydrogen production with fermentative bacteria [3]. One of the substrates that can be effectively used for microbial hydrogen production is glycerol, which is a by-product from the process of biodiesel production. Because of large quantities available of crude glycerol and the highly reduced nature of carbon in glycerol *per se*, microbial conversion of it seems to be economically and environmentally viable possibility,

especially because over the last several years the demand and production of biodiesel has remarkably increased [4, 5]. Recently several authors have investigated hydrogen production using glycerol as a substrate by fermentative bacteria. Mangayil et al. [6] investigated optimal conditions (pH 6.5; 40 °C and 1 g/L raw glycerol) for hydrogen production using crude glycerol with microbial consortium mainly dominated by *Clostridium* species. Environmental conditions like medium pH and temperature are the major parameters to be controlled in the hydrogen production because they affect the qualitative and quantitative content of bacterial produced gas and the hydrogen yield and rate. Hydrogen production using glycerol is 1.5-fold higher at pH 5.5 than at pH 6.5 [7]. Anaerobic conditions have to be maintained during the hydrogen production process, which are ensured by bubbling media with reducing agents such as argon or nitrogen [3].

Bacterial strains that have appropriate metabolic pathways for hydrogen production from glucose and glycerol substrates were chosen in this work. *Clostridia, Enterobacter* and *Escherichia* have been investigated for hydrogen production by many authors [8–11]. Bacterial strains used in the present work as hydrogen producers (except for *E. coli* BW25113 and *E. coli* MSCL 332) have been isolated in Latvia and the goal from this and further investigations is to use crude glycerol as a substrate from Latvia's biodiesel production leftovers. *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA* aceE::kan (from prof. T. K. Wood, USA) showed a 4.6-fold increase of hydrogen production from glucose [8] in the present work using glycerol as a substrate for hydrogen production.

MATERIALS AND METHODS

Fermentative bacteria strains

Aneurinibacillus aneurinilyticus Microbial Strain Collection of Latvia (MSCL) 1018, *Clostridium sporogenes* MSCL 764, *Enterobacter asburiae* MSCL 839, *Enterobacter cloacae* MSCL 778, *Eubacterium limosum* MSCL 840, *Kluyvera ascorbata* MSCL 732, *Paenibacillus pabuli* MSCL 1006, isolated in Latvia, *E. coli* MSCL 332 (ATCC 25922) and *Escherichia coli BW25113 hyaB hybC hycA fdoG frdC ldhA aceE::kan* (from prof. T. K. Wood, USA).

Growth media, cultivation and experimental set-up

Bacterial cultures were inoculated in 200 ml flasks containing Luria-Bertani (LB) (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride, 15 g/L Bacto agar) [12]. Strains were cultivated overnight aerobically in shaken flasks at 37 °C for 12 hours at 150 rpm using a multi-shaker PSU-20 (BioSan, Latvia) with the exception of *Clostridium sporogenes* (cultivated in the Thioglycollate resazurin broth (Bio-Rad, France) for two days without shaking). The optical density (OD) calibration curve was used to find out the number of cells in 1 mL of culture [13]. The bacteria concentration in the fresh culture was 10^7 CFU/mL. The overnight culture in the LB liquid medium was mixed (1:1) with phosphate buffered saline (PBS) pH 7.3 (0.8 g/l NaCl, 0.2 g/l KCl, 1.43 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4) [14] in a vessel sterilized for measurements. The PBS contained a complex trace element medium pH 5.5 (0.039 g/L $Fe(NH_4)_2 \cdot SO_4 \cdot 6H_2O$, 0.172 mg/L Na_2SeO_3, 0.02 mg/L $NiCl_2$, 0.4 mg/L $(NH_4)_6Mo_7O_{24}$) [15]. Crude glycerol (98% wt/wt, determined with HPLC analysis) from biodisel fuel production was used as a substrate, the final concentration of glycerol used was 240 mM. Glycerol and glucose were sterilized through 0.2 μm membrane filters. Substrates were added at the start of the anaerobic fermentations. Argon gas (99.99 % purity) bubbling through the media was used to sustain anaerobic environment.

Analytical methods

Liquid phase analysis

An experimental test-system with separate glass chambers for simultaneous measurements and hydrogen and oxygen Clark-type microsensors (Unisense, Denmark) were used to determine the hydrogen production rate in the liquid phase. Both hydrogen and oxygen microsensors were connected with the signal amplifier – a pico-ammeter and an A/D current converter connected to a PC using a USB port. Microsensors were calibrated in the liquid culture medium for zero concentrations. Sensors were calibrated in the LB medium using 99.999% pure hydrogen and 99.99% pure argon. A microsensor tip was sterilized using 96% ethanol, 0.1 M NaOH and distilled water every time when it was taken out from the sample [16].

Gas analysis

For hydrogen analysis in the gas phase the RGAPro-100 mass-spectrometer was connected to the experimental test-system (a prototype bioreactor system for hydrogen gas production). Evolved gases were collected from the test-system and tested in the mass-spectrometer RGAPro-100 (HyEnergy, Setaram, France) for hydrogen gas measurement. Argon gas (99.99% purity) was continuously flushed through a diffuser to maintain anaerobic environment. The bioreactor was kept in a water bath (Precisterm 2-110, 2L), in order to maintain temperature around 37 ± 2 °C. The data from the mass-spectrometer were analyzed by the RGA 3.0 Software for the SR Residual Gas Analyzers program.

RESULTS AND DISCUSSION

Using glucose as a substrate for hydrogen production in the liquid phase *E. asburiae, E. coli* BW25113, *E. coli* standard strain (332) and *E. cloacae* were tested and the highest

production was achieved using *E. coli* BW25113 (3 mmol H_2/l/h) and *E. coli* 332 (2 mmol H_2/l/h). With glucose as a substrate for hydrogen production in the gaseous phase *E. coli* 332 and *E. coli* BW25113 were tested from which the highest results were reached with *E. coli* BW25113 (2.5 mmol H_2/l/h). The septuple mutant strain (*E. coli* BW25113) shows that almost all hydrogen transforms into the gaseous phase (3.0 mmol H_2/l/h in the liquid phase, 2.5 mmol H_2/l/h in the gaseous phase), therefore it can be concluded that the liquid / gas phase proportion in the measuring system and constant flushing with argon gas maintain balanced transition from the liquid to gaseous phase. Bacterial strain hydrogen production rates using different substrate fermentation are shown in Table.

When comparing glucose and glycerol as hydrogen fermentation substrates in the liquid phase with bacterial strain *E. coli* BW25113, substantial differences were observed. The highest rates were gained using glucose as a substrate (maximum 3.0 hydrogen mmol/l/h), as compared to glycerol – maximum 0.13 hydrogen mmol/l/h. Glucose shows higher results compared to glycerol using *E. coli* because it is a very convenient substrate for fermentative bacteria metabolism. Isolated microorganisms can use glucose for fermentation up to 99.3% efficiency and reaching up to 64% hydrogen concentration in the gaseous phase [17] though bacterial strains with genetic modifications show higher results but grow comparatively slower [18].

The H_2 production rate with *C. sporogenes* in the liquid phase reached 1.50 mmol H_2/l/h, in the gaseous phase it was 1.42 mmol H_2/l/h. Measurements with *A. aneurinilyticus*, *E. limosum*, *K. ascorbata, P. pabuli* and *E. coli* showed that these strains were not producing a substantial amount of hydrogen using glycerol as a substrate, for example, *E. coli* BW25113 produced 0.125 mmol/l/h and *E. limosum* produced 0.07 mmol H_2/l/h. Hydrogen production measurements on the sample with the most productive strains (*E. coli* BW25113 and *C. sporogenes,* respectively) using glucose and glycerol as a substrate are shown in Figure.

Table. **H_2 production rates with all strains and substrates**

Bacterial strain	H_2 production rate in liquid phase, mmol/l/h	H_2 production rate in gaseous phase, mmol/l/h	Substrate	Substrate concentration, mmol/l
E. coli BW25113	3.00	2.50	Glucose	30
E. coli MSCL 332	2.00	0.10	Glucose	30
E. asburiae MSCL 839	0.002	NM	Glucose	15
E. cloacae MSCL 778	0.005	NM	Glucose	15
A. aneurinilyticus MSCL 1018	0.06	0.00	Glycerol	240
E. coli BW25113	0.125	0.04	Glycerol	240
K. ascorbata MSCL 732	0.09	0.04	Glycerol	240
C. sporogenes MSCL 764	1.50	1.42	Glycerol	240
E. limosum MSCL 840	0.07	0.18	Glycerol	240
P. pabuli MSCL 1006	0.08	0.00	Glycerol	240

NM – Not measured.

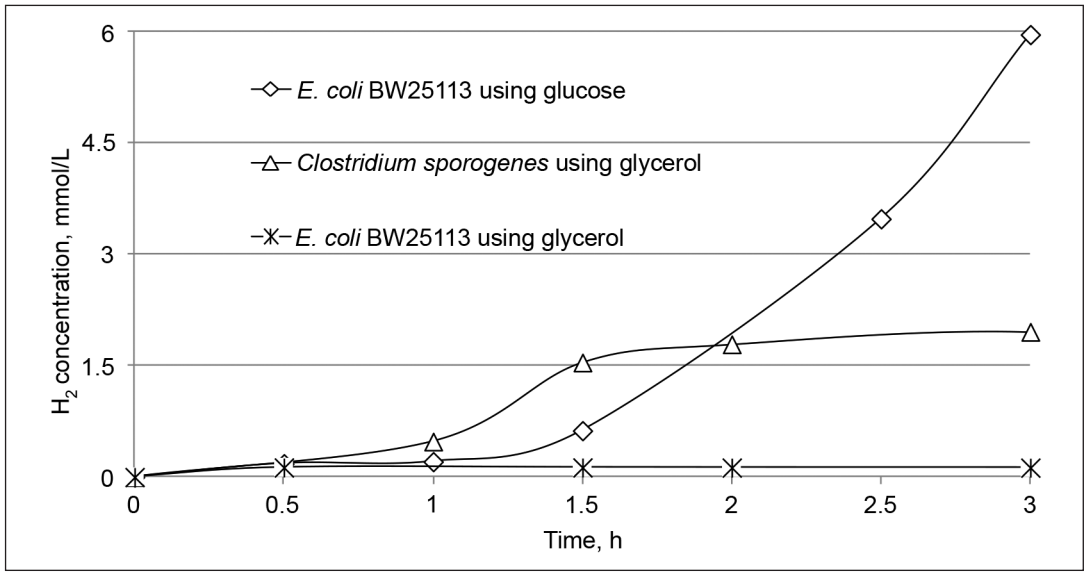

Figure. Hydrogen production measurements on the sample with *E. coli* BW25113 and *C. sporogenes* using glucose and glycerol as a substrate

E. coli BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE::kan* had the highest hydrogen production rates and almost all hydrogen transferred from the liquid to gaseous state (3.0 mmol/l/h in liquid and 2.5 mmol/l/h in gaseous phase, respectively) using glucose as a substrate. Our results (33 μmol hydrogen/mg protein/h) are comparable to the previous research by Maeda [18] where the same bacterial strain *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE::kan* produced 32 ± 6 μmol hydrogen/mg protein/h from glucose.

CONCLUSIONS

Several of the studied bacterial strains are good candidates for further investigations of bio-hydrogen production from glycerol, which is a perspective substrate to be used in fermentation process. *E. coli* BW25113 produced hydrogen with the maximum rate 0.13 hydrogen mmol/l/h (liquid phase), compared to the model substrate – glucose – hydrogen rate 3.0 hydrogen mmol/l/h. The maximum of hydrogen production with glycerol as a substrate was reached by *C. sporogenes* (1.5 H_2mmol/L/h). Test-systems were optimised to allow on-line estimation of substrate utilization and hydrogen production in liquid and gaseous phases and the factors, influencing the bacterial capability to produce hydrogen at fermentation of glycerol and glucose, as well as the possibilities to optimize the measuring process were investigated. Higher hydrogen concentration in the gaseous state was achieved by using constant liquid mixing and argon gas bubbling through the cultivation medium.

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BIOVANDENILIO GAMYBOS IŠ VANDENILIO IR GLIUKOZĖS FERMENTAVIMO BAKTERIJŲ VERTINIMAS

Santrauka

Fermentuojant organinius substratus mikroorganizmai geba išskirti vandenilį, todėl pramoninės atliekos gali būti naudojamos kaip žaliava vandenilį gaminančioms bakterijoms. Vienas iš substratų, kurį galima efektyviai naudoti mikrobiologiniam vandeniliui gaminti, yra glicerolis. Biodyzelino gamyboje glicerolis susidaro kaip šalutinis produktas. Šiame darbe gliukozė naudojama kaip pavyzdinis substratas. Įvairūs bakterijų izoliatai buvo naudojami siekiant nustatyti vandenilio išeigą iš gliukozės ir glicerolio. Tyrimams naudota bandymų sistema, sukurta mūsų laboratorijoje. Bandymų sistema optimizuota taip, kad būtų galima tinkamai įvertinti substratų ir bakterijų atmainų įtaką vandenilio išeigai skystoje ir dujinėje fazėse. Nustatyta, kad kelios bakterijų atmainos yra tinkamos biovandeniliui gaminti iš glicerolio substrato. Įvertinta, ar biovandenilio gamyba iš glicerolio yra ekonomiška ir tinkama aplinkos apsaugos požiūriu. Žaliavos kaina labai sumažėja dėl didelio glicerolio kiekio, susidarančio gaminant biodyzeliną, nors glicerolyje ir yra daug mažiau anglies.

Raktažodžiai: biovandenilis, fermentacija, substratai, bioreaktoriaus prototipas

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ОЦЕНКА ПРОИЗВОДСТВА БИОВОДОРОДА НА ОСНОВЕ ВОДОРОДА И ГЛЮКОЗЫ С ПРИМЕНЕНИЕМ МЕТОДА БАКТЕРИИ ФЕРМЕНТАЦИИ

Резюме

В процессе ферментации органических субстратов микроорганизмы в состоянии и способны выделить водород. В связи с этим промышленные отходы могут быть использованы в качестве сырья для синтеза бактерии по производству водорода. Один из субстратов, который можно эффективно использовать для производства микробиологического водорода является глицероль. В процессе синтеза глицероль синтезируется, как вторичный продукт. В данной работе глюкоза применяется как образцовый субстрат. Различные изоляты бактерии использовались с целью определения количества водорода, полученного из глюкозы и глицероля.

Система по проведению исследовании была создана в нашей лаборатории. Она была оптимизирована с целью, чтобы можно было достаточно точно оценить влияние видов субстратов и бактерии на количество водорода в жидкой и газовой фазах. Установлено, что некоторые виды бактерии способны производить биоводород из субстрата глицероля. В работе представлена оценка по определению экономичности и эффективности производства биоводорода из глицероля. Стоимость сырья значительно уменьшается из-за образования – синтеза больших количеств глицероля, образовавшихся в процессе производства, хотя в глицероле существует значительно меньше количества угля.

Ключевые слова: биоводород, ферментация, субстраты, прототип биореактора