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ORIGINAL PAPER

Comparison of different mixed cultures for bio-hydrogen production from ground wheat starch by combined dark and light fermentation

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Abstract Composition of the mixed culture was varied in combined dark-light fermentation of wheat powder starch in order to improve hydrogen gas formation rate and yield. Heat-treated anaerobic sludge and pure culture of *Clostridium beijerinckii* (*DSMZ 791*^{T}) were combined with two different light fermentation bacteria of *Rhodobacter sphaeroides* (RS-NRRL and RS-RV) in order to select a more suitable mixture resulting in high hydrogen yield and formation rate. A combination of the anaerobic sludge and RS-NRRL yielded the highest cumulative hydrogen (CHF = 140 ml), the highest yield (0.36 mol H_2 mol^{-1} glucose) and specific hydrogen formation rate (2.5 ml H_2 g^{-1} biomass h^{-1}). During dark fermentation (70 h) hydrogen was produced simultaneously by the dark and light fermentation bacteria using glucose from hydrolyzed starch. However, only light fermentation bacteria produced hydrogen from VFA's derived from dark fermentation after a long adaptation period.

Keywords Bio-hydrogen · Combined fermentations · Dark fermentation · Light fermentation · Mixed culture composition

Introduction

Hydrogen gas is a clean fuel and energy carrier with a high energy content of 122 kJ g^{-1}. Utilization of hydrogen gas as energy carrier and fuel reduces green house effects by eliminating CO_2, CO, SO_x and NO_x emissions derived from fossil fuels. Hydrogen gas can also be used in fuel cells for electricity generation. The major obstacle in the utilization of hydrogen gas is its unavailability in nature. Presently, hydrogen gas is produced by steam reforming of natural gas and other hydrocarbons requiring high energy inputs [14]. Fermentation of carbohydrate-rich raw materials for bio-hydrogen production offer significant advantages over costly chemical processes due to operation under mild conditions (30–35°C, 1 atm). However, low fermentation rates and hydrogen yields are the major problems requiring improvement for large-scale bio-hydrogen production [14, 16, 22, 24].

Renewable resources, such as starch and cellulose containing biomass, constitute an abundant, inexpensive and reliable raw material for bio-hydrogen production [14, 24]. Waste biomass such as agricultural and domestic wastes can be used as inexpensive raw materials for bio-hydrogen production at large scale. Bio-hydrogen production from carbohydrate-rich renewable resources requires dark and light anaerobic fermentations [14]. The first step in fermentative hydrogen production from biomass is the acid or enzymatic hydrolysis of starch or cellulose to highly concentrated sugar solution. After neutralization and nutrient balancing, glucose solution is subjected to dark fermentation by the acetogenic-anaerobic bacteria for production of volatile fatty acids (VFA), hydrogen and CO_2 [14, 16, 22]. Volatile fatty acids produced by the dark fermentation are further fermented by the photo-heterotrophic bacteria (light fermentation), such as *Rhodobacter* sp., to produce CO_2 and H_2 [14, 16, 22].

Pure starch was used in most of the reported studies on bio-hydrogen production [7, 9, 13, 17, 19, 20, 27–29]. Waste wheat constitutes a reliable renewable resource for bio-hydrogen production because of its high starch and gluten content (>95%). Studies on utilization of

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starch-containing wastes such as waste corn, wheat and rice for bio-hydrogen production by dark and light fermentations are limited [13, 29]. Partially hydrolyzed ground wheat starch was used as the substrate for bio-hydrogen production by dark and light fermentations in our previous studies [1–6, 15, 25].

Heat-treated anaerobic sludge and pure cultures of *Clostridia* and *Enterobacter* species were used for bio-hydrogen production by dark fermentation [13, 28]. Heat-treated anaerobic sludge was reported to perform better than the pure cultures in dark fermentation [4]. Most of the dark fermentation studies were done in batch culture using different substrate and anaerobic cultures [13, 17, 19, 20, 26–29]. Some recent studies utilized continuous culture for bio-hydrogen production from different substrates [7, 9, 18].

Dark and light fermentations for bio-hydrogen production can be used in sequential or combined modes. Combined dark-light fermentations for bio-hydrogen production may have advantages over sequential fermentation by reducing the fermentation time and increasing the hydrogen productivity. Most of the combined fermentations on bio-hydrogen production were carried out by batch operation [5, 6, 8, 11, 30]. In our previous studies on combined fermentations, we have used heat-treated anaerobic sludge and a mixture of *Rhodobacter* sp. for bio-hydrogen production by batch and fed-batch operations [5, 6]. Qualitative and quantitative composition of mixed culture affects the rate and extent of bio-hydrogen formation in combined fermentation. This aspect of combined fermentation has not been studied and reported in the literature. Therefore, this study was performed with different mixtures of dark and light fermentation bacteria in order to determine a suitable mixture with high hydrogen formation rate and yield in batch combined fermentation.

Materials and methods

Experimental setup and procedure

Serum bottles (Isolab-Germany Boro 3.3) with an initial fermentation volume of 200 ml (310 ml total volume) were used for batch combined dark and light fermentations. Silicone stoppers, screw caps and metal valves were used to avoid gas leakage from the bottles. The wheat powder used for the experiments contained ~97% (ww^{-1}) starch and gluten, 3.4 mg g^{-1} total nitrogen and 1.72 mg g^{-1} phosphate-P. The wheat powder solution was boiled for 1.5 h for partial hydrolysis of starch before being placed into serum bottles. Anaerobic conditions were maintained by adding 200 mg l^{-1} Na-thioglycolate and passing argon gas from the head space of the bottles for 5 min at the beginning of the experiments.

The bottles were illuminated with tungsten and halogen light (6,000 ± 500 lux) from the outer surface and incubated in an air-conditioned room at 30°C. The initial wheat starch concentration was 12.8 g l^{-1} with 8.6 g l^{-1} dissolved total sugar. The initial biomass (cell) concentration was constant at 1.15 g l^{-1} in all bottles. Six serum bottles were used, two of which contained only dark fermentation bacteria (anaerobic sludge and *C. beijerinckii)*, and the other bottles contained different combinations of dark and light fermentation bacteria with a D/L ratio of 1/2. Concentrations of the dark and light fermentation bacteria were $X_D = 0.38$ g l^{-1}, $X_L = 0.77$ g l^{-1} with a total biomass concentration of $X_T = 1.15$ g l^{-1}. Other nutrients, 10 mg l^{-1} $FeSO_4 \cdot 7H_2O$ with EDTA complex, 0.75 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, phosphate buffer (2.8 g l^{-1} K_2HPO_4 and 3.9 g l^{-1} KH_2PO_4) and 75 μg l^{-1} $NaVO_3$ were also added to the combined fermentation media to provide essential nutrients for *Rhodobacter* sp. The initial pH was adjusted to 7.5 with 10 M NaOH solution. The initial oxidation reduction potential (ORP) was −160 mV, which decreased to around −300 ± 20 mV at the end of fermentation period. pH was controlled between 6 and 7 in all experiments by manual pH adjustment. Serum bottles were prepared in duplicates.

Organisms

The anaerobic sludge (AN) was obtained from the acidogenic phase of the anaerobic wastewater treatment plant of PAK MAYA Bakers Yeast Company in Izmir, Turkey. The concentrated sludge was subjected to 1 h heat pre-treatment at 100°C after pH adjustment to 5.9. Heat treatment was used to select spore-forming and hydrogen-producing acidogenic bacteria and to eliminate hydrogen-consuming methanogens. The heat-treated anaerobic sludge was cultivated in a medium containing diluted molasses (total sugar = 5 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.25 g l^{-1}), K_2HPO_4 (2.8 g l^{-1}), KH_2PO_4 (3.9 g l^{-1}), L-cysteine-HCl·H_2O (0.1 g l^{-1}). *Clostridium beijerinckii*-DSMZ 791^T (CB) was cultivated in a medium containing 7.5 g l^{-1} glucose, 2 g l^{-1} nutrient broth, 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} KH_2PO_4, 0.2 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g l^{-1} L-cysteine-HCl·H_2O. AN and CB were cultivated in serum bottles at pH = 6.8 using an incubator at 37°C. Argon gas was passed through the cultivation medium before incubation. After 1 day of cultivation, the cells were centrifuged and re-suspended in distilled water to obtain high cell density inoculum culture.

Rhodobacter sphaeroides NRRL B-1727 (RS-NRRL) was obtained from USDA National Center for Agricultural

Utilization Research, Peoria, IL, USA. *R. sphaeroides*-RV (RS-RV) was obtained from Dr. J. Miyake of Japan [23]. The growth medium of the *Rhodobacter* sp. contained acetic acid (2 g l^{-1}), butyric acid (0.5 g l^{-1}), lactic acid (0.5 g l^{-1}), yeast extract (0.5 g l^{-1}), K_2HPO_4 (2.8 g l^{-1}), KH_2PO_4 (3.9 g l^{-1}), Na-glutamate (1.87 g l^{-1}), Na_2MoO_4 $2H_2O$ (0.75 mg l^{-1}), $FeSO_4{\cdot}7H_2O$ (10 mg l^{-1}) from $FeSO_4{\cdot}7H_2O$ EDTA complex and 75 μg l^{-1} Na V O_3, $MgSO_4{\cdot}7H_2O$ (0.2 g l^{-1}) at pH 7.0. *Rhodobacter* sp. was cultivated in serum bottles at 30°C using tungsten and halogen lamps with six klux light intensity. Argon gas was passed through the cultivation medium before incubation. After 5 days of incubation period, the cells were centrifuged and re-suspended in $MgSO_4{\cdot}7H_2O$ (0.25 g l^{-1}), K_2HPO_4 (1 g l^{-1}) and KH_2PO_4 (1 g l^{-1}) containing solution prior to inoculation.

Analytical methods

Starch (STR), total sugar (TSG) and total volatile fatty acid (TVFA) analyses were carried out on the samples removed from the liquid phase periodically. For starch analysis, the samples were acidified and boiled for 1.5 h for complete hydrolysis of starch to sugar, and the resulting total sugar concentration was determined by using the acid-phenol method [10]. Starch concentration was determined by dividing the total sugar concentration by 1.10 since the glucose-to-starch ratio is 1.10 in the hydrolysis reaction of starch. The samples were centrifuged at 7,000*g* to remove solids from the liquid media, and total sugar and TVFA analyses were carried out in clear supernatants. Total sugar content was determined by the acid-phenol spectrometric method [10]. TVFA analyses were carried out by using analytical kits (Spectroquant, 1.01763. 0001, Merck, Darmstadt, Germany) and a PC spectrometer (WTW Photolab S12). Analyses were carried out in triplicates with less than 3% deviation from the average.

Hydrogen gas was sampled from the head space of the bottles by using gas-tight glass syringes, and hydrogen concentration was determined by using a gas chromatograph (Agilent 6890). The GC column was Alltech, Hayesep D 80/100 6″× 1/8″ × 085″. Nitrogen gas was used as carrier with a flow rate of 30 ml min^{-1}. Temperatures of the oven, injection, detector and filament were 35, 120, 120 and 140°C, respectively. The amount of total gas produced was determined by water displacement method every day using sulfuric acid (2%) and NaCl (10%) containing solution. The cumulative hydrogen gas production was determined by using the following equation [21]:

$$V_{H2;i} = V_{H2;i-1} + V_W C_{H2;i} + V_{G;i} C_{H2;i} - V_{G;i-1} C_{H2;i-1} \quad (1)$$

where $V_{H2,\ i}$ and $V_{H2,\ i-1}$ are the volumes of cumulative hydrogen (ml) calculated after the *i*th and the previous measurement; V_W is the total gas volume measured by the water displacement method (ml); $C_{H2,i}$ is the concentration of H_2 gas in the total gas measured by the water displacement method (%); $V_{G,i}$ and $V_{G,i-1}$ are the volumes of the gas in the head space of the bottle for the ith and the previous measurement (ml); $C_{H2,i}$ and $C_{H2,\ i-1}$ are the percent H_2 in the head space of the bottle for the ith and the previous measurement. The amounts of released hydrogen gas and in the head space of the bottle were measured independently and added up to determine the cumulative H_2 formation for every period of sampling.

Biomass concentrations in the inocula were determined by filtering a 5-ml sample through a 0.45-mm milipore filter, drying at 105°C and determining the constant dry weight according to the standard methods [12]. pH and ORP of the fermentation medium were monitored by using a pH meter and ORP meter with relevant probes (WTW Scientific, Germany).

Results and discussion

Six batch combined fermentation experiments were performed by using different combinations of dark and light fermentation bacteria. The serum bottles contained the following bacteria mixtures: (1) Heat-treated anaerobic sludge (AN), (2) *Clostridium beijerinckii*-DSM-791^T (CB), (3) AN + *Rhodobacter sphaeroides* NRRL B-1727 (RS-NRRL), (4) AN + *Rhodobacter sphaeroides* RV (RS-RV), (5) CB + RS-NRRL and (6) CB + RS-RV.

Figure 1 depicts variation of cumulative hydrogen formation (CHF) with time for different mixtures of dark and light fermentation bacteria in combined fermentation. Dark fermentation was completed within 70 h (3 days) in all experiments. There was a negligible difference in cumulative hydrogen volumes (50–55 ml) produced by the anaerobic sludge (AN), CB, CB + RS-RV and AN + RS-RV within 70 h. AN + RS-NRRL and CB + RS-NRRL yielded lower CHF values (15–20 ml) within 70 h probably because of the low fermentative activity of RS-NRRL. Only in two of the fermentation media containing AN + RS-RV and AN + RS-NRRL did hydrogen production take place by light fermentation after a long lag period. In other fermentation media containing CB + RV-RS and CB + RS-NRRL, no hydrogen was produced by the light fermentation. Probably the *Rhodobacter* cultures in those mixtures were unable to ferment the VFAs produced by the CB culture. VFAs produced by the anaerobic sludge (AN) were partially fermented by the *Rhodobacter* sp. after a long adaptation period (100 h) similar to diauxic

growth. The light fermentation lasted for another 100 h, and hydrogen formation stopped after 275 h of incubation. The highest CHF (140 ml) was obtained with the AN + RS-NRRL mixed culture followed by the AN + RS-RV culture (110 ml). Apparently, the VFAs (acetic, butyric acids) produced by AN culture were fermentable, while the VFAs produced by the CB culture were not easily fermentable by the *Rhodobacter* sp. The difference in hydrogen production was less than 10% in duplicate experiments.

Both the dark and light fermentation bacteria produced hydrogen gas using carbohydrates from hydrolyzed starch within the first 70 h. Therefore, the metabolism of *Rhodobacter* sp was adjusted to glucose fermentation within this period. When glucose was depleted, it took a long time for the *Rhodobacter* sp. to switch their metabolism for fermentation of VFAs to produce hydrogen gas. In other words, the presence of glucose in the medium caused 'catabolite repression' on the synthesis of the enzymes used for fermentation of VFAs. The hydrogen gas productivity (l H_2 l^{-1} reactor h^{-1}) was low due to the long adaptation (lag) period between the dark and light fermentations.

Conversion of wheat starch to hydrogen gas by combined dark and light fermentations can be described as follows:

$$\text{Starch} \rightarrow \text{glucose} + \text{maltose} \rightarrow \text{VFA} + H_2 + CO_2 \rightarrow H_2 + CO_2$$

The first reaction is the enzymatic hydrolysis of wheat starch to glucose and maltose by the dark fermentation bacteria (AN and CB). *Rhodobacter* sp. do not have the capability of hydrolyzing starch. The second reaction is fermentation of glucose to a mixture of VFAs, H_2 and CO_2 by both the dark and light fermentation bacteria. The third step is fermentation of VFAs to H_2 and CO_2 by light fermentation bacteria (*Rhodobacter* sp.). After starch hydrolysis, produced carbohydrates were fermented by both the dark and photo-fermentation bacteria to produce hydrogen gas. Dark fermentation bacteria were probably more active than the *Rhodobacter* sp. in this conversion since AN, CB, AN + RS-RV and CB + RS-RV yielded almost the same CHF (~50 ml) within 70 h. However, only RS-NRRL and RS-RV cultures actively fermented VFAs produced by the AN culture for production of hydrogen by light fermentation. Therefore, inclusion of *Rhodobacter* sp. in fermentation media along with the anaerobic sludge (AN) helped to improve the hydrogen formation when the dark and light fermentation bacteria mixture was qualitatively and quantitatively balanced.

Variations of total sugar concentrations with time are depicted in Fig. 2 for different combined fermentation cultures. The rates of sugar depletion were all close to each other. However, the fastest sugar fermentation was observed with the AN + RS-RV, and the slowest was with the AN culture within the first 50 h. Total sugar concentration decreased from 8.8 g l^{-1} to less than 1 g l^{-1} after 175 h of fermentation time where hydrogen production was started by light fermentation. Total sugar was completely depleted in 200 h, and hydrogen formation for the last 3 days was due to light fermentation of the VFAs by the *Rhodobacter* sp. Apparently, the type of VFAs produced by the CB were not fermentable by the *Rhodobacter* sp. since no hydrogen gas was produced by the *Rhodobacter* sp. when cultivated with the CB culture. pH decreased from 7.0 to as low as 5.25 within the first 48 h and was between 6.5 and 7.2 for the rest of fermentation period. pH was controlled manually everyday by adding concentrated NaOH to the medium. ORP of the fermentation media decreased from −150 to −350 mV and fluctuated between −350 and −300 mV for the whole fermentation period.

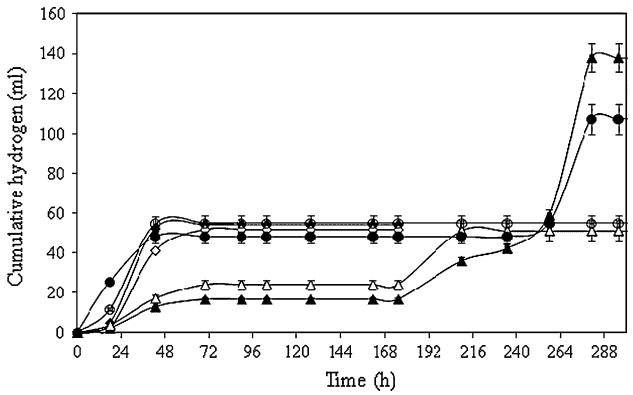

Fig. 1 Variation of cumulative hydrogen with time for different mixtures of dark and light fermentation bacteria in combined fermentation. *Filled diamond* AN, *open diamond* CB, *filled triangle* AN + RS-NRRL, *open triangle* CB + RS-NRRL, *filled circle* AN + RS-RV, *open circle* CB + RS-RV

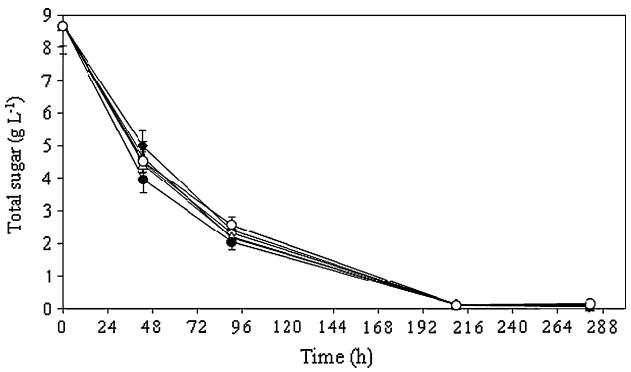

Fig. 2 Variation of total sugar concentration with time for different mixtures of dark and light fermentation bacteria in combined fermentation. *Filled diamond* AN, *opened diamond* CB, *filled triangle* AN + RS-NRRL, *open triangle* CB + RS-NRRL, *filled circle* AN + RS-RV, *open circle* CB + RS-RV

The data presented in the dark fermentation section of Fig. 1 (up to 70 h) were correlated with the Gompertz equation, and the constants were determined by regression analysis.

The Gompertz equation has the following form:

$$H(t) = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

where P is the maximum potential hydrogen formation (ml); R_m is the maximum rate of hydrogen formation (ml h^{-1}), and λ is the duration of the lag phase (h). Table 1 summarizes the Gompertz equation coefficients for different *Rhodobacter* species. The highest CHF (54.8 ml) and formation rate (1.73 ml h^{-1}) were obtained with the CB + RS-RV culture during the dark fermentation period. Duration of lag phase varied between 12 and 20 h, and the lowest was 13 h for the CB + RS-NRRL culture. Apparently, *C. beijerinckii* (CB) was very active in fermenting starch and glucose during the dark fermentation phase (70 h). Potential hydrogen formations (P, ml) obtained with the other cultures of AN, CB, AN + RS-RV and CB + RS-RV were close to each other (around 50 ml), indicating effective dark fermentation of starch by those cultures.

Variations of starch, total sugar and TVFA concentrations with time for the AN + RS-NRRL culture in combined fermentation are depicted in Fig. 3. Starch concentration decreased from 13 g l^{-1} to less than 0.1 g l^{-1} within 200 h. Total sugar followed the same trend and decreased from 8.8 g l^{-1} to less than 0.1 g l^{-1}. The results indicated complete fermentation of starch and glucose. However, hydrogen production did not follow the same trend. Hydrogen was produced within the first 70 h followed by a long adaptation period for nearly 100 h. Hydrogen production by light fermentation took place between 175 and 275 h of the fermentation period where the sugar concentration was very low. The results clearly indicated that the presence of glucose repressed fermentation of VFAs and hydrogen formation by light fermentation bacteria.

Table 1 Gompertz equation constants for the dark fermentation period (70 h) with different mixtures of dark and light fermentation bacteria

Bacteria mixtures	P (ml)	R_m (ml h^{-1})	λ (h)
AN	52.29	0.95	19.49
CB	54.16	0.95	17.29
AN + RS-NRRL	17.04	0.28	14.39
CB + RS-NRRL	25.85	0.39	12.88
AN + RS-RV	48.05	0.91	15.22
CB + RS-RV	54.82	1.73	15.67

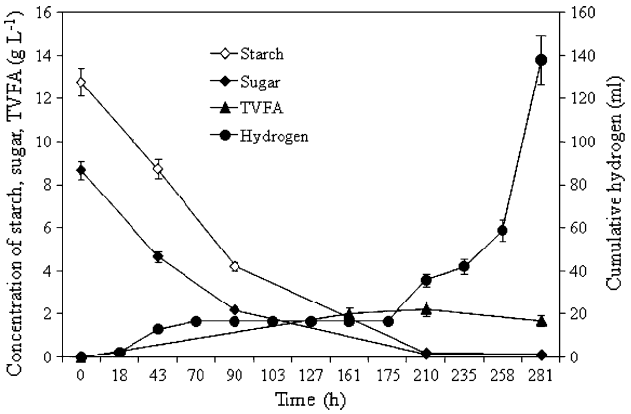

Fig. 3 Variation of starch, total sugar and TVFA concentrations with time for the AN + RS-NRRL culture in combined fermentation. *Open diamond* starch, *filled diamond* total sugar, *filled triangle* TVFA, *filled circle* cumulative hydrogen

Hydrogen yield and formation rate are the most important criteria for comparison of performances of different mixed cultures. Hydrogen yields (moles H_2 mol^{-1} glucose) were compared in Fig. 4 for different cultures. The highest hydrogen yield (0.36 mol H_2 mol^{-1} glucose) was obtained with the AN + RS-NRRL mixture. The AN + RS-RV mixture resulted in a yield of 0.28 mol H_2 mol^{-1}glucose. The yields with the other cultures were considerably lower than the aforementioned yields (0.14 ± 0.01 mol H_2 mol^{-1} glucose) since no light fermentation took place with those cultures. Apparently, *Rhodobacter* sp. was incapable of fermenting VFAs produced by the CB culture while fermenting VFAs produced by the anaerobic sludge (AN) and improving hydrogen yield considerably.

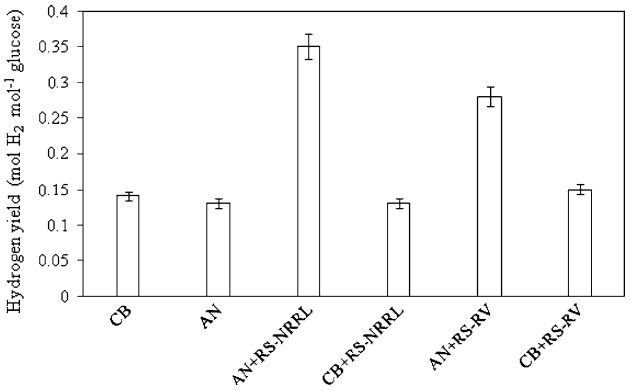

Fig. 4 Comparison of hydrogen yield coefficients for different combinations of dark and light fermentation bacteria in combined fermentation

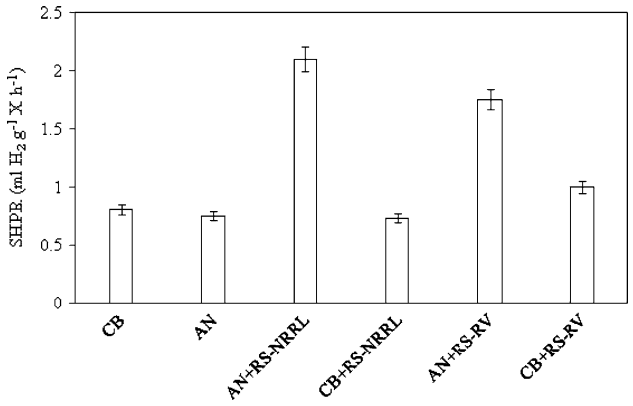

Fig. 5 Comparison of specific hydrogen production rates (SHPR) for different combinations of dark and light fermentation bacteria

Specific hydrogen production rates (SHPR) were calculated by dividing the CHFs by the fermentation time and the total amount of biomass.

$$\mathrm{SHPR} = \mathrm{CHF}/(t.V_oX_o)$$

where 't' is fermentation time, V_o is the initial fermentation volume, and X_o is the initial total biomass concentration. Figure 5 presents SHPRs for different mixed cultures in combined fermentation. Again the highest SHPR (2.13 ml g^{-1}biomass h^{-1}) was obtained with the AN + RS-NRRL mixture. AN + RS-RV mixture yielded a SHPR of 1.66 ml g^{-1}biomass h^{-1}. The other bacteria mixtures yielded considerably lower SHPRs (0.8 ± 0.02 ml g^{-1}biomass h^{-1}) because of no hydrogen formation by the light fermentation bacteria.

Most of the studies on combined fermentations were done by using pure cultures in suspended or immobilized forms [8, 11, 30]. There are no literature reports on combined fermentations comparing the performances of mixtures of different dark and light fermentation bacteria. In our previous studies on combined fermentations of ground wheat starch, we have used heat-treated anaerobic sludge and a mixture of four different *Rhodobacter* sp. instead of pure *Rhodobacter* sp. [5, 6]. As compared to our previous studies on combined fermentations [5, 6], we have obtained relatively high CHF in this study by using a mixture of AN + RS-NRRL and operating the system for a longer period to allow light fermentation to take place. The hydrogen yields obtained in this study are comparable to those reported in our previous studies on combined fermentation (0.4–0.6 mol H_2 mol^{-1}glucose). Hydrogen productivity of combined fermentation can be improved by eliminating the lag phase between the dark and light fermentations. This may be achieved by using only anaerobic sludge during the dark fermentation (first 3 days) and adding VFA-adapted *Rhodobacter* sp. into the fermentation medium when dark fermentation was completed.

Conclusions

Combined dark and light fermentations may have considerable advantages over sequential fermentations by reducing the fermentation time and eliminating some operations between the dark and light fermentations. The qualitative and quantitative composition of the bacterial cultures used in combined fermentation affects hydrogen yield and formation rates considerably. Two dark fermentation cultures (anaerobic sludge -AN and *C. beijerinckii*-CB) were used alone and in combination with the two light fermentation bacteria (*R. sphaeroides*-RV and *R. sphaeroides*-NRRL) in combined fermentation of powdered wheat starch for biohydrogen production. The highest CHF (140 ml) was obtained with the AN + RS-NRRL mixture. AN + RS-RV mixture also yielded comparable CHF (110 ml). Dark fermentation ended within 70 h (3 days), and the light fermentation of VFAs started after a lag period of 4 days and took nearly 3 days. Presence of glucose in fermentation medium repressed simultaneous fermentation of VFAs by *Rhodobacter* sp. and reduced hydrogen productivity.

The highest hydrogen yield and formation rates were also obtained with the AN + RS-NRRL mixed culture. The results clearly indicated that a mixture of anaerobic sludge and RS-NRRL is the most suitable culture in combined fermentation among the other mixtures tested. Qualitative and quantitative composition of the mixed dark-light fermentation bacteria should be further improved, and fermentation time should be reduced in order to improve hydrogen productivity.

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