Acceleration and increase of hydrogen production by simultaneous fermentation of *Clostridium butyricum* and *Rhodobacter sphaeroides* on wine-vinasse substrate

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A fermentation process for hydrogen production as a result of the simultaneous effect of *Rhodobacter sphaeroides* and *Clostridium butyricum* on a wine-vinasse substrate was realized in a single illuminated bioreactor. The kinetics of the cooperative process indicates rapid and enhanced production of hydrogen showing yield of 65.41 mmol/l vinasse with a mixed culture as compared to processes using the two bacteria separately that have yields of 27.41 and 25.49 mmol/l vinasse for *Rhodobacter* and *Clostridium*, respectively. The experiment with a mixture of the two bacteria revealed co-operative assimilation of almost all components studied in the following sequence: malic acid > lactic acid > residual sugars > tartaric acid > citric acid. The use of vinasse substrate for hydrogen production would be a significant ecological energy resource for enterprises producing wine brandies together with waste utilization.

**Key words:** hydrogen production, mixed fermentation, *Rhodobacter sphaeroides*, *Clostridium butyricum*, renewable energy resource, wine-vinasse.

**INTRODUCTION**

In the nearest future hydrogen is expected to find a wide application in both industry and transport because water is the only product of its burning. In addition to the labour-consuming and expensive methods of its chemical preparation, biological methods became very popular during the past years. Methods based on photo-fermentation and dark-fermentation H₂-producing bacteria on various substrates proved very important. One of the main problems with these bacterial processes is the substrate material which is, in most cases, an agricultural waste product, waste water, whey, etc., needing sometimes additional pre-treatment before being thrown away [1, 2]. The utilization of these products aimed at hydrogen production would result in cheap and pure energy, the polluting waste products being eliminated. Some studies used carbohydrate-containing substrates and dark-fermentation with the participation of bacteria, above all of the kind *Clostridium* [3, 4] as well as photo bacteria acting on substrates which contain mainly organic acids were carried out [5, 6]. Combination of the two processes has been achieved by successive utilization of a glucose-containing substrate with *Enterobacter cloacae* followed by photofermentation with *Rhodobacter sphaeroides* of the metabolites from the dark process which contain, mainly acetic acid and other products. This combination of the two processes significantly increases the hydrogen yield and the utilization of the substrate chemical energy. The hydrogen yield of the combined processes is found to be higher than that of a single process [7]. The development of an integrated biological hydrogen production process is described on the basis of unicellular green algae, which are driven by the visible portion of the solar spectrum, coupled with purple photosynthetic bacteria, which are driven by the near infrared portion of spectrum [8].

In the present study, we tried to obtain hydrogen by simultaneous photo fermentation and dark fermentation in a single bioreactor with combined action of *Clostridium butyricum* and *Rodobacter sphaeroides* on the waste substrate. The latter, called wine-vinasse, was a waste product formed during wine distillation before obtaining brandy as a final product. This product is rich in organic acids and residual sugars, amino acids and small amounts of other compounds coming from the grapes.

**MATERIALS AND METHODS**

*Cultivation of Rhodobacter sphaeroides and Clostridium butyricum*

*Clostridium butyricum* 1389 strain was supplied from the National Bank of Industrial Microorganis-
nisms and Cell Culture in Sofia. The initial *Rhodo-

bacter sphaeroides* strain was bought from the firm

NCIMB, UK with an authentic certificate.

*Rhodobacter sphaeroides* was cultivated in M22

medium [9] containing sodium lactate, succinate,

glutamate and aspartic acid as carbon and nitrogen

sources as well as minerals and vitamins. Cultiva-

tion was performed under anaerobic conditions in

light at a temperature of 30°C and pH 6.5. The cells

grown were colored in red. Prior to its use, the

culture was adapted in vinasse, at first in a 1:1 ratio,

and then on pure vinasse.

*Clostridium butyricum* was precultured at 37°C

in a basal medium (pH 7.0) containing (g/l) casein

hydrolyzate 15 g, L-cystein 0.5 g, glucose 5.0 g,

yeast extract 5 g, sodium thioglycolate 0.5 g,

sodium chloride 2.5 g and agar 0.75 g. Before using

the culture to obtain hydrogen from vinasse, it was

adapted in a nutrient medium and vinasse in a 1:1

ratio, after which it was transferred to pure vinasse.

Vinasse substrate was prepared by distillation of

white wines and consisted mainly of tartaric acid,

citric, malic and lactic acids, amino acids, residual

sugars as well as other compounds of lower

contents. When vinasse from red wines were used,

the residue after the alcohol distillation was deco-

loured with active carbon in order to eliminate
dyeing substances.

**Assays**

Analysis of acids were performed with a HPLC

chromatography (HPLC Waters column Lichrosper

100, RP-C18). The sugars were determined spectro-

photometrically as reducing substances by means of

3,5-dinitrosalicylic acid [10]. Quantitative estima-

tion was made on the basis of a standard straight

line obtained using glucose and the above method.

The cell concentration in this case was also
determined nephelometrically at 600 nm and then
recalculated per mg dry weight with the use of a
standard calibrating curve.

Hydrogen gas was estimated using an electro-

chemical gas sensor TGS-FIGARO Engineering

Inc., based on tin dioxide as sensing material. The
output signal displayed the percentage volume of H2
in a biogas mixture. The system was calibrated once
in two days using pure hydrogen calibration gas.

**Procedure of hydrogen production**

Hydrogen is produced from both single pure
cultures and the mixed cultures in the laboratory
installation shown in Fig. 1.

Vinasse substrate (150 ml) was placed in a glass
bioreactor with a volume of 200 ml and flat walls
ensuring better illumination. Inoculated and adapted

*Rhodobacter sphaeroides* culture was added during
the single experiments in amounts of 20 ml so that
the final concentration in the total working volume
was about 0.4 mg/ml dry cells. In the case of

*Clostridium butyricum*, an inoculate of its cells
adapted to wine-vinasse in a volume of 20 ml was
added to 150 ml of the substrate with a view to
achieving a final concentration of about 0.2 mg·ml⁻¹
dry cells in the working volume of the reactor. The
biomass concentration in the mixed culture of the
two microorganisms was the same as with the
experiments with one bacterial kind (*R. sphaeroides*
0.4 mg·ml⁻¹ *C. butyricum* 0.2 mg·ml⁻¹, i.e. a ratio of
2:1 between them taking into consideration that
organic acids content is higher in vinasse than
reducing sugar compounds). With both kinds of
experiments the pH value was 6.5 and this value
was maintained during the whole process at a
temperature of 30°C. Immediately after introducing
the inoculate, the whole system was blown through
with argon for 15 min to ensure an anaerobic
medium. After this procedure, the halogen lamp of
500 W was switched on in order to illuminate the
reactor. The bioreactor content was stirred with a
magnetic stirrer and the adsorption of carbon
dioxide before hydrogen accumulation proceeded in
a 10% Ca(OH)₂ solution.

**Fig. 1. Laboratory scheme for photo production of
hydrogen by Clostridium butyricum and Rhodobacter
sphaeroides. 1 - argon; 2 - microbial filter; 3 - pH-meter;
4 - photobioreactor; 5 - temperature control; 6 - magnetic
stirrer; 7 - halogen lamp; 8 - CO2 trap (10% Ca(OH)2);
9 - gasholder.**

**RESULTS AND DISCUSSION**

**Photo fermentation process for hydrogen
production using Rhodobacter sphaeroides and
waste wine-vinasse substrate**

Fig. 2. shows the kinetics of hydrogen produc-
tion in a periodic photo fermentation process.

Hydrogen evolution began approximately during
the fifth hour from the process beginning and had its
highest rate of 163.79 µmol·h⁻¹ until the 23th hour,
then continued with a rate of 49.05 µmol·h⁻¹ up to the 68th h. After that, the rate of biohydrogen production gradually dropped until abating of the process 96 h after its start probably due to exhaustion of the substrate. During these 96 h the volume of hydrogen amounted to 5.612 mmoles, i.e. the mean rate of its generation was 59.03 µmol·h⁻¹. Along with the fermentation process, the increase in biomass was also followed. The cell biomass introduced with the inoculate, was found to grow from 0.42 mg·ml⁻¹ to 0.997 mg·ml⁻¹ in the exponential phase of the growth curve, these investigations being also presented in Fig. 2. The productivity curve (Fig. 2) shows the maximum in the late exponential phase of the microbial growth followed by sharp decrease, with a relatively low productivity in the stationary phase.

**Hydrogen production using Clostridium butyricum and waste wine-vinasse substrate**

The batch fermentation process of hydrogen production by means of *Clostridium butyricum* was realized under conditions identical to those of the above process, however without illumination. Fig. 3, illustrating the process kinetics, evidences noticeable hydrogen production about the 10th h. The process continued with a constant high rate of 120.55 µmol·h⁻¹ till the 32th h, after which a rapid rate drop was noticed. The process preserved its intensity till the 50th h (Fig. 3). The same plot demonstrates a biomass increase from 0.2 mg·ml⁻¹ to 0.71 mg·ml⁻¹ at the end of the process. For the strain *Clostridium butyricum* the maximum productivity was observed in the all phases of growth, with a maximum in the stationary one (Fig. 3)

Probably, for hydrogen production in the stationary phase a substrate available in the growth phase is necessary.

**A periodic photo fermentation process for hydrogen production using the joint effect of Rhodobacter sphaeroides and Clostridium butyricum on wine-vinasse as a substrate**

Vinasse substrate in an amount as already used separately with the two microorganisms (*Rh. sphaeroides* and *C. butyricum*) was placed in the above reactor together with them taking into account the necessary condition of their having a 2:1 ratio in the working volume. The process was accompanied by illumination. Fig. 4 shows the kinetics of hydrogen production in the presence of both bacteria as well as biomass growth and productivity.

Evidently, the production of hydrogen begins relatively soon (during the 5th h after the process beginning) and the rate increases quickly till the 26th h with an average rate of 326.74 µmol·h⁻¹. Hydrogen generation was observed for 50 h. but already with a lower rate of 117.64 µmol·h⁻¹. The total gas production was 9.811 mmoles, i.e. much more than the productions in the previous batch processes. During the 75th h the intensity of hydrogen production was still high.
It is obvious that hydrogen production takes place within the whole time for culture growth tending to zero when the substrate is probably exhausted. The results show the possibility of cooperating the two bacteria in order to enhance the hydrogen production and achieve better utilization of the vinasse substrate.

**Analysis of the utilized components of the vinasse substrate**

Table 1 shows some of the more important vinasse components followed separately in the presence of Rhodobacter sphaeroides and Clostridium butyricum as well as of mixed cultures during the fermentation processes. Some of the main vinasse components such as tartaric acid, lactic acid, malic acid and citric acid were subjected to analysis. The residual sugars were determined as reducing agents. Rhodobacter sphaeroides showed intense utilization of malic and lactic acids, and partial utilization of tartaric and citric acids. This bacterium was also found to use residual sugars relatively well. Clostridium butyricum was characterized by intense utilization of residual sugars as well as by consumption of some of the acids such as lactic and malic acids.

**Table 1.** Components content of initial vinasse and their residual concentrations at the end of different fermentation processes. In parentheses – the percentage molar consumption of a certain substrate; reducing substances are presented as glucose.

<table>
<thead>
<tr>
<th></th>
<th>Tartaric acid (g·l⁻¹)</th>
<th>Lactic acid (g·l⁻¹)</th>
<th>Malic acid (g·l⁻¹)</th>
<th>Citric acid (g·l⁻¹)</th>
<th>Reducing subst. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinasse</td>
<td>1.77</td>
<td>0.77</td>
<td>0.89</td>
<td>0.168</td>
<td>5.5</td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>1.578</td>
<td>0.017</td>
<td>0.103</td>
<td>0.146</td>
<td>1.3</td>
</tr>
<tr>
<td>sphaeroides</td>
<td>(10.8%)</td>
<td>(77.9%)</td>
<td>(85.5%)</td>
<td>(13.1%)</td>
<td>(76%)</td>
</tr>
<tr>
<td>Clostridium</td>
<td>1.7</td>
<td>0.031</td>
<td>0.091</td>
<td>0.16</td>
<td>1.1</td>
</tr>
<tr>
<td>butyricum</td>
<td>(4%)</td>
<td>(59.7%)</td>
<td>(90.8%)</td>
<td>(4.8%)</td>
<td>(80%)</td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>0.74</td>
<td>0.004</td>
<td>0.017</td>
<td>0.142</td>
<td>0.5</td>
</tr>
<tr>
<td>+Clostridium</td>
<td>(41.8%)</td>
<td>(94.8%)</td>
<td>(98.1%)</td>
<td>(15.4%)</td>
<td>(90.9%)</td>
</tr>
</tbody>
</table>

The experiment with a mixture of the two bacteria revealed co-operative assimilation of almost all components in the following sequence: malic acid > lactic acid > residual sugars > tartaric acid > citric acid

Taking into account the fact that Rhodobacter and Clostridium exist in nature as cooperative population in various kinds of habitats both in water basins and in soil [11] we assumed the probability for them to participate simultaneously in the fermentation processes, utilizing the components of the substrates used and showing mutual tolerance. On the one hand, our studies showed that waste wine-vinasse was a suitable substrate for hydrogen production in the presence of both Rhodobacter sphaeroides and Clostridium butyricum, and on the other, organizing a fermentation process with the simultaneous participation of the two organisms, resulted in accelerated and increased hydrogen yield – 65.41 mmol/l vinasse in comparison with separated participation of Rhodobacter – 27.41 mmol/l vinasse and Clostridium – 25.49 mmol/l vinasse, respectively.

As it is known anoxygenic photosynthetic bacteria as Rhodobacter sphaeroides is photoheterotrophs that can grow anaerobically utilizing sunlight and short chain organic acids as substrate. Photosynthetic bacteria utilizing the enzyme nitrogenase, which catalyze to conversion of molecular nitrogen to ammonia as well as evolution of hydrogen according to the Eqn. (1):

\[
\text{N}_2 + 10\text{H}^+ + 8\text{e}^- \rightarrow 2\text{NH}_4^+ + \text{H}_2
\]  

(1)

In the absence of N₂ gas the enzyme acts as ATP-depending hydrogenase and simply reduce protons to generate H₂ [12].

On the other hand dark anaerobic fermentative bacteria like Clostridium butyricum utilize carbohydrate substrate and H₂ is one of the end products of their metabolism according to the Eqn. (2):

\[
\text{Glucose} + 2\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{CO}_2 + 2\text{acetates}
\]  

(2)

Depending of bacterial species and organic nutrients the fermentation results in generation of small organic acids as malate, lactate, acetate etc. [13, 14]

Based on analysis of the substrate components in our experiments, it may be assumed that they are not assimilated as a simple sum but, more probably, as a result of a cooperative process. Probably, Rhodobacter sphaeroides assimilates small organic acids in the substrate-vinasse as well as the metabolite products of Clostridium butyricum. The consumption of tartaric acid is of interest. Its assimilation by Clostridium butyricum is weak and that by Rhodobacter sphaeroides, medium. In the simultaneous presence of the two bacteria, however, drastic exhaustion of this acid is observed. This maybe due to its transformation into a metabolite which is assimilated quickly. The results obtained also showed that both anaerobic fermentations runs together and the light does not disturbs C. butyricum fermentation.

In an industrial scheme, the single hydrogen bioreactor with both bacteria can be placed in the same enterprise producing brandy and the illumination could be realized by sun.
CONCLUSION

The main advantages of the present investigation are:

• realization of a fermentation process yielding hydrogen with the use of the simultaneous effect of Rhodobacter sphaeroidis and Clostridium butyricum on waste vinasse substrate in a single bioreactor, leading to a quick and enhanced hydrogen production as compared to processes with the two bacteria used separately;

• simultaneous utilization of the substrate and the metabolite products by the microorganisms demonstrated the mutual tolerance between them;

• utilization of waste vinasse substrate for the production of hydrogen would be a significant ecological energy resource for wine processing enterprises.

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REFERENCES


УСКОРЯВАНЕ И ПОВИШАВАНЕ НА ПРОДУКЦИЯТА НА ВОДОРОД С ЕДНОВРЕМЕНА ФЕРМЕНТАЦИЯТА НА Clostridium butyricum И Rhodobacter Sphaeroides ВЪРХУ СЪБСТРАТ ОТ ВИНАНА ВИНАСА

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(Резюме)

Ферментационен процес за получаване на водород беше организиран като резултат от едновременния ефект на Rhodobacter sphaeroides и Clostridium butyricum в единичен реактор с осветяване и използване на винена винаса като субстрат. Кинетиката на кооперативния процес показва бърза и повишена продукция на водород с добив от 65.41 mmol/l винаса със смесената култура, в сравнение с процесите, където бактериите се използват поотделно и показат добиви от 27.41 и 25.49 mmol/l винаса, съответно за Rhodobacter и Clostridium. Експериментът със смеса от двете бактерии, показва кооперативно асинерализирание на почти всичките изследвани компоненти в следния ред: жълтена киселина > млечна киселина > остатъчни захари > винена киселина > лимонена киселина. Използването на винаса като субстрат за продукция на водород би представлявало значителен екологичен енергийен ресурс за предприятиета, които произвеждат винено бренди, заедно с оползотворяването на отпадъка винаса.