Journal of the Islamic University of Gaza, V. 10 no.1 P21-P32, 2002

Photoproduction of Hydrogen by Rhodobacter sphaeroides O.U. 001 in a Column Photoreactor: effect of Halobacterium halobium

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الانتاج الضوئي لغاز الهيدروجين بواسطة بكتيريا الرودوباكتر سفيرويدس: تأثير إضافة بكتيريا الهلوبكتيريوم هالوبيوم

ABSTRACT **In the present study, a photobioreactor was designed and constructed for investigating hydrogen production by *Rhodobacter sphaeroides* O.U. 001 grown under anaerobic conditions. In the reactor, growth characteristics, pH measurements, and total hydrogen production were determined.**

In the second part of the study, *Halobacterium halobium* cells were added to the photoreactor, containing *Rhodobacter sphaeroides* O.U. 001 anaerobically grown at stationary phase, in order to determine its effect on hydrogen production at this growth phase. It was observed that *H. halobium* could enhance the hydrogen production rate of *R. sphaeroides* O.U. 001. Average rate of hydrogen production before and after addition of *H. halobium* cells was determined as 1.370 micromole/ liter-min and 1.610 micromole/liter-min, respectively.

ملخص **خلال هذه الدراسة تم تصميم وإنشاء مفاعل ضوئي حيوي لإنتاج غاز الهيدروجين بواسطة بكتريا رودوباكتر سفيرويدس (عزلة رقم O.U. 001) تحت ظروف لاهوائية و تم تحديد كل من خصائص النمو و الرقم الهيدروجيني والكمية الكلية لغاز الهيدروجين المنتج داخل المفاعل.**

في الجزء الثاني من الدراسة، تم بحث تأثير بكتريا هالوبكتريوم هالوبيوم على إنتاج غاز الهيدروجين بإضافتها للمفاعل الذي يحتوي على الرودوباكتر سفيرويدس وهي في مرحلة النمو القصوى. أشارت الدراسة إلى أن بكتريا هالوبكتريوم هالوبيوم أدت إلى زيادة سرعة إنتاج غاز الهيدروجين وكان ترتيب معدل سرعة إنتاج غاز الهيدروجين بواسطة رودوباكتر سفيرويدس قبل وبعد إضافة بكتريا

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Photoproduction of Hydrogen by Rhodobacter sphaeroides

هالوبكتريوم هالوبيوم 1.37 هو ميكرومول/لتر–دقيقة و1.61 ميكرومول/لتر–دقيقة.

INTRODUCTION

Rapid advancement of biotechnology in recent years has drawn attention to technologies for production of hydrogen gas by using microorganisms and solar energy. Currently, the amount of biophotoproduction of hydrogen energy produced is not enough for daily human use although many research centers are available throughout the world (Mao *et al*, 1986; Sasikala *et al*, 1990; Hustede *et al*, 1993; Eroglu *et al*, 1999).

Hydrogen is one of the most basic raw materials in the chemical industry. It is used in large quantities as a reduction gas in refining processes, and as a feedstock gas for ammonia/methanol synthesis. It is also widely used as a reduction gas in manufacturing semiconductors, glass and ceramics, and as an ambient in the metal industry (Turkarslan, 1999).

Hydrogen is a clean and highly efficient fuel, since it returns to water after burning without generating any pollutant. The fossil fuels (coal, oil, and natural gas) are presently the main energy sources of the industrial world. The resources of fossil fuels, however, are limited. In addition to that, the deleterious environmental factors associated with burning fossil fuels and even nuclear energy have forced the world to search for alternative energy sources. As a logical consequence of these arguments, the feasibility of energy economy based on hydrogen energy has been widely considered (Turkarslan, 1999).

The photosynthetic bacteria are aquatic gram-negative organisms found in a wide range of environments, including marine and fresh water systems. This type of bacteria is able to utilize solar energy for the fixation CO_2 and N_2. They have one photosystem (a bacteriochlorophyll) and perform anaerobic photosynthesis associated with hydrogen production (Pfenning and Truper, 1981). Several species of microorganisms that have been found to produce hydrogen include chemotrophs and phototrophs. The phototrophic organisms include cynobacteria, photosynthetic bacteria (purple green bacteria) and green algae. Rates of hydrogen production can vary greatly in different species. Screening of photosynthetic bacteria from different ecosystems may provide suitable hydrogen producers. Thus, more research should be undertaken with different kinds of bacterial strains, culture media and growth conditions.

Anaerobic hydrogen production by photosynthetic bacteria has several advantages over other microorganisms (Gunduz *et al*, 2000). Among the photosynthetic bacteria, *R. sphaeroides* is the most promising one

because of its high activity in hydrogen production under anaerobic conditions (Yigit et. al, 1999).

Enzymes that catalyze hydrogen production in photosynthetic bacteria are nitrogenase and hydrogenase systems (Turkarslan, 1999 and Gunduz *et al*, 2000). Electrons utilized by hydrogenase for the reduction of protons come from organic carbon substrates such as, lactate, malate and others. This reaction is activated by light energy. Nitrogenase system reduces the molecular nitrogen into ammonia, a reaction that is also associated with hydrogen gas production. The physiological substrate for nitrogenase is nitrogen. However, it is versatile and can reduce a variety of nitrogen sources, e.g., glutamate (Turkarslan, 1999).

H. halobium is also a photosynthetic bacterium, which belongs to the *Halobacteriaceae* family. In the purple membrane (pm) of the bacteria, the retinal protein bacteriorhodopsin (br) acts as a light driven proton pump (Zabut, 1997a). *H. halobium* cells show proton pumping activity upon illumination, however, illumination of isolated pm fragments cause br to release proton from the extracellular side of the membrane and uptake proton from the intracellular side (Zabut, 1997b*). H. halobium* lacks both hydrogenase and nitrogenase systems that are essential for production of molecular hydrogen. Therefore, a proton reduction system should be coupled with *H. halobium* for hydrogen production (Khan and Bhatt, 1992).

The main aim of this preliminary study was to construct a photoreactor for hydrogen production by *R. sphaeroides* O.U. 001 and to study the effect of adding *H. halobium* cells on the capability of hydrogen gas production of this bacterium.

MATERIALS AND METHODS

Pre-activation of *R. sphaeroides* O.U. 001

Anaerobic preactivation of *R. sphaeroides* O.U. 001 was performed in a medium containing L-malic acid/ sodium glutamate in 7.5/10 molar ratio (medium I), vitamins and trace elements solution as described by Bieble and Pfenning (1981). Illumination of the preactivated bacteria was provided for 48 hrs by a 1000 W tungsten lamp (Philips) placed 20 cm from the culture bottle.

Construction of a Column Photoreactor

Photoproduction of hydrogen was studied in a glass column photoreactor with a water jacket. The volume of the reactor is 300 ml with a height of 35.5 cm, an inner diameter of 4.0 cm and an outer diameter of 5.5 cm. The reactor was filled by a culture medium containing L-malic acid/sodium glutamate in 15/2 molar ratio (medium II) enriched also with

vitamins and trace elements (Bieble and Pfenning, 1981). The reactor, then, closed tightly with rubber stopper and together with other connected parts was autoclaved at 120 ^{0}C for 20 min. The final volume of culture medium II in the reactor was ~ 240 ml. After autoclaving the setup was constructed and sealed with silicone to prevent any leakage. The Photoreactor and its connected parts are illustrated in Figure 1. Under anaerobic conditions, ~20% (50 ml) of pre-activated culture was inoculated into the reactor. The temperature was maintained at 32^{0}C by using circulating water. Illumination was provided by a 150 W lamp (Philips) from a 40-cm distance. About 3 ml samples were collected at 12 hr intervals from the sample outlet while the reactor was flushed by argon gas to maintain complete anaerobic conditions. The pH and optical density (OD) at 660 nm of samples were measured. The evolved gas was collected in a gas measuring burette by reversible displacement of water. The hydrogen gas produced was analyzed by gas chromatography (Hewlett Packered 5890, series II).

Effect of Adding *H. halobium* on Hydrogen Production.

Preparation of *H. halobium*

H. Halobium cells (strain S-9) were grown according to the method of Oesterhelt and Stoeckenius (1974). Growth was followed by measuring the OD of cells at 660 nm. The cells were left to grow for 7 days. The cell pellets were collected by centrifugation at 6600 rpm for 20 min. in a Sorval centrifuge (GSA rotor). The centrifugation was repeated once after washing the cell pellets by sterile medium II. The packed cells were then weighed, suspended in medium II and shaked well until a homogenous suspension was obtained.

Addition of *H. halobium*

In this stage of the study, three identical photoreactors, constructed in parallel, were used. The volume, preparation and other characteristics of bioreactors were as described above. When the anaerobic growth of *R. sphaeroides* O.U. 001 inside the bioreactors was in stationary phase, 0.3 g and 0.6 g of the *H. halobium* packed cells (each suspended in 40 ml of sterile medium II) were added to two of the reactors and the third reactor served as a control. For the control, when the growth of the bacteria was in stationary phase, 40 ml of sterile medium II were added. All operations were carried out under strict anaerobic conditions.

RESULTS AND DISSCUSION

R. sphaeroides O.U. 001 was grown under sterile and anaerobic conditions in the minimal medium of Bieble and Pfenning supplemented

with malic acid, sodium glutamate and vitamin solution (Thiamin, Niacin, and Biotin). The initial pH of the medium was 7.0. Anaerobic conditions were maintained through flushing out the medium by argon gas. Tungsten lamp was selected for illumination since anaerobic growth was the best as compared to other light sources (Arik *et al*, 1996). The growth characteristics of the bacteria in the column photoreactor were as illustrated in Figure 2. The growth of *R. sphaeroides*, under the employed conditions, starts after a lag phase of about 20 hrs. The pH as well as the OD_{660} in the reactor increased. The increase in OD_{660} (Figure 3-B) is due to the growth of bacteria while the increase in pH (Fig. 3-A) is due to the hydrogen gas formation by hydrogenase enzyme system. The hydrogen gas production started at about 26 hrs and continued until about 100 hrs as is indicated in Figure 3-C. The total volume of hydrogen produced is 360 ml. The rate of hydrogen production is 1 micromole/liter-min. *R. sphaeroides* produces hydrogen gas both in exponential and stationary phases of growth. However, our results showed that most hydrogen was produced in the stationary phase and these results are consistent with those reported by other authors (Arik *et al*, 1996; Gunduz *et al*, 2000).

In the second stage of the experiments, the effect of adding *H. halobium* on rate of hydrogen production by *R. sphaeroides* O.U.001 at stationary phase was studied. Figure 3 shows the effect of addition of 0.6 g of *H. halobium*. The hydrogen production ceased for 2 hrs and then restarted again. During hydrogen production both the OD_{660} and the pH increased. *R. spaeroides* produces hydrogen in the presence and absence of *H. halobium*. However, the rate of hydrogen production increased with addition of *H. halobium* cells, whereas the total amount of hydrogen produced is not significantly changed. This implies that *H. halobium* presence may enhance the production rate of hydrogen. The rate of hydrogen production before and after addition of *H. halobium* was determined to be 1.461 micromole/ liter-min. and 1.735 micromole/ liter-min, respectively. The rate of hydrogen production by this coupled system was about 16% higher than that of *R. sphaeroides* system alone.

Figure 4 shows the effect of addition of 0.3 g of *H. halobium* to the biophotreactor. Also the hydrogen production ceased for 2 hrs and restarted again. The rate of hydrogen production before and after addition of *H. halobium* was 1.287 micromole/ liter-min. and 1.478 micromole/ liter-min, respectively. In this case the rate of hydrogen production has increased by almost 13 % as compared to that of *R. sphaeroides* alone.

Figure 5 shows the effect of addition of *H. halobium* free medium to the biophotreactor. The hydrogen production also ceased for 2 hrs and restarted

again. The rate of hydrogen production before and after addition of *H. halobium* cell-free medium (medium II) was 1.365 micromole/ liter-min. and 1.500 micromole/ liter-min., respectively. The control experiment shows that the cessation of hydrogen production for a short period was due to the addition of the medium. Moreover, it seems that there is a slight increase in the rate of hydrogen production by about 9%, due to the addition of the fresh medium. The difference in the increased rate of hydrogen production in the two columns to which *H. halobium* was added, as compared to the control, is due to the presence of *H. halobium*. In a separate experiment, *H. halobium* shows proton releasing activity upon illumination and uptake when the light is switched off (data is not shown).

Currently, it is not clear whether there is a difference in hydrogen rate production between *R. sphaeroides* systems coupled to H. halobium and those coupled to pm fragments. In a study carried out by Kaya (1995) no difference was observed in hydrogen-producing systems of *E. coli* coupled to pm alone or coupled to *H. halobium*. However, the use of packed cells of *H. halobium* seems much more economical since there is no need for the isolation of pm. The slight increase in the rate of hydrogen production in the presence of *H. halobium* is due to the proton releasing activity of pm fragments of the bacterium under continuous illumination.

Conclusion

The results of this preliminary study of coupling of *H. halobium* with *R. sphaeroides* show slight increase in the rate of hydrogen production by the latter. Further studies are recommended for investigating the significant effect of different concentrations of. *H. halobium* on the hydrogen production ability of R. sphaeroides.

Acknowledgement: Experimental part of this work was carried out at METU, Ankara, Turkey during summer 2000. Many thanks are extended to Prof. Meral Yucel (head of hydrogen group) and her colleagues at METU for permitting us to use their facilities.

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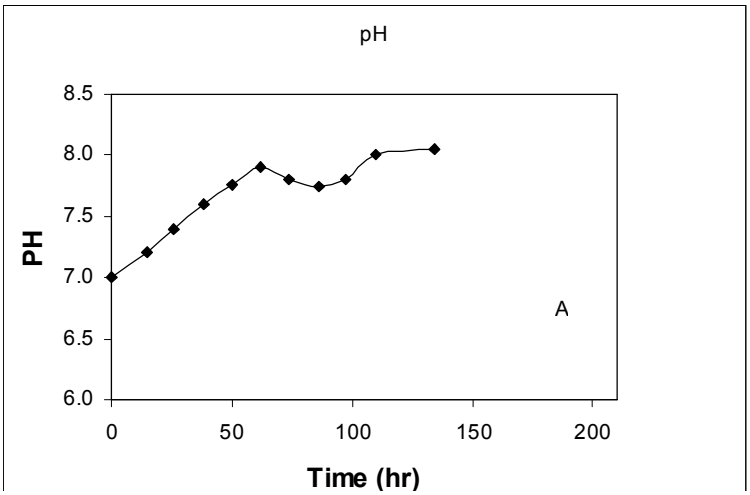
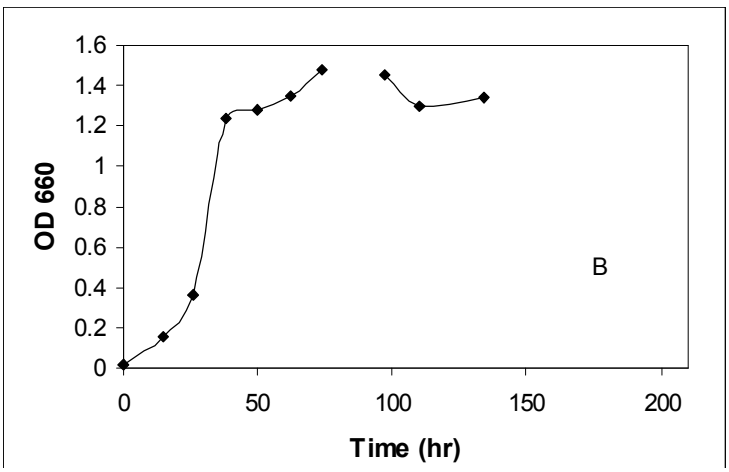
Photoproduction of Hydrogen by Rhodobacter sphaeroides





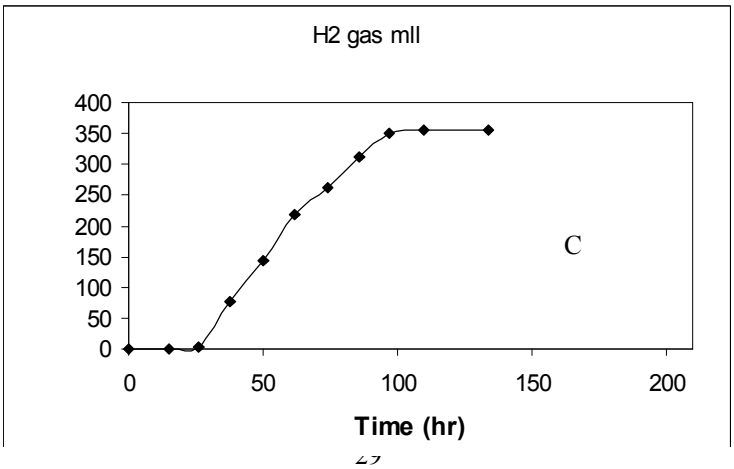
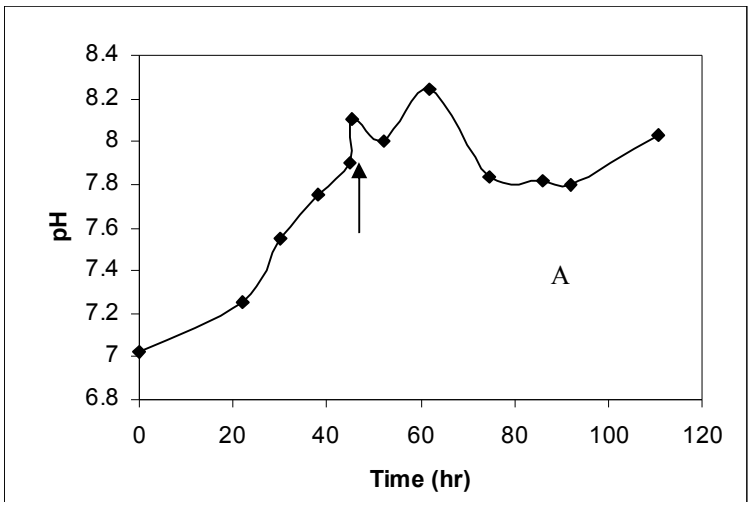
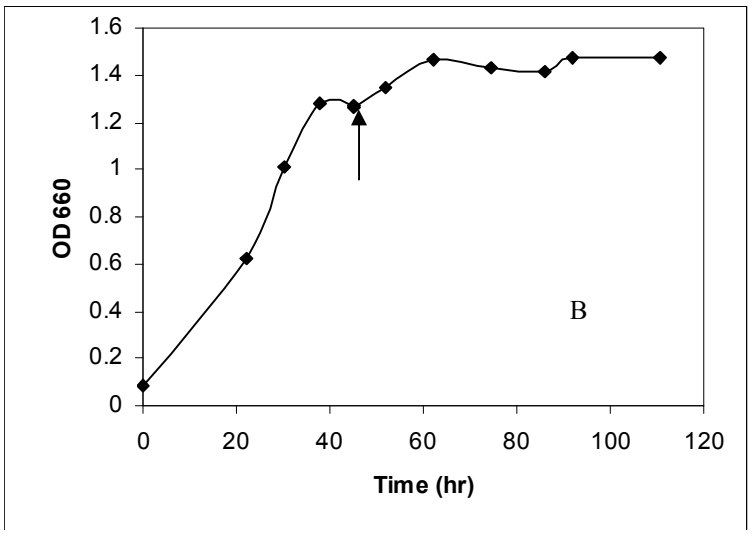


Figure 2: pH, growth, and total amount of hydrogen gas produced by *R. sphaeroides* O.U. 001.

Photoproduction of Hydrogen by Rhodobacter sphaeroides





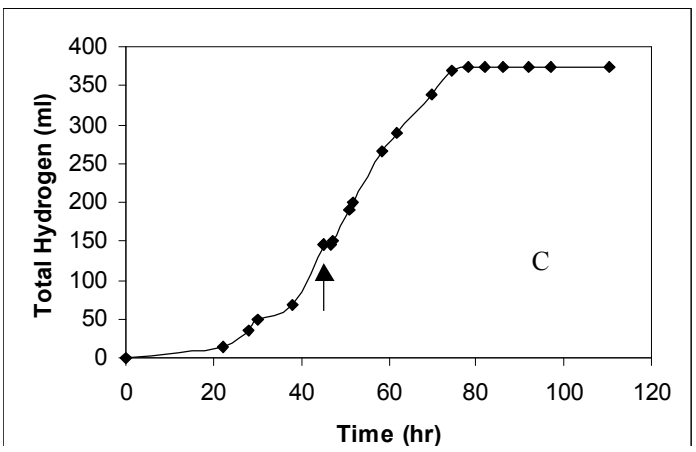
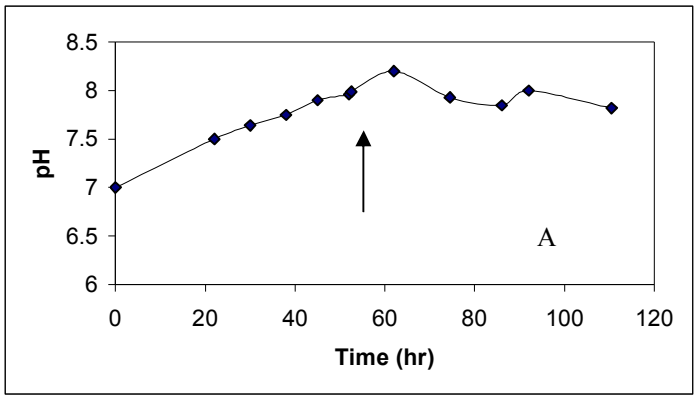
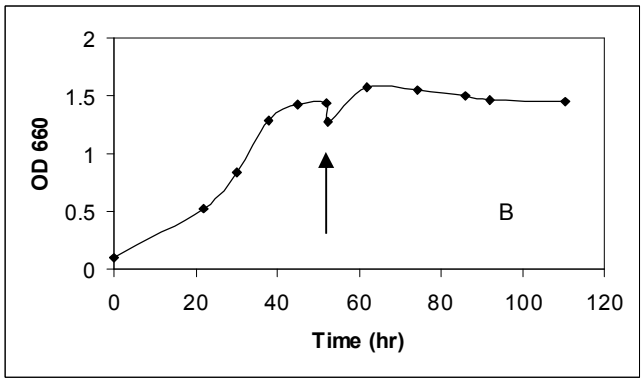


Figure 3: The effect of addition of 0.6 gm *H. Halobium* packed cells on pH change, growth, and total amount of hydrogen gas produced by *R. sphaeroides* O.U. 001 strain (arrows indicate addition of *H. halobium*).





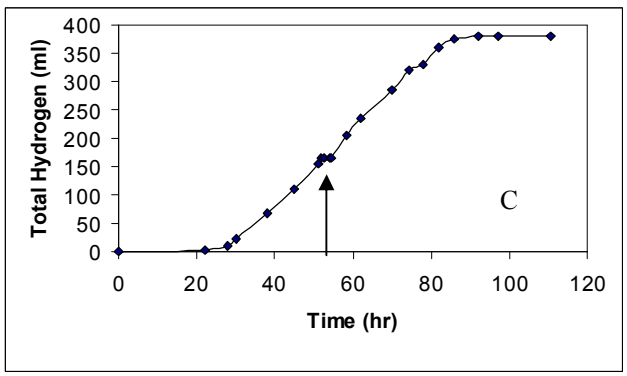


Figure 4: The effect of adding 0.3 g of *H. Halobium* packed cells on pH, growth, and total amount of hydrogen gas produced by *R.sphaeroides* O.U. 001 strain. The arrows indicate addition of *H. halobium*.

Photoproduction of Hydrogen by Rhodobacter sphaeroides

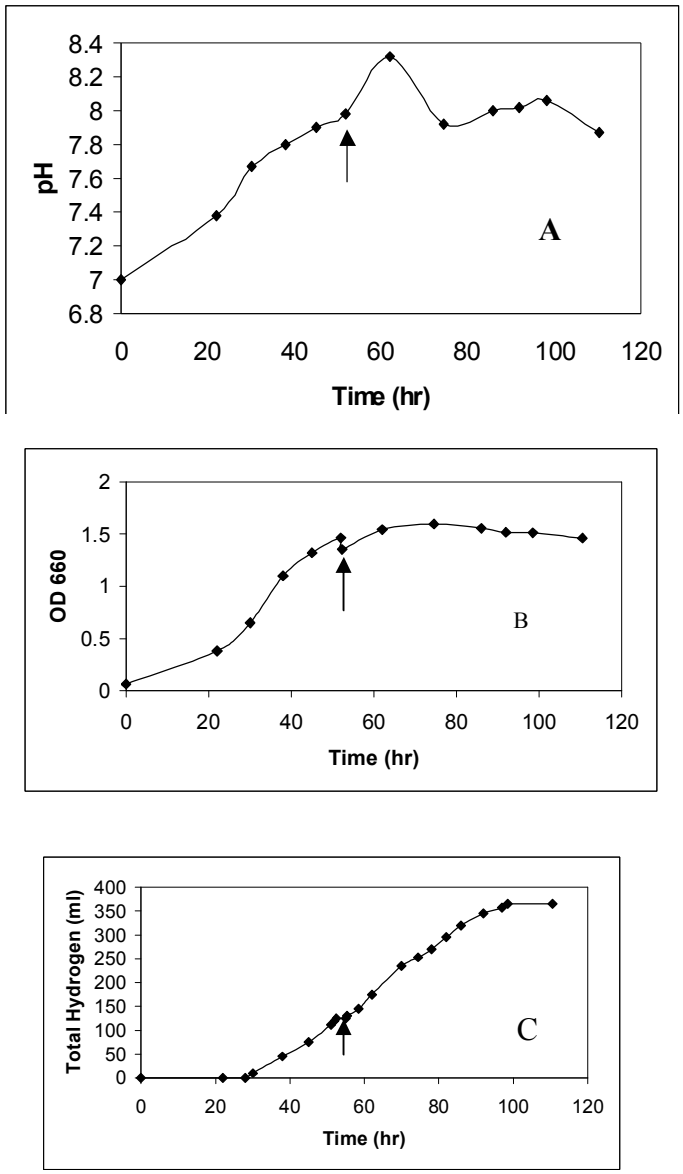


Figure 5: pH, growth, and total amount of hydrogen gas produced by *R.sphaeroides* O.U. 001 strain. The arrows indicate the addition of *H. Halobium* cell-free medium.