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ENHANCED BIOPRODUCTION OF HYDROGEN BY ALGINATE IMMOBILIZED CELLS OF A PURPLE NON-SULPHUR BACTERUM *RHODOCYCLUS TENUIS KU 017*.

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ABSTRACT

Preliminary investigations on photoproduction of hydrogen by free viable cells of *Rhodocyclus tenuis* KU 017, and immobilized cells in sodium alginate matrix, under different cultural conditions with various carbon and nitrogen sources were carried out. Growing cells produced more amount of hydrogen under aerobic dark than aerobic light conditions. In contrast, under anaerobic conditions, hydrogen production was more in light than dark. Actively growing cells were more efficient and sustained hydrogen production for longer period than resting cells. Resting cells preferred light anaerobic conditions for hydrogen production. Resting cells required more lag period for hydrogen production, while growing cells produced more hydrogen during early phase of growth. Immobilization of bacterial cells not only enhanced hydrogen production but enabled the cells to produce hydrogen over an extended period. Among carbon sources formate induced maximum production of hydrogen at 96h and among nitrogen sources ammonium chloride induced maximum production at 192h incubation period. Lactate and L-tyrosine were poor carbon and nitrogen sources respectively for hydrogen production.

KEYWORDS: ***Rhodocyclus tenuis*, Immobilization, Hydrogen production, Light/dark, Anaerobiosis/aerobiosis.**

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INTRODUCTION

Modern power generation is based on the use of natural sources of fuel viz, coal, oil and natural gas. All over the world consumption of power is increasing while the natural sources are depleting fast and at the same time production costs are increasing. The result is a wide and unbridgeable gap between production and consumption in foreseeable future[1]. Development of new technologies allowing clean and renewable energy is one of the main aims of biotechnology and chemical industry. In this context, hydrogen is looked upon as one of the most promising fuels of 21st century. Serious attempts are being made to explore the microbiological methods of hydrogen production[2]. The advantage with these organisms is that they produce clean and efficient hydrogen fuel utilizing solar energy and waste waters. Among different photosynthetic microorganisms, anoxygenic purple non-sulphur bacteria produce molecular hydrogen under a wide range of environmental conditions[3]. High rate of hydrogen production for longer periods of time by immobilized cells was reported in many photosynthetic purple non-sulphur bacteria[4]. Advantages of application of phototrophic cells in immobilized form *vis-a-vis* their free state were previously demonstrated by Tsygankov (1994)[5]. Various immobilization techniques are being thoroughly investigated all over the world to improve the yields and to stabilize photoproduction of hydrogen by whole cells[6,7]. In this regard, a variety of gels were used to immobilize the bacterial cells and to enhance hydrogen production several fold[8]. In the present communication, we report bioproduction of hydrogen by alginate immobilized cells of *Rhodocyclus tenuis* KU 017 and compared with that of free cells. Further, the effect of different carbon and nitrogen sources on the photoproduction of hydrogen by immobilized cells under aerobic/anaerobic and light/dark conditions was also investigated.

MATERIALS AND METHODS

ORGANISM

Rhodocyclus tenuis KU 017 isolated from paper industry effluents, Kaghaznagar, Andhra Pradesh, was used in these investigations.

CULTURE MEDIA AND GROWTH CONDITIONS

Pure culture of *Rc. tenuis* was maintained in Biebl and Pfennig's (BPM)[9] medium containing in mg/lit medium KH_2PO_4, 500; Mg SO_4.7 H_2O, 200; NaCl, 400; NH_4Cl, 400; $CaCl_2.2H_2O$, 50; carbon source (citrate), 1000; yeast extract, 200; ferric citrate solution (0.01g/l) 5 ml; membrane filtered trace element solution (0.01g/l) 1 ml and cyanocobalamin (Vit B_{12} solution 1.0 mg/100 ml) 1 ml; distilled water 1000 ml. Trace element solution contained (mg/lit) : $ZnCl_2$, 70; $MnCl_2$.$4H_2O$,100; H_3BO_3, 60; $CaCl_2$ $6H_2O$, 200; $NiCl_2$. $6H_2O$, 20; $CuCl_2$. $2H_2O$, 20; $NaMO_4$. $2H_2O$, 40 and HCl (25% v/v) 1 ml. The pH of the medium was adjusted to 6.8-7.0 with the help of 2M HCl/2M NaOH. In one experiment, equivalent amounts of formate, lactate, glucose and sorbitol were added separately as substitutes for carbon source (citrate) and similarly in another experiment, thiourea, L-tyrosine and L-glutamic acid were added as substitutes for nitrogen source (NH_4Cl). The respective media were sterilized at 15lbs pressure for 15 min. and after sterilization membrane filtered trace elements and vitamin solution was added aseptically. The media were inoculated with organism and incubated at 30 ± 2^0C for 168 to 240 h under aerobic/anaerobic, and light/dark conditions as the case may be.

PREPARATION OF GROWING CELLS

Cultures of *Rc. tenuis* in logarithmic phase were inoculated (1% v/v) into basal medium containing different carbon sources (1%) along with ammonium chloride (0.5%) as nitrogen source. Similarly, when different nitrogen sources were tested, citrate was used as a source of carbon.

PREPARATION OF RESTING CELLS

Cells of *Rc. tenuis* grown in BPM medium containing citrate and ammonium chloride as carbon and nitrogen sources respectively until mid-log phase were harvested by centrifugation at 10,000xg for 15min. The resultant pellet was washed thrice in saline (0.3% w/v in distilled water) and suspended in basal medium. The cultures were grown anaerobically in fully filled screw cap tubes (15ml) and incubated under 2000 lux light or dark conditions at 30±2^{0}C.

HYDROGEN PRODUCTION STUDIES OF FREE AND IMMOBILIZED CELLS

The basic technique used in the assay of hydrogen production was that of Vincenzini *et al.* (1982)[10]. Five ml of bacterial culture was harvested by centrifugation at 10,000xg for 10 min, washed thrice with 0.3% saline and the cells were suspended in the basal medium devoid of carbon source/electron donor and nitrogen source. Depending on the experimental conditions, different electron donors and nitrogen sources were added at required concentrations. To test the hydrogen production ability, the washed cell suspension was inoculated into 8ml of the medium in 15ml capacity rimless test tubes sealed with subaseals and anaerobic conditions were created by evacuating and flushing with 100% nitrogen. Alginate immobilized beads were prepared by dropping bacterial-alginate suspension (prepared by mixing washed bacterial suspension with 3% sodium alginate solution at 1:1 ratio) into calcium chloride (2%w/v) solution through a syringe[4]. Beads (2-3mm) thus obtained were washed repeatedly (after 1h of curing in calcium chloride solution) with sterile distilled water and used for hydrogen production assay. Precautions were taken to maintain the biomass of the free and immobilized cells identical. Hydrogen produced was measured by injecting 0.5ml of the gas phase from the reaction vessels with an air tight syringe into a gas chromatograph (Mak Analytica, India make) fitted with a molecular sieve 5A column (2Mx1/8" ODSS) to a thermal conductivity detector (TCD). Gas analysis was done with oven temperature at 60^{0}C with argon as carrier gas (flow rate 30ml/min), 120mA detector current. Integrator and recorder were used at highest sensitivity. Before withdrawing each sample for assay, 0.5 ml of nitrogen was injected into the vessel to maintain positive pressure. The amount of hydrogen liberated by the photosynthetic bacterium was calculated from the peak height of the recorder with reference to calibration curve prepared using ultra pure hydrogen. The results obtained were subjected to statistical analysis using SPSS package 12.0 version. The results are presented in table 1 and figures 1-8.

RESULTS AND DISCUSSION

HYDROGEN PRODUCTION BY GROWING CELLS

A critical perusal of Table 1 reveals that *Rc. tenuis* started hydrogen production from 24h of incubation and it continued to produce up to 168h. Anaerobic conditions appear to be more favorable than aerobic conditions. Similarly, light conditions were more favorable for hydrogen production than dark conditions. The growing cells produced hydrogen up to only 96h under aerobic light condition however, under aerobic dark conditions they continued to produce hydrogen up to the end of observation period with a maximum production at 96h of incubation. Growing cells exposed to anaerobic light conditions produced maximum amount of hydrogen (1.71±0.32) by 120h of incubation period. Under aerobic dark and anaerobic dark conditions 96h of incubation period was found to be optimum. On the other hand, cells exposed to anaerobic light conditions ceased to produce hydrogen after 144h incubation.

HYDROGEN PRODUCTION BY RESTING CELLS

Resting cells also preferred anaerobic conditions over aerobic conditions. Interestingly, light conditions were more favorable than dark conditions for resting cells. The production of hydrogen by resting cells was comparatively less than those of actively growing cells.

HYDROGEN PRODUCTION BY IMMOBILIZED CELLS

It is also evident from table-1 that the immobilized cells also preferred aerobic dark condition over aerobic light. Under aerobic light conditions, bacterial cells produced hydrogen upto 120h with a peak around 48h and subsequently the production declined. However, the cells exposed to aerobic dark conditions produced increasing amount of hydrogen upto 144h of incubation. Immobilized cells preferred anaerobic light conditions over anaerobic dark. Thus, the cells of *Rc. tenuis* behaved differently with the growth stage of cells and prevailing aerobic/anaerobic and light/dark conditions. Such response was also reported with several bacteria studied by Sasikala and Ramana (1995)[11]. Dependence of phototrophic bacteria on light for hydrogen production was also demonstrated by several workers[12,13]. Immobilization of cells has shown a significant enhancement effect on hydrogen production (fig. 1-4). However the degree of stimulation varied with the carbon source available. Formate induced more amounts of hydrogen both under aerobic and anaerobic light conditions with the progress of incubation period (Fig 1), while hydrogen production was significant only during early phase of incubation under dark aerobic and anaerobic conditions. Similarly, aerobic light was more favorable than dark aerobic conditions. In lactate medium also aerobic light was more conducive over dark aerobic conditions (Fig 2). Similar trend was observed under anaerobic light condition under the influence of glucose (Fig 3). *Rc. tenuis* produced more hydrogen when it was exposed to aerobic light conditions than the aerobic dark conditions. However, during later part of the incubation, dark aerobic conditions were more conducive than light aerobic for hydrogen production in the presence of sorbitol (Fig 4). On the other hand, *Rc. tenuis* preferred anaerobic light conditions to dark anaerobic conditions for hydrogen production.

Ammonium chloride enhanced maximum hydrogen production at 192h when the cells of *Rc. tenuis* were immobilized under anaerobic light conditions (Fig 5). Similarly, anaerobic dark conditions were preferred than aerobic dark. Thiourea also induced more hydrogen under aerobic light than aerobic dark (Fig 6). Tyrosine also induced hydrogen upto the end of the incubation period with maximum production at 192h (Fig.7). Anaerobic light conditions were preferred over anaerobic dark. L-glutamic acid also induced good amount of hydrogen with maximum production at 168h (Fig 8). Anaerobic light and aerobic dark conditions were more favorable than dark conditions. Among all the nitrogen sources investigated, ammonium chloride followed by L-tyrosine was more favorable for hydrogen production. L-glutamic acid and thiourea were the next preferred nitrogen sources for production of hydrogen under different conditions. The present investigations clearly demonstrate that growing cells produce comparatively more hydrogen than resting cells. It was well established that growing cultures under constant anaerobic conditions normally produce hydrogen at higher rates than those by resting cells[14]. The utilization of substrate into hydrogen is relatively less since a part of the substrate must have been used for the synthesis of cellular constituents. Segers and Verstraete (1983)[15] observed a conversion efficiency of 78% for *Rhodomicrobium vannielii* metabolizing lactate during first ten days. However, during next ten days when the cells were in non-growing stage, a conversion efficiency of 100% was noted. These observations lead them to conclude that resting cells are more efficient in hydrogen production. Thus there is a need to optimize the conditions for both the growing and resting cells. Anaerobic conditions were more favorable than aerobic conditions. Growing cells produced more amount of hydrogen under aerobic dark conditions, while anaerobic light conditions induced more amount of hydrogen and reverse was true with resting cells. The efficiency of hydrogen production by *Rc. tenuis* increased several fold when they were immobilized. Immobilization also resulted in increased production of hydrogen under aerobic and anaerobic dark condition and anaerobic light. Enhancement in the hydrogen evolution by alginate immobilized whole cells of *Rc. tenuis* compared with that of free cells was also reported by several workers[7,16].

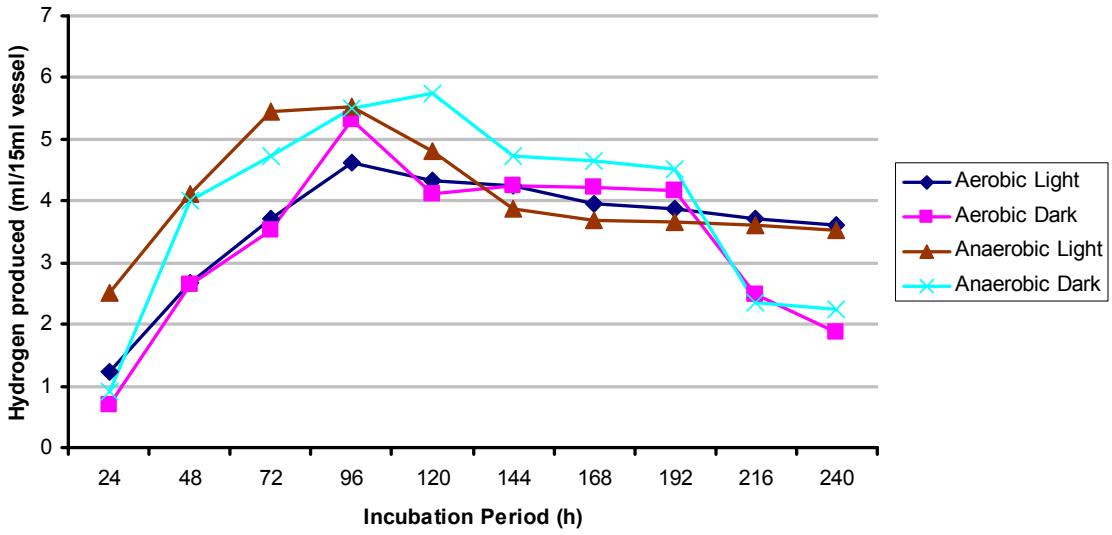

Figure 1
Influence of formate on photoproduction of hydrogen by immobilized cells of Rc. tenuis

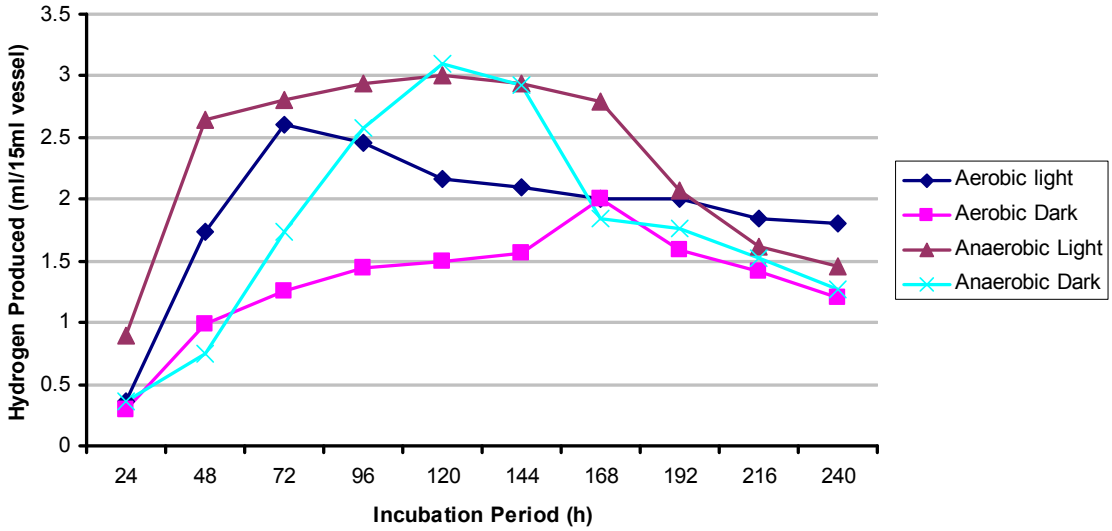

Figure 2
Influence of lactate on photoproduction of hydrogen by immobilized cells of Rc. tenuis

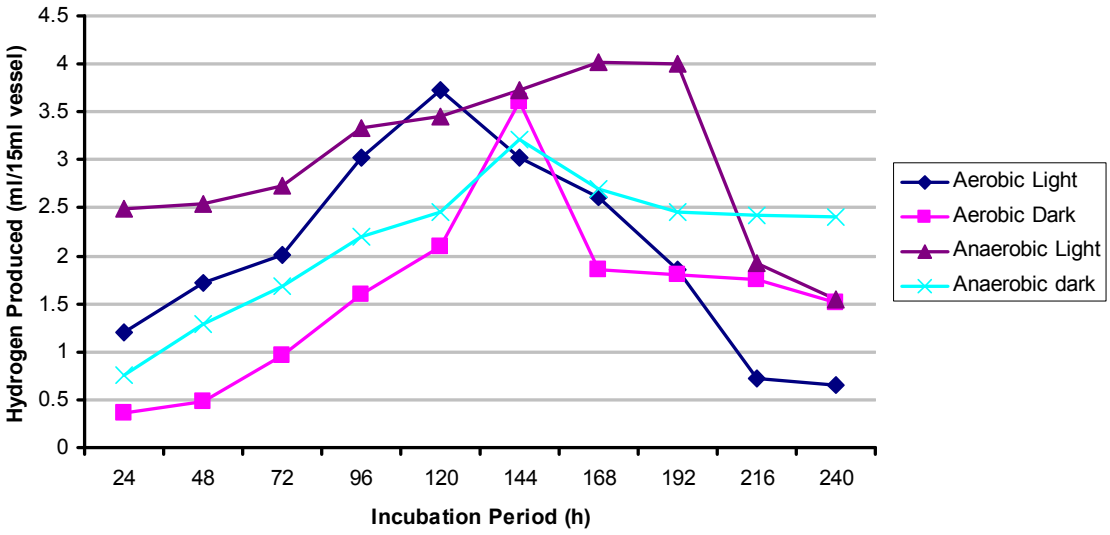

Figure 3

Influence of glucose on photoproduction of hydrogen by immobilized cells of Rc. tenuis

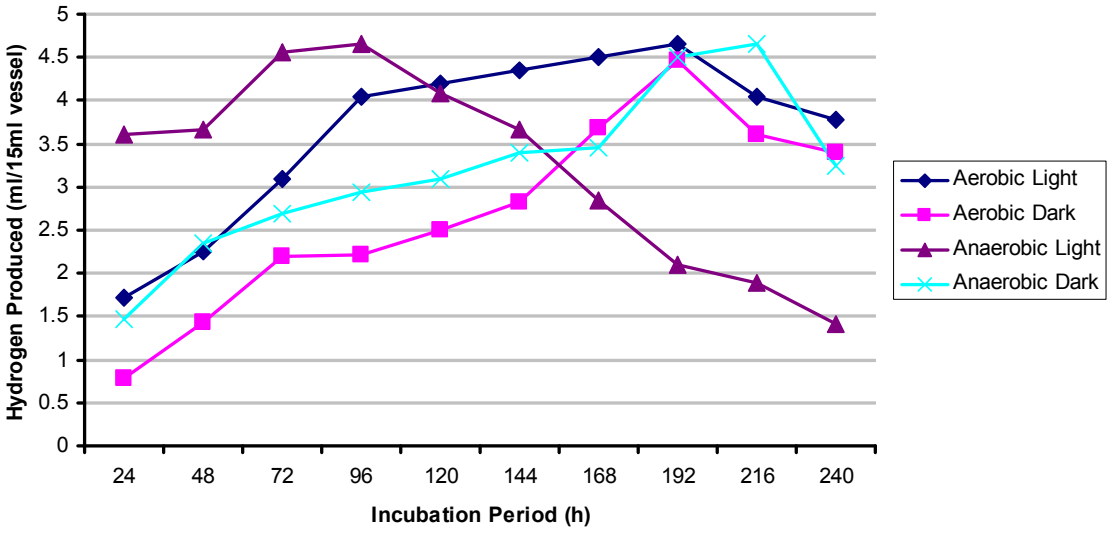

Figure 4

Influence of sorbitol on photoproduction of hydrogen by immobilized cells of Rc. tenuis

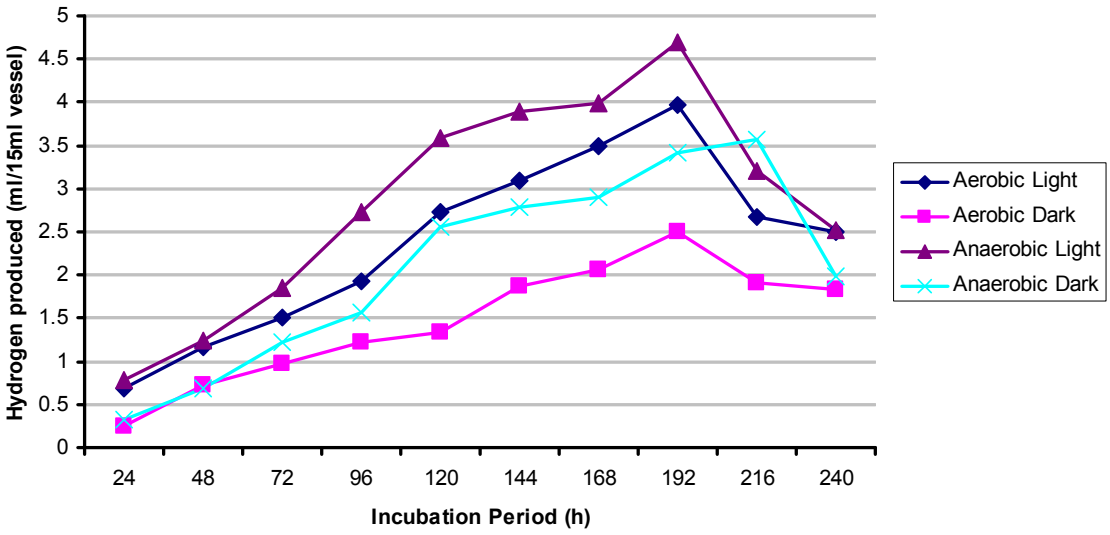

Figure 5
Influence of ammonium chloride on photoproduction of hydrogen by immobilized cells of Rc. tenuis

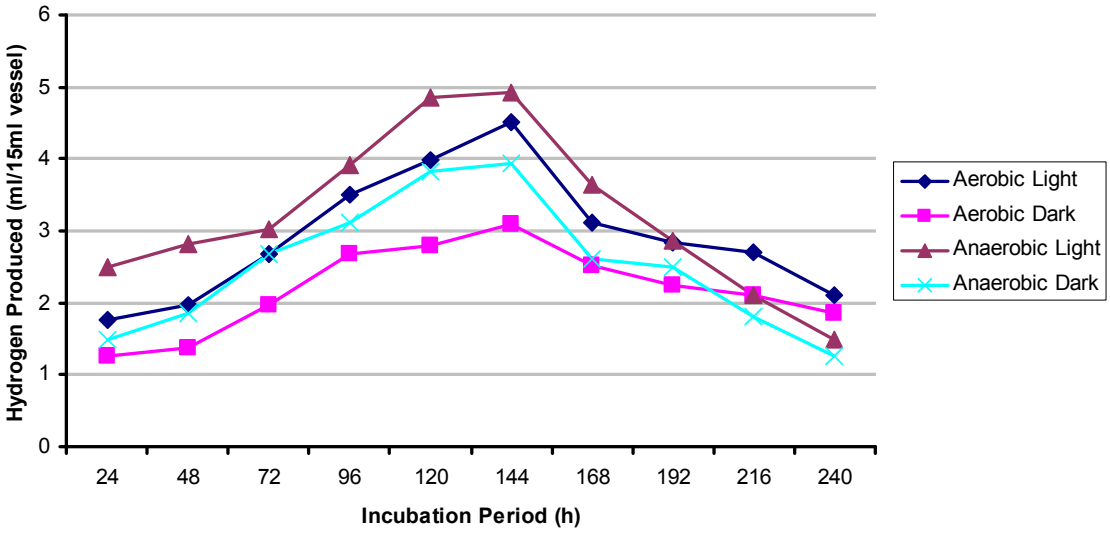

Figure 6
Influence of thiourea on photoproduction of hydrogen by immobilized cells of Rc. tenuis

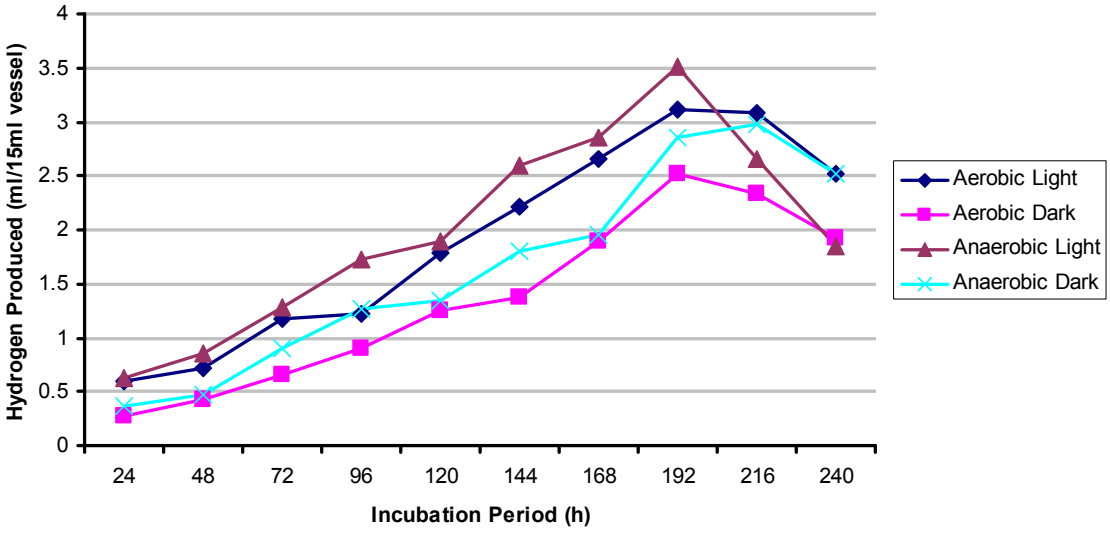

Figure 7
Influence of L-tyrosine on photoproduction of hydrogen by immobilized cells of Rc. tenuis

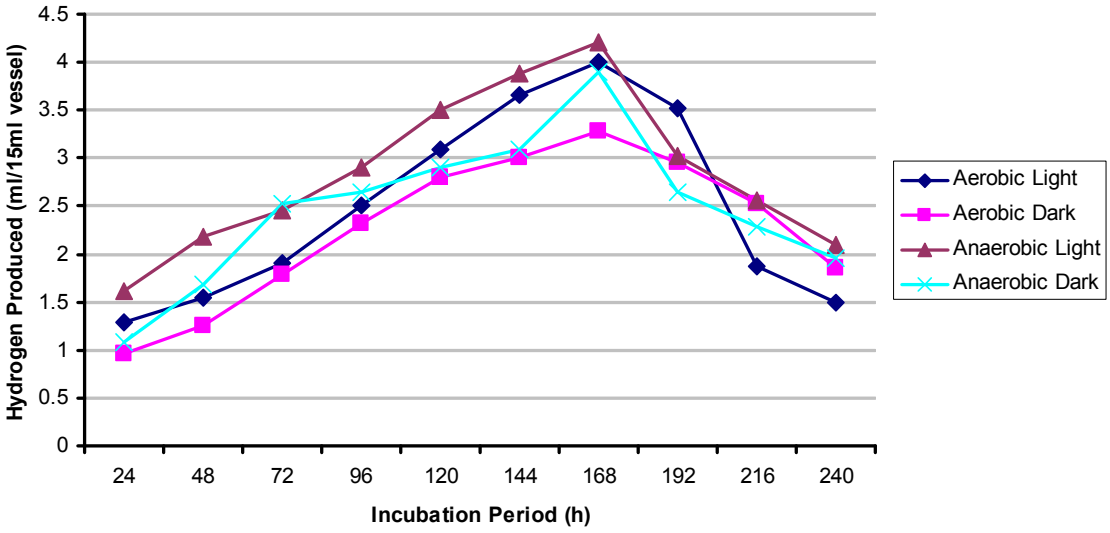

Figure 8
Influence of L-glutamic acid on photoproduction of hydrogen by immobilized cells of Rc. tenuis

Table 1
Photoproduction of hydrogen (ml/15ml vessel) by Rc. tenuis under different cultural conditions

Incubation Period (in h)	Growing cells				Resting cells				Immobilized cells			
	Aerobic		Anaerobic		Aerobic		Anaerobic		Aerobic		Anaerobic	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
24	0.10±0.06	0.08±0.02	0.78±0.08	0.06±0.02	0.11±0.09	0.03±0.01	0.75±0.06	0.01±0.01	0.19±0.02	0.06±0.04	0.04±0.02	0.10±0.02
48	0.12±0.08	0.18±0.06	1.11±0.22	0.18±0.08	0.24±0.08	0.11±0.06	0.08±0.018	0.03±0.02	1.02±0.018	0.45±0.04	1.62±0.14	0.42±0.08
72	0.05±0.02	0.35±0.08	1.20±0.14	0.19±0.10	0.08±0.03	0.10±0.05	1.14±0.32	0.05±0.02	0.57±0.13	0.70±0.08	1.71±0.18	0.50±0.22
96	0.01±0.01	0.54±0.24	1.41±0.18	0.22±0.14	0.06±0.02	0.07±0.03	1.08±0.12	0.07±0.04	0.24±0.10	0.93±0.12	1.56±0.20	0.75±0.18
120	–	0.30±0.14	1.71±0.32	0.16±0.08	0.02±0.01	0.04±0.02	1.00±0.08	0.07±0.02	0.13±0.06	1.47±0.18	1.50±0.14	1.02±0.32
144	–	0.18±0.08	1.62±0.28	0.13±0.08	0.02±0.01	–	0.50±0.06	0.03±0.01	–	1.56±0.28	1.20±0.88	0.48±0.12
168	–	0.16±0.06	0.10±0.04	0.12±0.06	–	–		0.02±0.01	–	1.49±0.30	1.00±0.08	0.32±0.08
F-values*	1.588235	0.320523	0.080775	0.014126	2.581894	0.01	4.547002	0.099338	0.002615	0.115864	1.085103	0.114126

–= No hydrogen production
**The F-values are means of the triplicates*

CONCLUSION

Thus the present investigations clearly indicate that immobilized cells of *Rc. tenuis* KU 017 produce more hydrogen than growing and resting cells. However, in between resting cells and growing cells, the later supported more hydrogen production. In all the cases, anaerobic light conditions favored more hydrogen production than aerobic conditions. Under aerobic conditions light supported the hydrogen production. Among the carbon and nitrogen sources tested, formate and thiourea supported more hydrogen production.

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