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Influence of Nitrogen Sources on Photoproduction of Hydrogen by *Rhodocyclus tenuis* KU 017 Isolated from Paper Industry Effluents

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ABSTRACT

Influence of different nitrogen sources on photoproduction of hydrogen by *Rhodocyclus tenuis* KU 017 was investigated. Among 16 different nitrogen sources investigated ammonium chloride, thiourea, L-tyrosine and L-glutamic acid were observed to be good sources of nitrogen as they induced maximum production of hydrogen. However, the optimum concentration for hydrogen production varied with the nitrogen source. Anaerobic light and aerobic dark conditions were found to be more favourable than anaerobic dark and aerobic light conditions. Immobilization of bacterial cells not only enhanced hydrogen production but enabled the cells to produce hydrogen over an extended period. Resting cells for hydrogen production required more lag period, while actively growing cells produced more hydrogen during early phase of growth.

INTRODUCTION

Energy is vital to global prosperity, yet dependence on fossil fuels as our primary energy source contributes to global climate change, environmental degradation and health problems (Bockris, 2002). Hence, it became necessary to find alternative forms of energy that can be produced using the non-conventional sources. Among different nonconventional sources hydrogen offers a tremendous potential as a clean and renewable energy. Photoproduction of hydrogen is considered as a promising biological process (Weaver and Seibert, 1980). Biological production of hydrogen has been observed in a large number of microbial species (Patel et al., 2012). Studies on light dependent hydrogen production by blue green algae, photosynthetic bacteria and cell free systems have been reviewed (Kumazawa and Shimamura, 1993).

Photosynthetic bacterial systems possess the maximum efficiency (5%) of all the proposed biological systems in terms of energy efficiency and it equally competes with that of electrolytically produced hydrogen. The efficiency need to be scaled up to 10% if it has to become more competitive with the current price of hydrogen being produced from natural gas or coal (Debabrata Das et al., 2008). Hence the need for intensive research to enhance the efficiency of photosynthetic bacterial systems is clearly identified. The initial observation of hydrogen evolution by photosynthetic bacteria was made by Roelofsen (1934) and Nakamura (1939) who reported the liberation of hydrogen by *Chromatium minutissimum* and *Rhodobacillus palustris* under dark anaerobic conditions. The nature of nitrogen source for the growth of phototrophic bacteria is reported to play an important role in hydrogen production also. Molecular nitrogen enhanced the hydrogen evolution by many photosynthetic bacterial species (Yongzhen et al., 2008; Tekucheva and Tsygankov, 2012). The importance of nitrogen source for photoproduction of hydrogen has also been emphasized by other workers (Lakshmi and Polasa, 2001; Eroglu et al., 2006).

Various immobilization techniques are being explored to enhance and stabilize the photoproduction of hydrogen by photosynthetic bacteria. Recycling of biological hydrogen photoproduction system using immobilized cells has a promising potential in applied bioenergy (Mizuno et al., 2000). Singh et al., 1994 reported that immobilized cells of *Rhodopseudomonas sp*. have evolved more hydrogen (1.5 fold) than the free cells.

Our preliminary investigations proved that Rc. tenuis a photosynthetic bacterium, isolated from paper industry effluents is able to produce hydrogen. In view of significance of nitrogen sources and cell immobilization process on hydrogen production the present investigations were taken up on *Rc. tenuis*.

METERIALS AND METHODS

Organism

Rhodocyclus tenuis KU 017 isolated from paper industry effluents, Kaghaznagar, Telangana state was used in these investigations. The bacterium was enriched by inoculating into the medium and incubating anaerobically in the 2000 lux light intensity. The bacterium thus isolated was identified with the help of cultural characteristics (colour, size and shape) carbon and nitrogen requirements, vitamin requirements, absorption spectral analysis, bacteriochlorophylls and carotenoids and following the key provided in Bergey's Manual of Systematic Bacteriology (Stanley et al., 1989).

Culture media and growth conditions

Pure culture of *Rc. tenuis* was maintained in Biebl and Pfennig's (BPM) (Biebl and Pfennings, 1981) medium [mg/lit medium KH_2PO_4, 500; $MgSO_4$.7 H_2O, 200; NaCl, 400; NH4Cl, 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 B12 solution 1.0 mg/100 ml) 1 ml; distilled water 1000 ml]. Trace element solution contained (mg/lit) : ZnCl2, 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. Influence of incubation period on hydrogen production was monitored. Different nitrogen sources in 15ml of basal medium were substituted for ammonia chloride so as to supply equivalent amounts of nitrogen. The respective media were sterilized at 15lbs pressure for 15 min. and after sterilization membrane filtered trace elements and vitamin solution were added aseptically. The media were inoculated with organism and incubated at 30±2^{o}C for 168 to 240 h under aerobic/anaerobic, and light/dark conditions as the case may be.

Preparation of immobilized cells

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 (Munjam and Jyothi Rani, 2014). 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 production

The basic technique used in the assay of hydrogen production was that of Vincenzini et al. (1982). Five ml of log phase bacterial suspension 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 nitrogen source. Depending on the experimental conditions, different 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 and incubated

under light intensity of 2000 lux and temperature at 30±2°C.

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 Make) fitted with a molecular sieve 5A column (2M x 1/8" ODSS) to a thermal conductivity detector (TCD). Gas analysis was done with oven temperature at 60°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 are presented in tables 1-4 and figures 1-4.

RESULTS AND DISCUSSION

The perusal of table 1 and 2 reveals that among different nitrogen sources investigated thiourea and L-tyrosine were the most favourable nitrogen sources for hydrogen production by *Rc.tenuis*. In contrary sodium nitrate ammonium nitrate and barium nitrate did not to induce hydrogen production. With regard to different cultural conditions, anaerobic light conditions appear to be more favourable than anaerobic dark conditions of the different incubation periods, 120 hrs incubation

Table 1: Effect of nitrogen source on hydrogen production (ml/15ml vessel) by *Rc. tenuis* under anerobic light and dark conditions

Nitrogen source	Condition	Incubation time (in hrs)						
		24	48	72	96	120	144	168
Sodium nitrate	A	0.03	0.08	0.21	0.15	0.12	0.06	—
	B	0.03	0.10	0.15	0.10	0.08	0.06	—
Barium nitrate	A	—	—	—	—	—	—	—
	B	—	—	—	—	—	—	—
Ammonium nitrate	A	—	—	—	—	—	—	—
	B	—	—	—	—	—	—	—
Ammonium chloride	A	0.51	1.02	1.32	1.50	2.07	1.71	1.40
	B	0.02	0.08	0.08	0.06	0.02	0.02	--
Ammonium molybate	A	0.06	0.45	0.60	0.72	0.84	0.99	0.92
	B	0.07	0.10	0.51	0.58	0.42	0.30	0.10
Ammonium sulphate	A	0.21	0.42	0.57	0.81	0.93	0.90	0.82
	B	0.10	0.03	0.08	0.10	1.10	1.12	0.10
Urea	A	0.10	0.15	0.60	0.75	0.84	0.90	0.82
	B	0.02	0.02	0.08	1.10	1.26	1.00	—
Thiourea	A	0.90	1.41	2.25	2.61	3.21	3.72	3.57
	B	0.05	0.12	0.25	0.31	0.95	1.05	1.00
L-aspargine	A	0.05	0.30	0.36	0.40	0.54	0.60	0.52
	B	0.13	0.84	0.24	0.18	0.10	0.05	0.02
L- glutamine	A	0.18	0.30	0.60	0.75	1.14	1.56	1.32
	B	0.08	0.30	0.42	0.48	0.32	0.28	0.12
L-glycine	A	0.33	0.39	0.66	1.02	1.68	1.72	0.62
	B	0.04	0.03	0.48	0.18	0.06	0.01	—
D- arginine	A	—	0.36	0.40	0.68	0.92	1.08	0.90
	B	0.02	0.02	0.08	0.09	0.09	0.03	0.02
DL -alanine	A	0.06	0.51	0.54	0.63	0.75	1.00	0.84
	B	0.01	0.08	0.10	0.12	0.09	0.08	0.05
L- histidine	A	0.06	0.12	0.18	0.20	0.28	0.32	0.18
	B	0.17	0.78	1.05	1.20	1.08	0.05	0.04
L-tyrosine	A	0.60	0.87	1.23	2.31	2.80	3.36	2.99
	B	0.78	0.24	0.69	0.10	0.08	0.07	0.04
L-glutamic acid	A	0.03	0.63	0.84	1.14	1.52	1.62	1.50
	B	0.01	0.03	0.05	0.08	0.08	0.06	—
Control	A	0.69	0.93	1.08	1.20	1.38	1.44	1.17
	B	0.02	0.03	0.03	0.02	0.01	—	—

A = Anaerobic light conditions, B = Anaerobic dark conditions, — = No hydrogen produced

Table 2: Effect of nitrogen source on hydrogen production (ml/15ml vessel) by *Rc. tenuis* under aerobic light and dark conditions

Nitrogen source	Condition	Incubation time (in hrs)						
		24	48	72	96	120	144	168
Sodium nitrate	A	—	—	—	—	—	—	—
	B	—	—	—	—	—	—	—
Barium nitrate	A	—	—	—	—	—	—	—
	B	—	—	—	—	—	—	—
Ammonium nitrate	A	—	—	—	—	—	—	—
	B	—	—	—	—	—	—	—
Ammonium chloride	A	0.03	0.04	0.03	0.02	0.02	0.01	—
	B	0.04	0.08	1.23	1.42	1.08	0.01	—
Ammonium molybate	A	0.02	0.08	0.08	0.06	0.05	0.03	—
	B	0.04	0.06	0.09	0.06	0.02	0.01	—
Ammonium sulphate	A	0.02	0.01	0.01	—	—	—	—
	B	0.02	0.08	0.10	0.10	1.40	1.00	—
Urea	A	0.03	0.09	0.12	0.10	0.06	—	—
	B	0.03	0.10	0.08	1.10	1.26	1.00	0.10
Thiourea	A	0.08	0.10	0.52	0.61	0.75	1.02	0.90
	B	0.08	0.10	0.18	0.16	0.75	0.07	0.03
L-aspargine	A	0.03	0.09	0.03	0.02	—	—	—
	B	—	0.10	0.12	0.32	0.58	0.60	0.47
L-glutamine	A	1.26	0.38	0.06	0.02	0.01	—	—
	B	0.08	0.30	0.42	0.48	0.32	0.28	0.12
L- glycine	A	0.02	0.02	0.01	—	—	—	—
	B	0.03	0.05	0.08	0.10	0.08	0.06	0.02
D-arginine	A	0.02	0.02	0.01	—	—	—	—
	B	0.03	0.06	0.10	1.09	0.09	0.05	0.03
DL - alanine	A	—	—	0.01	0.04	0.03	--	--
	B	0.02	0.03	0.05	0.10	0.20	0.37	0.25
L-histidine	A	0.02	0.03	0.01	—	—	—	—
	B	0.02	0.08	0.08	0.10	0.28	0.41	0.20
L-tyrosine	A	0.02	0.02	0.03	0.02	0.01	--	0.20
	B	0.08	0.10	0.22	0.54	0.28	0.10	0.09
L-glutamic acid	A	0.05	0.08	0.15	0.20	0.75	1.00	0.50
	B	0.02	0.27	0.72	0.87	0.93	0.72	0.32
Control	A	0.03	0.05	0.04	0.03	0.02	0.02	0.01
	B	0.04	0.03	0.03	0.02	0.01	0.01	—

A = Aerobic light conditions, B = Aerobic dark conditions, — = No hydrogen produced

Table 3: Photoproduction of hydrogen (ml/15ml vessel) by immobilized cells of Rc. tenuis under different cultural conditions

Incubation period (in hrs)	Aerobic		Anaerobic	
	Light	Dark	Light	Dark
24	0.19	0.06	0.04	0.10
48	1.02	0.45	1.62	0.42
72	0.57	0.70	1.71	0.50
96	0.24	0.93	1.56	0.75
120	0.13	1.47	1.50	1.02
144	—	1.56	1.20	0.48
168	—	1.49	1.00	0.32

— = No hydrogen produced

Table 4: Effect of different nitrogen sources on photoproduction of hydrogen (ml/15ml vessel) by immobilized cells of *Rc. tenuis*

Nitrogen source	Incubation period (in hrs)	Aerobic		Anaerobic	
		Light	Dark	Light	Dark
Ammonium chloride	24	0.68	0.25	0.78	0.32
	48	1.17	0.72	1.25	0.68
	72	1.50	0.97	1.86	1.22
	96	1.92	1.22	2.72	1.56
	120	2.72	1.34	3.58	2.56
	144	3.10	1.87	3.90	2.78
	168	3.50	2.06	3.98	2.90
	192	3.97	2.50	4.70	3.42
	216	2.68	1.91	3.21	3.56
	240	2.50	1.84	2.52	1.98
Thiourea	24	1.76	1.25	2.50	1.50
	48	1.96	1.38	2.81	1.86
	72	2.68	1.96	3.02	2.68
	96	3.51	2.67	3.92	3.11
	120	3.98	2.80	4.85	3.82
	144	4.50	3.10	4.92	3.95
	168	3.12	2.52	3.65	2.62
	192	2.85	2.24	2.86	2.50
	216	2.70	2.10	2.10	1.80
	240	2.10	1.85	1.50	1.25
L-tyrosine	24	0.59	0.28	0.62	0.36
	48	0.72	0.42	0.85	0.48
	72	1.18	0.65	1.28	0.90
	96	1.22	0.90	1.72	1.26
	120	1.78	1.25	1.90	1.35
	144	2.22	1.38	2.60	1.80
	168	2.65	1.90	2.85	1.95
	192	3.12	2.52	3.51	2.86
	216	3.08	2.33	1.85	2.52
	240	2.52	1.93	1.85	2.52
L-Glutamic acid	24	1.28	0.97	1.62	1.08
	48	1.55	1.25	2.18	1.69
	72	1.90	1.78	2.45	2.52
	96	2.50	2.32	2.90	2.65
	120	3.10	2.80	3.50	2.90
	144	3.65	3.00	3.89	3.10
	168	4.00	3.28	4.21	3.90
	192	3.52	2.96	3.02	2.65
	216	1.88	2.52	2.56	2.28
	240	1.50	1.85	2.10	1.96

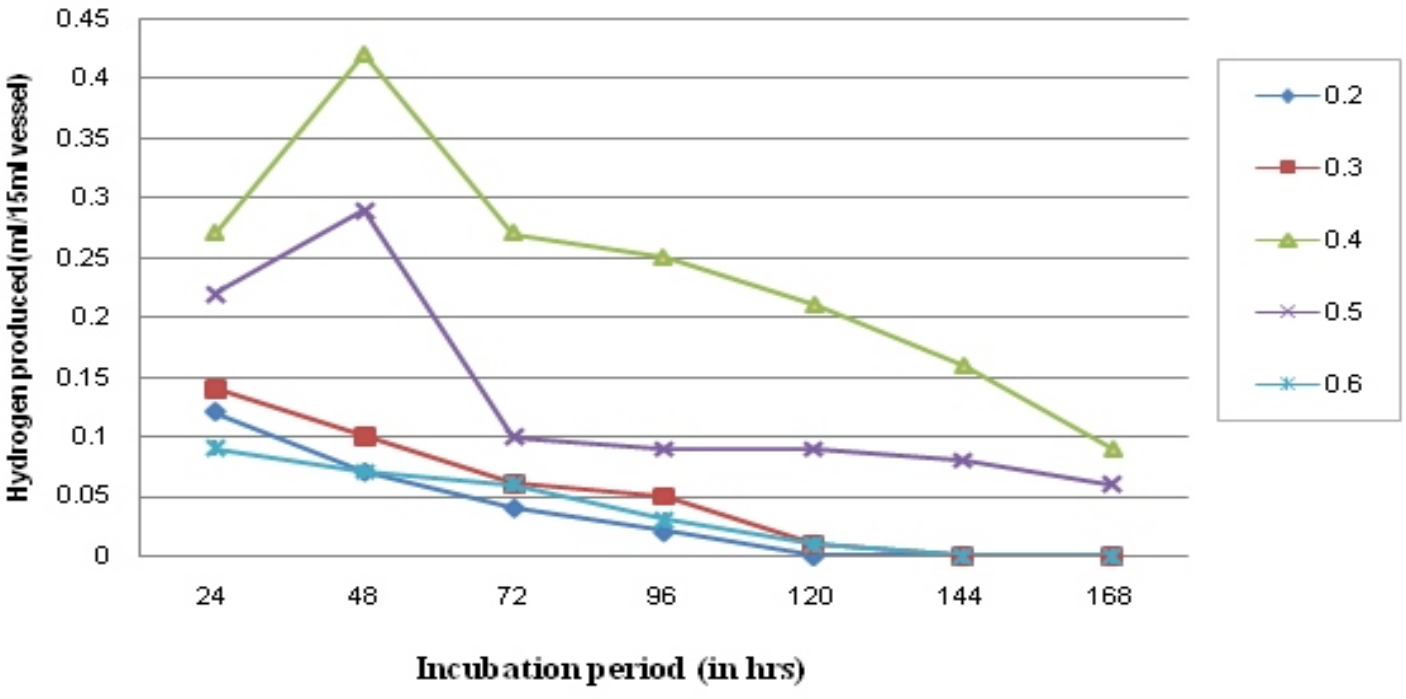

Fig.1: Influence of different concentrations of ammonium chloride on photoproduction of hydrogen by *Rc. tenuis*

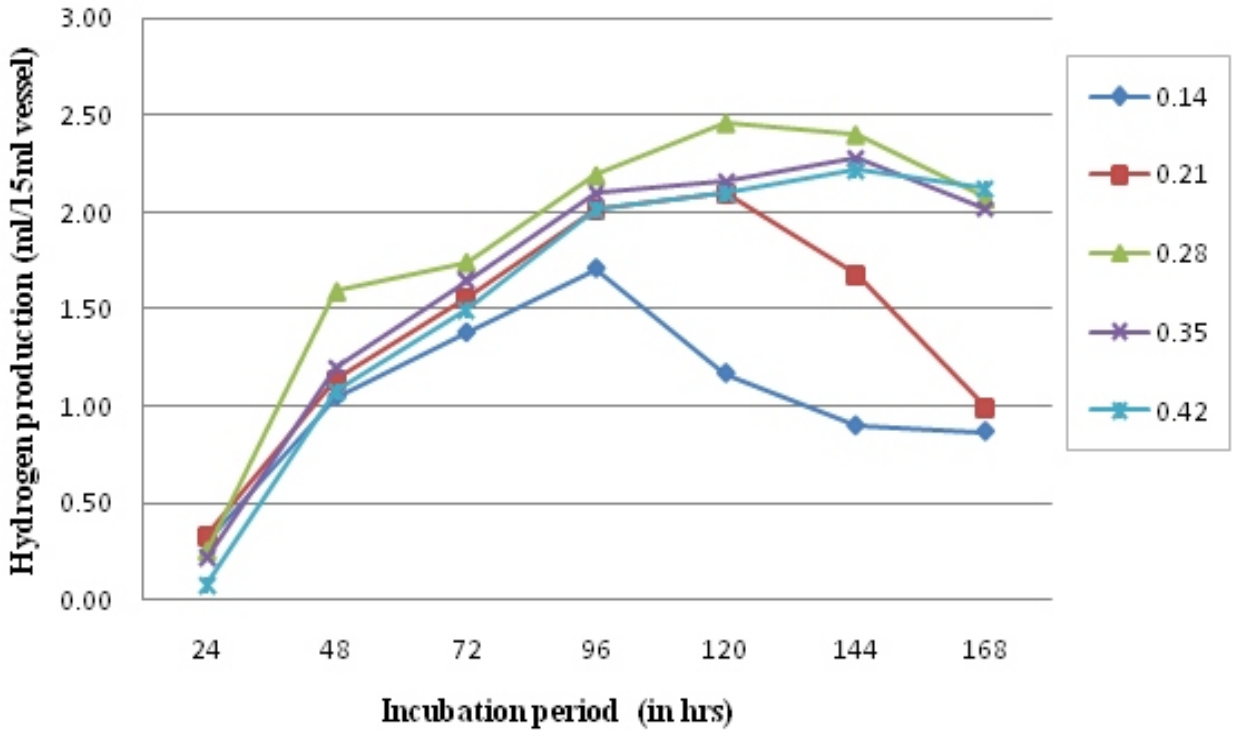

Fig. 2: Influence of different concentrations of thiourea on photoproduction of hydrogen by *Rc. tenuis*

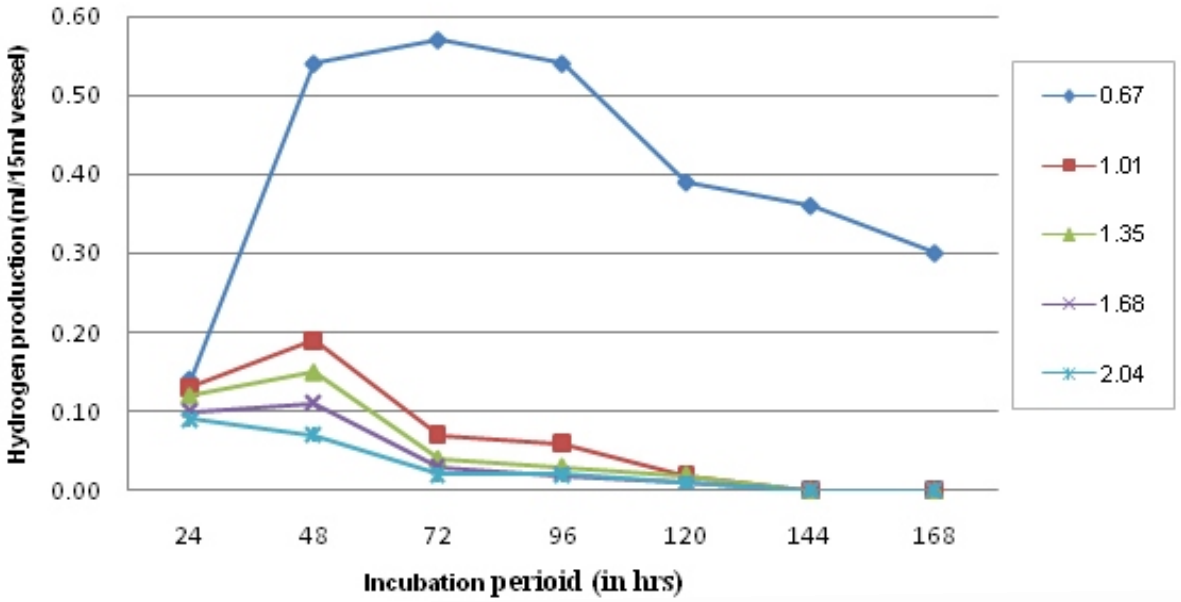

Fig. 3: Influence of different concentrations of L-tyrosine on photoproduction of hydrogen by *Rc. tenuis*

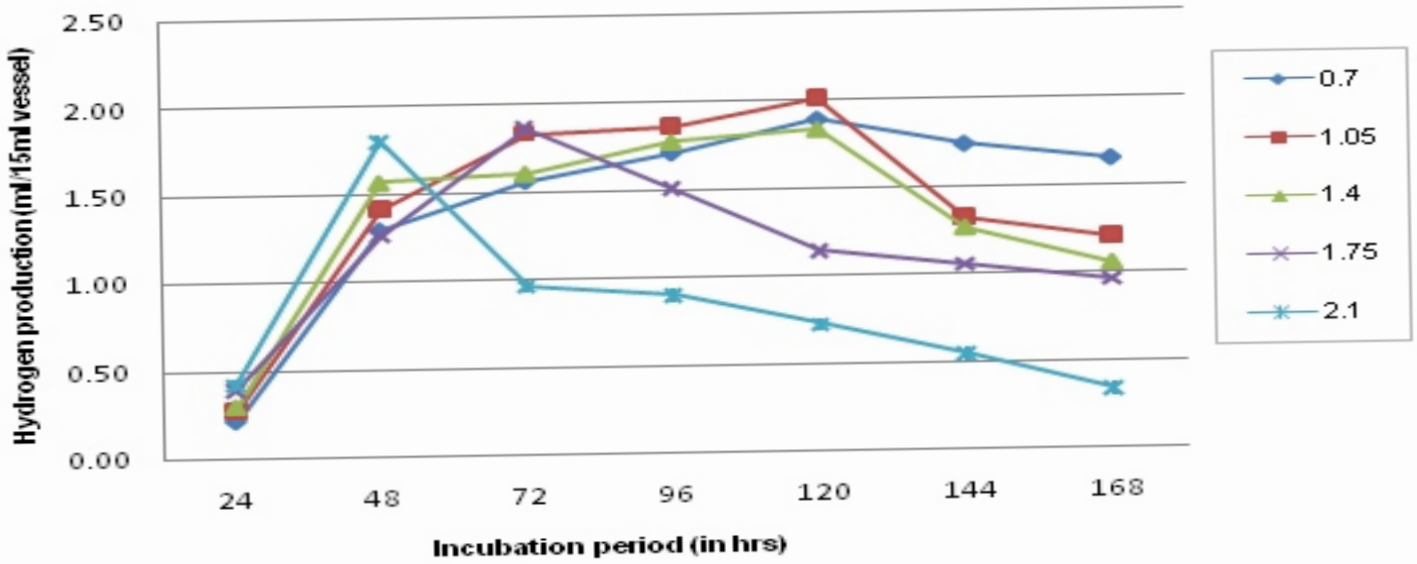

Fig. 4: Influence of different concentrations of L-glutamic acid on photoproduction of hydrogen by *Rc. tenuis*

As ammonium chloride, thiourea, L-tyrosine and L-glutamic acid induced maximum amount of hydrogen production, an attempt was made to optimize the concentrations of these amino acids and results are presented in fig 1-4. It is evident from figure 1 that hydrogen production was optimum at 0.4 mg/ml followed by 0.5 mg/ml. For all levels of concentrations, the test organism produced maximum hydrogen at 48 hrs of incubation. Perusal of figure 2 reveals that hydrogen production with thiourea was observed throughout the incubation period. Hydrogen production was optimum at 0.28mg/ml concentration. Interestingly, in contrast to ammonium chloride, later periods of incubations yielded more hydrogen. L-tyrosine also induced good amount of hydrogen throughout the incubation period. (fig 3). At 0.67 mg/ml concentration hydrogen production was recorded till the end of the incubation period with optimum at 72 hrs. At more than 1.0 mg/ml concentrations hydrogen production was poor. Though the maximum production was almost same at all concentrations, but the incubation periods varied with the concentrations. For instance, at 1.4 mg/ml concentration maximum hydrogen was recorded at 48 hrs incubation, whereas at 1.05 mg/ml concentration maximum production was recorded at 12 hrs. Hydrogen production was recorded at all the concentrations of L-glutamic acid (fig. 4).

The immobilized cells also preferred aerobic dark conditions over aerobic light conditions (Table 3). Immobilized cells produced maximum hydrogen at the end of 48 hrs incubation period and ceased to produce hydrogen after 120 hrs incubation. Interestingly immobilized cells preferred anaerobic light conditions rather than anaerobic dark conditions. Thus the cells of *Rc. tenuis* behaved differently with the growth stage of cells and aerobic/anaerobic and light/dark conditions. Such response was also noticed by earlier investors (Sasikala and Ramana, 1995; Yongzhen et al., 2008; Tekucheva and Tsygankov, 2012). Light dependent hydrogen production was also demonstrated by several workers while working with different bacteria (Yang et al., 2006; Yongzhen et al., 2008).

Among four nitrogen sources investigated ammonium chloride induced maximum hydrogen production (Table 4) when the immobilized cells of Rc.tenuis were incubated under anaerobic light conditions than aerobic light conditions. Similarly, anaerobic dark conditions were preferred rather than aerobic dark. Thiourea also induced more hydrogen under aerobic light than aerobic dark. Similarly, anaerobic light conditions were preferred over anaerobic dark by *Rc. tenuis* for hydrogen production. In general, aerobic light and anaerobic light conditions were more conducive than aerobic and anaerobic dark for all four nitrogen sources investigated. However, degree of hydrogen production varied with the incubation period. For immobilized cells thiourea followed by L-glutamic acid was more favourable for production of hydrogen. On the other hand, ammonium chloride and L-tyrosine are next preferential nitrogen sources for production of hydrogen under different cultural conditions.

CONCLUSIONS

Among 16 different nitrogen sources investigated ammonium chloride, thiourea, L-tyrosine and L-glutamic acid were observed to be good sources of nitrogen as they induced maximum production of hydrogen. However, the optimum concentration for hydrogen production varied with the nitrogen source. Anaerobic light and aerobic dark conditions were found to be more favourable than anaerobic dark and aerobic light conditions. Immobilization of bacterial cells not only enhanced hydrogen production but enabled the cells to produce hydrogen over an extended period. Resting cells for hydrogen production required more lag period, while actively growing cells produced more hydrogen during early phase of growth.

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