Int.J.Curr.Res.Aca.Rev.2017; **Special Issue-4***: 173-179*

International Journal of Current Research and Academic Review

ISSN: 2347-3215 Special Issue-4 (October-2017)

Journal home page: http://www.ijcrar.com

Mutation studies with *Citrobacter freundii* for enhanced hydrogen

P. Sumithira[1*] and R. Gowrisankar[2]

[1]Department of Microbiology Dr. MGR Janaki College of Arts & Science for Women, Chennai, Tamil Nadu, India
[2]PG Department of Microbiology, Sri Paramakalyani College, Azhwarkurichi, Tirunelveli, Tamil Nadu, India

Corresponding author

KEYWORDS

Citrobacter freundii,
Mutation,
Strain improvement,
Hydrogen,
bio-fuel

A B S T R A C T

Strain improvement for amplifying the expression of desired metabolic activity or product is an actively pursued branch in microbial technology. Many physical and chemical methods are employed to generate mutants with desired characteristics. An attempt is being made in this study to genetically manipulate *Citrobacter freundii* for enhanced hydrogen production. In the present study, *Citrobacter* field isolate was characterised and select *C. freundii* isolates were mutagenized with ultra violet radiation (UV). Distinct morphological difference and tetracycline resistance (tet^r) were used to differentiate mutants from the wild type *C. freundii*. Two mutants of *Citrobacter* namely *C. freundii* SPK02M, *C. freundii* SPK03M were found to exhibit higher hydrogen output over the wild type from a wide variety of carbon source. Environmental friendly renewable alternate to fossil fuels is actively perused worldwide. Microbe driven bio-fuels, bio hydrogen are increasingly becoming popular as the renewable energy source. Genetic manipulations of target microbial fuel cell should be pursued to maximize the scope of microbial bio-hydrogen for various applications.

Introduction

Shrinkage of fossil-fuel resource and excessive utilization of these non-renewable resources has placed the ultimate existence of mankind in jeopardy on the basis of energy crisis. This fast depletion of fossil-fuel has opened the doors for an aggressive search for an alternate energy source to ensure the continuance of technological development and existence of life. The concept of bio-energy is being actively pursued due to its renewable and other advantageous features.

Among the alternate energy sources, hydrogen is one of the possible alternate energy source. Hydrogen gas, a main stay of the chemical industry and much discussed potential fuel is central to life form that inhabit virtually all anaerobic environment including lake sediments, deep sea,

hydrothermal vents and the human intestine (Jannasch and Motti 1985). Microbial hydrogen production is a promising line of research as the microbes are easy to propagate. Further, due to their wide substrate conversion ability, they can be grown on cheap renewable resources which includes organic wastes, thereby opening up the possibility for developing cost effective system with an added advantage of recycling the possible pollutants. (Zajic *et al.,* 1978).

Hydrogen production by microbes was demonstrated in 1950s itself and the hydrogen evolving hydrogen evolving hydrogenase of *Clostridia* was first purified and bio-chemically analyzed 30 years ago. Many workers have demonstrated the production of hydrogen by a variety of aerobic, anaerobic and phototrophic organisms. Many members of *Enterobacteriaceae* were found to produce hydrogen through fermentation of a wide variery of carbon sources. This includes representatives belong to the genera of *Clostridia*, *Escherichia*, *Citrobacter* etc., Among these, *Citrobacter* is an important genus capable of producing hydrogen gas, CO_2 and other chemicals.

Citrobacter is an important genera found in most ecological niches. Genus *Citrobacter* includes a variety of species which are Gram negative, non-spore forming bacilli found frequently in soil, river sediment and the alimentary tract of man and animals. Most of the members of these genera are commensals but are frequently observed to be opportunistic pathogens (Toranzo *et al.*, 1994). *Citrobacter freundii*, *C. diverses* and *C. amalonaticus* are the most important Citrobacter's.

In view of the increasing importance of bio-energy and the relevance of *Citrobacter* as the promising candidate for hydrogen production, this study was taken up to access the native *C. freundii* for its carbohydrate utilization and hydrogen productivity. In order to enhance the rate of carbohydrate conversion to achieve an increase in hydrogen production, mutation studies were also carried out with the *C. freundii* isolate and the mutants were evaluated for their hydrogen productivity.

Materials and Methods

Characterisation of *Citrobacter freundii*

Citrobacter field isolates were characterized based on their microscopic, morphological, biochemical, physiological and antibiotic resistance characteristics in accordance to the recommendations of Bergey's manual of systemic bacteriology (Sakazaki, 1984).

Test for Carbohydrate utilization

The Oxidation –Fermentation (OF) basal medium (Hi-Media, India) with inverted Durham's tube was prepared. After sterilization, filter sterilized solution of glucose, sucrose, lactose and cellulose at 10% (v/v) concentration was added to these tubes in aseptic condition. The tubes were inoculated with the overnight test culture (0.1ml) and incubated for 24 - 48 hours at 37^{o}C. To maintain anaerobic condition after inoculation a layer of sterile mineral oil was added on the broth surface. Change in media colour and collection of gas in Durham's tube was recorded for acid and gas production respectively.

Mutation of *Citrobacter freundii* isolates

Physical mutagen, ultra violet radiation (UV) was used to generate *Citrobacter freundii* mutants. The bacterial isolate was spread plated on air dried LB plates and exposed to UV for 3 minutes maintaining

the distance between the culture and the UV source at 70 cms. After this, the UV exposed plates were covered with black cloth to prevent photoractivation and incubated in dark at 37^{o}C for 24 hrs.

Selection of *Citrobacter freundii* mutants

From the UV exposed LB plates, well isolated colonies were picked and pure cultured on sterile nutrient agar. After overnight incubation, the individual colonies were stored in sterile nutrient slants. From this, a loopful was inoculated into sterile nutrient broth, incubated overnight and cross checked for altered phenotype which is taken as a marker to recognise mutants.

Among the various characteristics, carbohydrate utilization and antibiotic resistance pattern of the mutants were chosen as the indication of mutation and were compared with that of the wild type.

Mutants were scored and stored on NA slant. The mutants of *C. freundii* were sub cultured repeatedly to check for the stable inheritance of mutant characters over several generations. Stable *C. freundii* mutant cultures were analysed for hydrogen production.

Screening of *Citrobacter freundii* strains for hydrogen production

Of basal medium with glucose or lactose (0.5%) was prepared and 100 ml aliquots were hermetically sealed with rubber cork, and autoclaved. *C. freundii* test culture (5ml) was injected into the OF glucose vial and incubated at 37^{o}C. At 24 and 48 hrs time points, 1ml was drawn from the vials and tested for hydrogen production using gas chromatography (Agilent, US) with MS5A column, thermal conductivity detector (TCD) and argon carrier gas.

Results and Discussion

Citrobacter field isolates were characterized and strains identified as *Citrobacter freundii*. *Citrobacter freundii* is one of the most important and most frequented member of *Citrobacter* sp. in a soil ecosystem. Toranzo *et al.,* (1984) have remarked the incidence of *Citrobacter freundii* in different ecological niches. Prevalence of *C. freundii* in soil was reported by Montgomery (1995). Interestingly, both the *Citrobacter freundii* isolated were found to hydrolyse gelatin which is not a frequently observed characteristic.

Antibiotics sensitivity is often recommended as one of the important characteristics of *Citrobacter freundii* which is even employed in the differentiation of the members of *Citrobacter*. Both the isolates of *Citrobacter freundii* in this study were found to be sensitive to cotrimoxazole and chloramphenicol (Table 1) Lund *et al.,* (1974) reported the incidence of ampicillin sensitive *Citrobacter*. Toranzo *et al.,* (1994) have reported streptomycin resistance in *Citrobacter freundii* isolates drawn from sediment sample. The differential pattern of antibiotic resistance among the *Citrobacter freundii* isolates which includes our strain is not surprising as there has been an increased incidence of chromosomally and plasmid mediated drug resistance among *Citrobacter* genera (Hayashi *et al.,* 1982 Mc pearson *et al.,* 1991).

Both *Citrobacter freundii* SPK01W and *Citrobacter freundii* SPK 02W were found to ferment a range of sugars which includes monosaccharide (glucose) disaccharide (sucrose, lactose) polysaccharide (cellulose). This property of fermentation was not observed to be influenced by the presence or absence of oxygen (Table 3) Even though all

four carbohydrates were feremented, not all fermentation process have resulted in the evolution of gas. *Citrobacter freundii* SPK 02W was able to produce gas from glucose and lactose only in anaerobic condition whereas *Citrobacter freundii* SPK 01W didn't produce gas with glucose and sucrose as substrates (Table 4). This observation correlates with the reports of Sakazaki (1984). Babusha (1996) has reported such variation in fermentation efficiencies among *Citrobacter freundii* isolates. He has also reported the preferential utilization of disaccharides over glucose. Such ability to utilize wide range of carbohydrates was reported by many workers which includes Lutgen and Gottschalk (1982); Washington *et al.*, (1970) and Montgomery *et al.*, (1995).

Table.1 Antibiotic sensitivity pattern of *Citrobacter freundii* – Wild type strains

Antibiotic tested	Drug concentration (mcg)	Zone of inhibition (mm)	
		SPK01W	SPK02W
Chloramphenicol	30	30	30
Ampicillin	30	24.5	25
Gentamycin	10	30	24
Tetracycline	30	15	13
Co-trimoxazole	30	32	33

Table.2 Antibiotic sensitivity pattern of *Citrobacter freundii* – Mutants

Antibiotic tested	Drug concentration (mcg)	Zone of inhibition (mm) – Strain SPK							
		SPK 1M	2M	3M	4M	5M	6M	7M	8M
Chloramphenicol	30	28	32	29	21	38	24	34	32
Ampicillin	30	31	27	R	17	19	21	20	17
Gentamycin	10	30	25	28	30	25	26	25	23
Tetracycline	30	R	R	R	R	13	R	14	R
Co-trimoxazole	30	33	43	18	18	33	30	36	30

R = Resistant

Table.3 Carbohydrate utilization pattern of *Citrobacter freundii* – Wild type strains

Substrates	*Citrobacter freundii*			
	Anaerobic condition		Aerobic condition	
	SPK01W	SPK02W	SPK01W	SPK02W
Glucose	A	AG	A	A
Sucrose	A	A	A	A
Lactose	AG	AG	A	A
Cellulose	A	A	AG	A

A = Acid; G = Gas

Table.4 Carbohydrate utilization pattern of *Citrobacter freundii* – Mutants

Substrates	*Citrobacter freundii* SPK															
	Anaerobic condition								Anaerobic condition							
	1M	2M	3M	4M	5M	6M	7M	8M	1M	2M	3M	4M	5M	6M	7M	8M
Glucose	*	(A)	A	(A)	*	A	(A)	*	A	A	A	A	A	A	A	A
Sucrose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Lactose	A	A	A	(A)	*	A	*	(A)	A	A	A	A	A	A	A	A
Cellulose	A	A	(A)	(A)	(A)	A	A	(A)	*	*	*	*	*	*	*	*

A = Acid production; G = Gas production; (A) = Weak acid; * = No acid & gas

Fig.1 Hydrogen production with glucose - *Citrobacter freundii* – Wild & Mutant strains

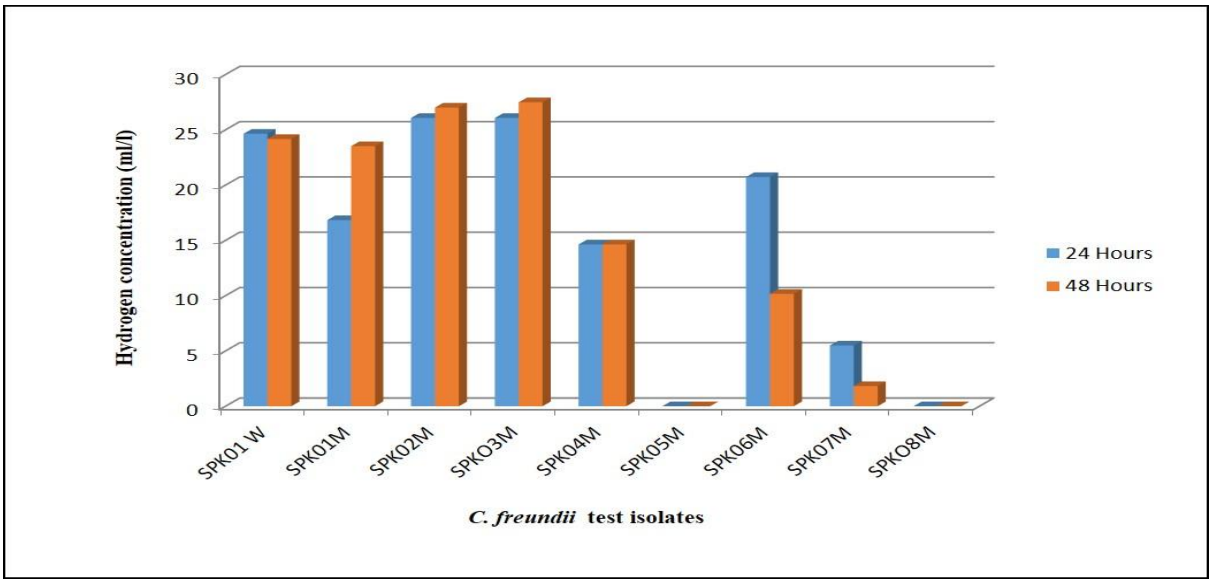
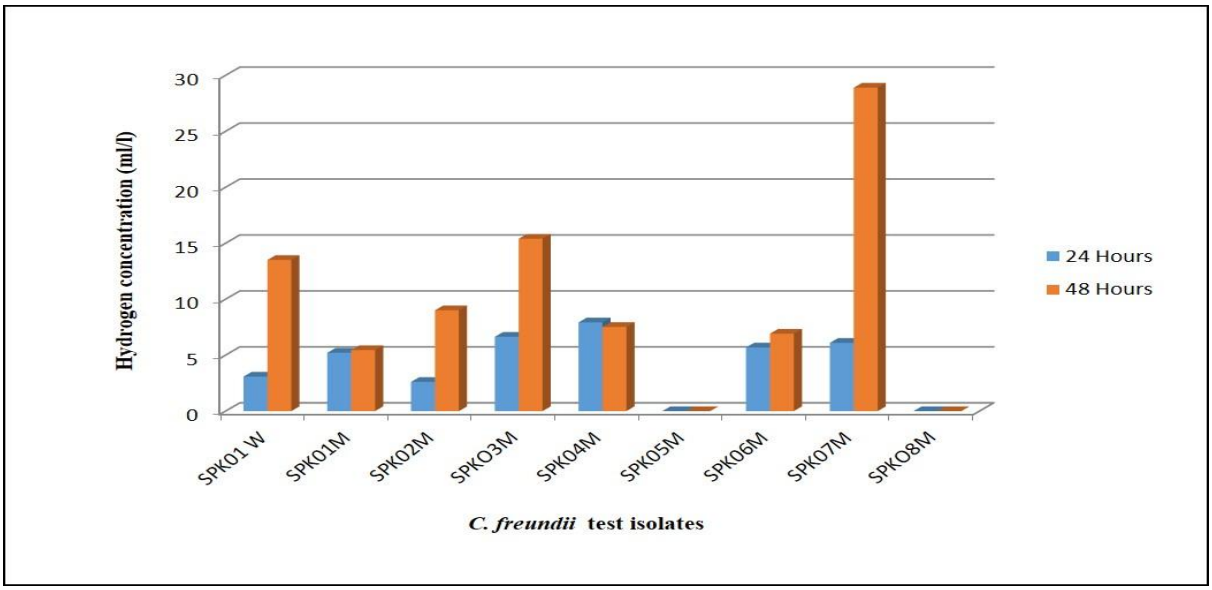


Fig.2 Hydrogen production with lactose - *Citrobacter freundii* – Wild & Mutant strains



Citrobacter freundii SPK 01W was exposed to UV and mutants were scored on their antibiotic pattern. Of the 8 *C. freundii* mutants, 6 were found to be tetracycline resistant. (Table 2). Further, analysis of carbohydrate utilization pattern of *C. freundii* mutants has revealed similar variation in fermentation. Contrast to the wild type parent, none of the mutants were found to be poor cellulose utilizers. Loss of ability to ferment glucose by *Citrobacter freundii* SPK01M, *Citrobacter freundii* SPK03M, *Citrobacter freundii* SPK08M and lactose by *Citrobacter freundii* SPK03, *Citrobacter freundii* SPK07M clearly confirms the mutagenic effects of UV on *C. freundii*. Both the wild type and mutants were screened for hydrogen production with glucose and lactose as carbon source. Maximum hydrogen production was observed with glucose as carbon source when compared to lactose.

Interestingly, *Citrobacter freundii* mutant SPK02M and *Citrobacter freundii* SPK03m were found to exhibit higher hydrogen production over wild type. On the contrary, *Citrobacter freundii* SPK08M were found to have lost the ability to produce hydrogen from both glucose and lactose. *Citrobacter freundii* mutant SPK07M even though has failed to produce appreciable amount of hydrogen with glucose, it was the most efficient strain in fermenting lactose with extremely high hydrogen production (28.90%) which is over double the concentration of that of wild type (13.51%). Among the mutant cultures, *C. freundii* SPK03M was found to be consistently efficient in hydrogen production both in the presence of glucose and lactose which is superior to that of the wild type (Fig 1 & 2).

Role of *C. freundii* in hydrogen production is well documented and is a reason for its consideration in bio-energy production. Babusha (1996) has reported a significantly higher hydrogen production with monosaccharide (glucose) over lactose. Ramesh Kumar and Vatsala (1989) have reported a time bound increase in gas production with C*itrobacter freundii* isolated from sewage. Neilson and Allard (1985) has reported such difference in hydrogen production with various carbohydrates. Maru *et al.,* (2013) have reported biohydrogen production by enterobacter and citrobacter strains suing glycerol as the substrate. This variation in hydrogen production could be attributed to the change is fermentation environment due to the accumulation of various fermentation byproducts.

This study clearly indicates the possibility of mutagenic improvement of *Citrobacter freundii* for increased hydrogen production efficiency. Further understanding on the molecular changes in *Citrobacter freundii* in response to mutation would throw open the avenues for an efficient and cost effective hydrogen production which is the need of the hour.

References

Alasdair H. Neilson and Ann-Sofie Allard 1985. "Acetylene reduction (N_2 – fixation) by Enterobacteriaceae isolated form industrial waste waters and biological treatment systems". *Appl. Microbiol. Biotechnol*. 23: 67 - 74

Babusha. S. T., 1996 "Studies on hydrogen productivity of *Citrobacter freundii* strains isolated and characterised from various sources". P.G. Department of Microbiology. Sri Paramkalyani College, Alwarkurichi p54

Holmes, P., and M.Freischel. 1978. "Hydrrogen producing bacteria in digestive sewage sludge isolated on

simple defined media". *Appl. Environ. Microbiol*. 36: 394 -395

Jannasch H.W., and M.J. Motti 1985. *Science* 229:717.

Lutgens M and Gottschalk. G, 1982. "Cell and ATP yields of *Citrobacter freundii* growing with fumerate and H_2 or formate in continous culture". *J.Gen Microbiol*. 128: 1915 -1921

Marlys E. Lund, John M.Matsen and Donna J. Blazevic. 1974. "Biochemical and Antibiotic suspectibility studies of H_2S negative citrobacter". *Appl. Microbiol*. 28: 22 -25

Maru BT, Constanti, M, Stchigel, AM, Medina, F, and Sueiras, JE, 2013. Biohydrogen production by dark fermentation of glycerol using Enterobacter and Citrobacter Sp. *Biotechnol Prog*. 29(1):31-8.

Montgomery M., C. R. Dean Wiffen, and E.Macaskie, 1995. "Phosphatase production and activity on *Citrobacter freundii* and a naturally occurring heavy metal accumulating Citrobacter sp". *Microbiol*. 141: 2433 -2441

Ramesh Kumar.G and T.M.Vatsala, 1989. "Hydrogen production from glucose by *Citrobacter freundii*". *Ind.J. Exp. Biol*. 27: 824 -825

Sakazaki, R. 1984. "Family *Enterobacteriaceae* In. Bergys manual of systemic bacteriology, N.R. Krieg and J.G. Holt (Eds) Williams and Wilkins, Baltimore, London. 1: 458 – 461

Toranzo, AE, JM. Cutrin, BS. Roberson, S. Nunez, JM. Abell, FM. Hetrick, and AM. Baya. 1994. "Comparision of the taxonomy, serology, drug resistance transfer and virulence of *Citrobacter freundii* strains from mammals and poikilothermic hosts". *Appl. Environ. Microbiol*. 60: 1789 -1797

Washington II, J.A., PKW. Yu, and WJ Martin 1970 "Hydrogen sulphide negative varient of *Citrobacter*". *Appl. Microbiol*. 20: 587 -589

Zajic, J.E., Kosaric, and JD. Brosseau. 1978(A). "Microbiol production of hydrogen". *Adv. Biochem. Engineering*. 9: 57 -109.

How to cite this article:

Sumithira P. and Gowrisankar R. 2017. Mutation studies with *Citrobacter freundii* for enhanced hydrogen. *Int.J.Curr.Res.Aca.Rev.* Special Issue-4: 173-179.