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L-glutamic acid production by immobilized wild and mutant *Bacillus* species

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ABSTRACT: The effect of Ultra-Violet (UV) irradiation and acridine orange dye on L-glutamic acid producing *Bacillus subtilis* and *B. megaterium* was investigated. The selected mutant strains and wild types of *B. subtilis* and *B. megaterium* were immobilized and used for L-glutamic acid production. The *Bacillus* strains were exposed to UV irradiation and treated with acridine orange dye respectively. The survived *Bacillus* strains were found to reduce as the exposure time and concentration of the mutagens increased in this study. Four mutants of *B. subtilis* and two mutants of *B. megaterium* were selected for L-glutamic acid production. Mutant strain of *B. subtilis* (MAIR4) treated with acridine orange dye yielded the highest L-glutamic acid (4.62 mg/mL) at 72 hours. L-glutamic acid production by mutant wild and strains of *B. subtilis* and *B. megaterium* immobilized on sodium alginate, Agar-agar matrix and poly urethane foam ranged from 1.65 to 4.03 mg/mL, 2.04 to 3.98 mg/ mL and 1.89 to 3.39 mg/mL, respectively with *B. megaterium* (MUSO17) on sodium alginate producing the highest L-glutamic acid. Sodium alginate was the best supporting matrix for the production of L-glutamic acid in this research. Immobilization of mutant strains of *Bacillus megaterium* (MASO17*)* exposed to UV irradiation using sodium alginate supported the L-glutamic acid production.

Keywords: *B. subtilis, B. megaterium*, immobilization, L-glutamic acid, mutation, polyurethane foam (PUF).

INTRODUCTION

Amino acids production has been the second most important in quantity within “white biotechnology” after antibiotics production which is taking the lead (Shyamkumar *et al*., 2014). Since the 1950s, the fermentation method of amino acid production has become a vital technology in the area of industrial microbiology (Abou-taleb, 2014; Paloyan *et al*., 2022). Microorganisms involved in amino acid fermentation are found not to excrete amino acid in reasonable amounts due to the regulatory mechanism. Therefore, to produce a surplus of these amino acids, there is need to generate mutants that have the capacity of overproducing the respective amino acids. Mutagenesis occurs when there is a change in the metabolic process and thus has become the most extensively used tool for industrial microorganisms. Mutations can be produced chemically or by exposing the microorganisms to irradiation.

Microorganisms used in the fermentation of amino acids can be categorized into 4 groups, namely, the wild-type, auxotrophic mutant, regulatory mutant, and auxotrophic regulatory mutant (Zelder *et al*., 2005). Auxotrophic mutant which is found to be resistant to analogues is widely employed in large scale fermentation of amino acids. Hopwood (1970) reported the use of penicillin enrichment for auxotrophic mutants’ isolation and which can also be employed to modify a particular auxotrophic isolate.

Lopez *et al*. (1997) defined immobilization as the process by which particles are attached or entrapped, which is basically applied to all types of biocatalysts including, cellular organelles, enzymes, and plant and animal cells. Immobilization of cells is one of the methods employed in the field of biotechnology to improve amino acid production (Amin *et al*., 2010).

Microbial cell immobilization is found useful in the field

of industrial as well as environmental processes. There has been a significant reduction in cost production when microorganisms retained on a carrier are used in both continuous and semi-continuous production processes since the biocatalyst does not need to be refilled (Park and Chang, 2000; Mrudula and Shyam, 2012). Some bacteria (wild type) have the capacity of producing amino acids reasonably. In addition to the use of the wild type strains for amino acid biosynthesis, different strain improvement techniques i.e., cloning, mutagenesis and protoplasm fusion are used (Musa *et al.,* 2016). Therefore, the objective of this research was to determine the effect of mutation (physical and chemical mutagens) and immobilization using different supporting matrices for the enhancement of L-glutamic acid by *Bacillus* strains.

MATERIALS AND METHODS

Bacterial strains

The *Bacillus* spp. which had been identified and previously screened for production of L-glutamic acid were collected from the Microbial Physiology and Biotechnology Laboratory, Department of Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria. The isolates were re-cultivated under aerobic conditions by plating on Nutrient agar medium and Trytose Soy agar fortified with 1% L-glutamic acid containing 0.5% w/v, peptone 0.3% w/v, beef extract, and 1.5% w/v, agar-agar, pH 7.2 for confirmation.

Mutation using UV irradiation

Ten millilitres of each of the Tryptic soy broth cultures of *B. subtilis* and *B. megaterium* was transferred into a sterile Petri-dish (10 mm diameter) and exposed to ultra-violent light (UV) of wavelength of 245 nm at a distance of 30 cm at different time interval of 5, 10, 15, 20, 25, 30 and 35 minutes. After exposure to UV, the cultures were wrapped in black polyethene bags to avoid photo-reactivation and then incubated at 30°C for 6 hours. After incubation, seven successive 10-fold dilutions of the irradiated cells were taken and diluent (0.1 ml) from each dilution 10^{-6} and 10^{-7} were inoculated on Dextrose Peptone Agar (DPA) plates for growth. After which the colonies were incubated for 24 hours at 30°C were on minimal agar medium: glucose, 4 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.001 g, $MnSO_4.H_2O$ 0.001 g; $FeSO_4$.$7H_2O$ 0.001 g; $CaCO_3$ 2g; $(NH_4)_2SO_4$, 2 g; water 1 litre; pH 7.0. Mutant strains which showed no growth on the minimal medium were selected and stored on Nutrient agar slants at 4°C. The mutant strains were further screened for L-glutamic acid production.

Mutation using acridine orange dye

Chemical mutagenic study of the isolates was done using the method of Ahmed *et al*. (2013). 10 mL of culture broth was placed in 3 test tubes containing different concentration (10, 25, 50, 75, 100 and 150 µl) of 1% acridine orange dye solution was introduced into each of the test tubes. The test tubes were then incubated for 6 hours in a dark place by covering the tubes with dark polythene bag. After incubation, serial dilutions up to 10^{-7} were prepared and 0.1 ml of the bacteria culture was taken from dilution 10^{-6} and 10^{-7} and spread over Nutrient agar plates. The plates were then incubated for 48 hours at 37°C. Further total viable colony count was measured and plated out on minimal agar medium composed of glucose 4 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.001, $MnSO_4.H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $CaCO_3$ 2 g; $(NH_4)_2SO_4$ 2 g; water 1 litre; pH 7.0. Mutant strains with no growth on the minimal medium were selected.

Screening of the mutant strains for L-glutamic acid production

Mutants obtained from UV radiation and acridine orange were screened for L- glutamic acid production. 2 mL of the mutants were grown in 20 mL of Fermentation Medium containing: K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MnSO_4.H_2O$, 0.001 g; $FeSO_4$.$7H_2O$, 0.001 g; $MgSO_4.7H_2O$, 0.001 g; $CaCO_3$, 50 g; 1 litre of water at pH 7.2. The inoculated fermentation broth was incubated at 35°C for 96 hours on a rotary shaker at 180 rpm. Samples were taken at every 24 hours and centrifuged at 5000 rpm for ten minutes. Supernatant were then examined for L-glutamic acid (Ganguly, 2019).

Immobilization

The selected wild type and mutant strains of *Bacillus* were immobilized on various supporting matrix. Adsorption method was used for Polyurethane foam (0.4 - 0.6 mm pore size) (Kirkpatrick *et al*., 1990). The foam was cut into 1.2 cm^3 (1.38 g dry weight), washed and sterilized in the autoclave using deionized water prior to use. The immobilization matrix was inoculated with 5 mL of inoculum and incubated at 37°C on a rotary shaker (200 rpm) for up to 5 days until the surface of the foam appeared to be uniformly colonized, as determined by visual inspection.

Entrapment method was employed for sodium alginate immobilization (Pasha *et al*., 2011). 2 g of sodium alginate was dissolved in 100 mL of 0.9% sodium chloride and solubilization was done by warming the solution gently. The solution was sterilized for 15 minutes at 121°C. 10 mL of inoculum was gradually introduced into the prepared solution, followed by sodium alginate solution which was drop-wisely introduced (using a burette) with continuous stirring to a 0.1 M $CaCl_2$ solution. The gel beads formed were left in solution for 1 hour before being filtered off. The formed beads were then stirred in a 0.9% NaCl per 50 mM

Table 1. Effect of UV irradiation on the colony forming unit and survival rate of *B. subtilis* and *B megaterium*

B. subtilis					***B megaterium***				
Exposure time (min)	Dilution	Colony forming unit	Survival rate (%)	Average survival rate (%)	Exposure time (min)	Dilution	Colony forming unit	Survival rate (%)	Average survival rate (%)
0	10^{-6}	178	100	100	0	10^{-6}	180	100	100
	10^{-7}	154	100			10^{-7}	184	100	
5	10^{-6}	164	85	84	5	10^{-6}	150	97	95
	10^{-7}	132	83			10^{-7}	135	93	
10	10^{-6}	74	50	46	10	10^{-6}	83	45	42
	10^{-7}	68	42			10^{-7}	71	39	
15	10^{-6}	56	36	39	15	10^{-6}	76	33	32
	10^{-7}	33	43			10^{-7}	45	31	
20	10^{-6}	22	25	24	20	10^{-6}	43	12	12
	10^{-7}	18	23			10^{-7}	53	13	
25	10^{-6}	20	7	8	25	10^{-6}	15	7	6
	10^{-7}	10	9			10^{-7}	15	5	
35	10^{-6}	2	2	2	35	10^{-6}	5	1	1
	10^{-7}	3	2			10^{-7}			

$CaCl_2$ solution for 20 minutes to allow the diffusion of access calcium. The washed, cell-coated beads were then used.

Entrapment of cells on Agar-agar matrix was carried out according to the method of Ishola and Adebayo-Tayo (2018) with slight modifications. 4% agar solution was prepared in 25 mM sodium acetate buffer (pH 5.5) by autoclaving it at 50°C. After cooling to room temperature, 1 mL inoculum volume (approximately 10^8 CFU) was mixed with 9 mL agar solution (the total volume of matrix and cell mixture being 10 mL) and immediately casted on preassembled glass plates. After solidification at room temperature, the gel was cut into small beads of 5 × 5 mm size and washed several times before use to remove any cell attached to the gel surface. The beads were stored in 25 mM sodium acetate buffer (pH 5.5) and at 4°C.

Production of L-glutamic acid by immobilized and un-immobilized wild and mutant *Bacillus* strains.

Immobilized and unimmobilized wild and mutant *Bacillus* strains were used for L-glutamic acid production. 2 mL of the mutants and wild types were grown in 20 mL of Fermentation Medium. The inoculated Fermentation broth was incubated at 35°C for 96 hours on a rotary shaker at 180 rpm. L-glutamic acid was quantitatively determined by the acidic-ninhydrin method. The optical density was recorded at 460 nm for L-glutamic acid. Standard curves were prepared by taking a known concentration of different L-glutamic acids (Troll and Cannan, 1953).

Statistical analysis

The results from this research were statistically subjected to analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Mutagenesis study of *Bacillus* species

The number of colony forming units (CFU) observed after exposure to UV and treatment with different concentration of acridine orange dye at different time intervals is shown in Tables 1 and 2. Count at 0 hour and 0 mg/mL represent the control experiment in which the *Bacillus* strains were not exposed or treated with any mutagen. It was observed that as the exposure time increases and acridine orange dye concentration increases, the colony forming units decreases. Reduction in colony forming units is in line with the work of Adebayo-Tayo *et al.* (2017), in which decrease in colony forming units of *Lactobacillus plantarum* when exposed to UV light was observed and reported. Four and two mutant strains were obtained from *B. subtilis* and *B. megaterium* respectively, when exposed to UV radiation at different time intervals, ranging from 0 to 35 minutes and acridine orange dye at different concentration ranging from 0 to 150 mg/mL. For UV irradiation, the control produced 1.78 x 10^8 CFU/mL and 1.80 x 10^8 CFU/mL for *B. subtilis* and *B. megaterium* at 10^{-6} dilution. With acridine orange treatment, the control produced 1.78 x 10^8 CFU/mL and 1.82 x 10^8 CFU/mL at 10^{-6} dilution which corresponds to

Table 2. Effect of acridine orange dye on the colony forming unit and survival rate of *B. subtilis* and *B. megaterium*

B. subtilis					*B megaterium*				
Concentration (mg) of acridine	Dilution	Colony forming unit	Survival rate (%)	Average survival rate (%)	concentration (mg) acridine orange	Dilution	Colony forming unit	Survival rate (%)	Average survival rate (%)
0	10^{-6}	182	100	100	0	10^{-6}	178	100	100
	10^{-7}	174	100			10^{-7}	167	100	
10	10^{-6}	61	60	63	10	10^{-6}	60	48	46
	10^{-7}	70	66			10^{-7}	64	44	
25	10^{-6}	63	31	32	25	10^{-6}	56	38	36
	10^{-7}	56	33			10^{-7}	53	34	
50	10^{-6}	46	27	26	50	10^{-6}	46	28	26
	10^{-7}	50	25			10^{-7}	44	24	
75	10^{-6}	28	16	14	75	10^{-6}	25	20	16
	10^{-7}	19	12			10^{-7}	22	12	
100	10^{-6}	5	5	4	100	10^{-6}	6	1	2
	10^{-7}	3	4			10^{-7}	7	3	
150	10^{-6}	0	3	2	150	10^{-6}	1	0	0
	10^{-7}	0	1			10^{-7}	2	0	

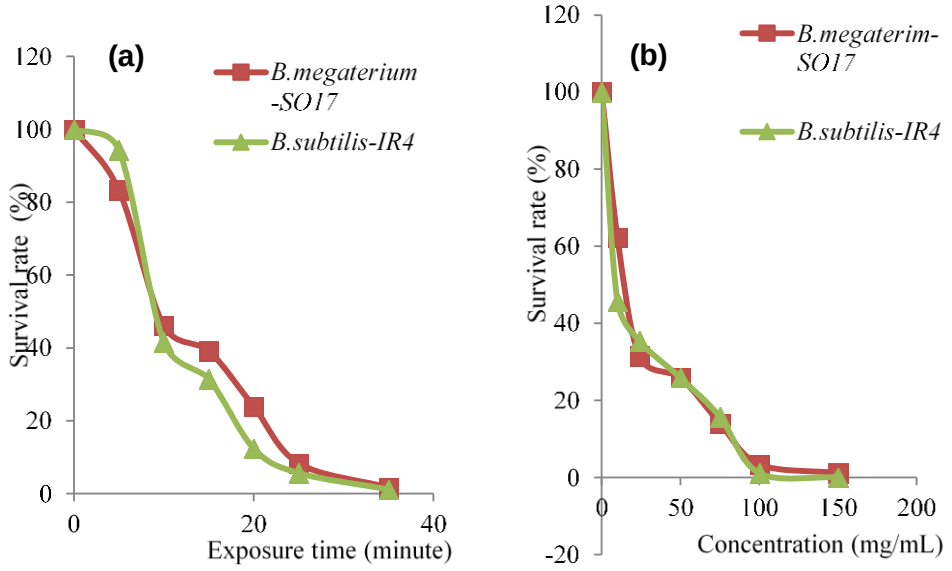

Figure 1. Survival rate of the *Bacillus* strains (a). Exposure to UV-light (b). Treatment with acridine orange dye.

100% survival. This is in agreement with the work done by Ahmed *et al*. (2013). Pasha *et al*. (2011) reported that both physical and chemical mutagens are useful in strain improvement techniques and thus capable of producing higher yields of L-glutamic acid. Also, increase in glutamic acid production by *C. glutamicum* was also reported when treated with Ethyl Methane Sulfonate (EMS) mutagen (Khattab *et al*., 2018).

The survival rate of the *Bacillus* strains after exposure to UV-irradiation (254 nm) and treatment with acridine orange dye is shown in Figures 1a and 1b. It was observed that all the treatments investigated had different degrees of lethal effect (which varies with time) on the survival of the *Bacillus subtilis* and *B. megaterium*. As the time of exposure (to different treatments) increased, there was a decrease in the survival rate of the treated isolates to about 1% at 35 minutes of exposure to UV-light and 150 mg/mL of acridine orange dye.

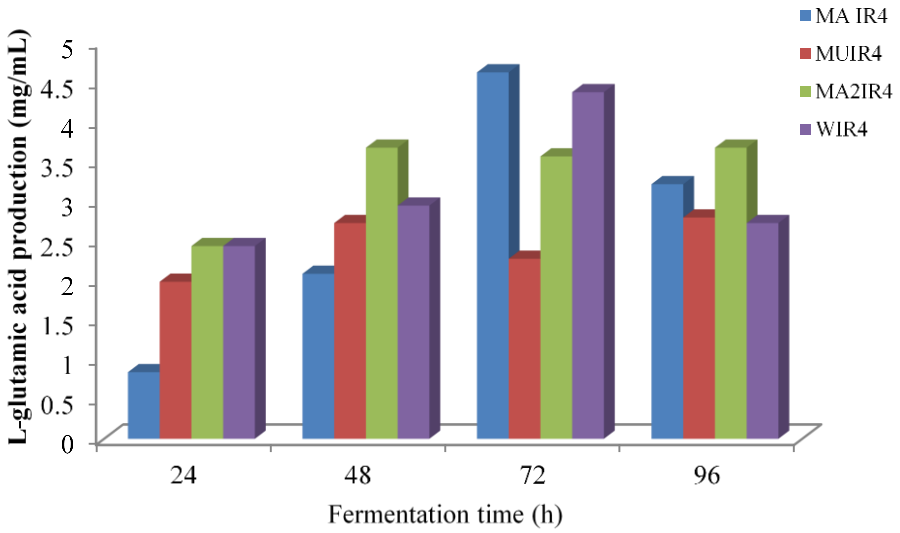

Figure 2a. L-glutamic acid production by selected mutants strains and wild *Bacillus subtilis.* **Legend:** MA IR4 (*B. subtilis*-IR4 mutant using acridine orange)**,** MUIR4 (*B. subtilis*- IR4 mutant using UV)**,** MA2IR4 (*Bacillus subtilis*-IR4 mutant using acridine orange), MU2IR4 (*Bacillus subtilis*- IR4 mutant using UV)**,** WIR4 (*B. subtilis* –IR4 wild type).

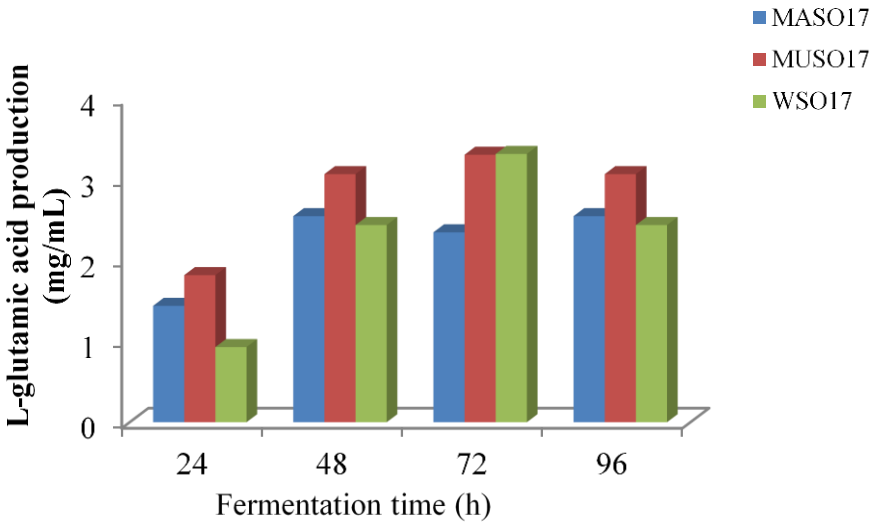

Figure 2b. L-glutamic acid production by selected mutants strains and wild *Bacillus megaterium*. **Legend:** MASO17 (*B. megaterium* –SO17 mutant using acridine orange)**,** MUSO17 (*B. megaterium*-SO17 mutant using UV), WSO17 (*B. megaterium* –SO17 wild type).

L-glutamic acid production by the selected mutants and wild type of *Bacillus subtilis* and *B. megaterium*

L-glutamic acid production by the selected mutant strains is shown in Figures 2a and 2b. The production by selected mutants and wild type *B. subtilis* ranged from 0.84 to 3.32 mg/mL. The highest was produced by MAIR4 at 72 hours while the least was produced by MAIR4 at 24 hours. There was a significant difference in L-glutamic acid production by the wild and mutant strains of *B. subtilis*. In *B. megaterium,* L-glutamic acid production by selected mutants and wild type ranged from 0. 93 to 3.32 mg/mL. The highest L-glutamic acid production was produced by WSO17 at 72 hours while the least was produced by WSO17 at 24 hours. Mutant *B. subtilis* was observed to accumulate different quantities of L-glutamic acid and was

Table 3. Production of L-glutamic acid (mg/mL) by d wild strains and d mutant *Bacillus* strains

Support matrix	Isolate	L-glutamic acid production (mg/mL)/ fermentation time (h)			
		24	48	72	96
Sodium alginate	MUSO17	2.21^{f}	3.39^{a}	4.03^{a}	3.49^{b}
	WSO17	3.13^{bc}	3.13^{b}	3.22^{e}	3.27^{e}
	MAIR4	2.99^{c}	3.15^{b}	3.13^{f}	3.03^{f}
	WIR4	1.65^{j}	3.39^{a}	3.19^{ef}	3.07^{f}
	MA2IR4	3.37^{a}	3.36^{a}	3.32^{d}	2.99^{g}
Agar -Agar	MUSO17	2.04^{h}	2.85^{f}	3.94^{b}	3.98^{a}
	WSO17	2.44^{e}	2.71^{g}	3.07^{g}	3.42^{bc}
	MAIR4	3.18^{b}	2.98^{d}	3.21^{e}	3.28^{e}
	WIR4	2.82^{d}	2.89^{e}	3.32^{d}	3.46^{b}
	MA2IR4	2.07^{h}	2.95^{d}	3.14^{f}	3.44^{bc}
Polyurethane foam	MUSO17	1.98	2.19^{h}	3.13^{f}	3.00^{g}
	WSO17	2.06^{h}	2.75^{g}	3.43^{c}	3.39^{d}
	MAIR4	2.13^{g}	3.02^{c}	2.98^{h}	3.03^{f}
	WIR4	2.22^{f}	2.97^{d}	3.08^{g}	3.39^{d}
	MA2IR4	1.89^{i}	2.04^{i}	2.98^{h}	2.78^{h}

Legend: MUSO17 - *B. megaterium*-SO17 mutant using UV; WSO17 -*B. megaterium* –SO17 wild type; MAIR4 - *B. subtilis*-IR4 mutant using acridine orange; WIR4 - *B. subtilis* –IR4 wild type; MA2IR4 - *Bacillus subtilis*-IR4 mutant using acridine orange; SA – Sodium alginate*;* AA-Agar Agar; PU-Polyurethane foam.

observed to produce L-glutamic acid in higher yield than the wild strain.

The ability of the mutant *B. subtilis* to produce different quantities of L-glutamic acid is in line with the work of Nakazawa *et al.* (1996). Also, Ganguly (2019) reported that UV irradiation appeared to be more effective mutagen on *Corynebacterium glutamicum* with increase in L-glutamic acid accumulation, up to 7.4 mg/ml. While in *B. megaterium*, the wild type produced L-glutamic acid more than the mutants strains, this variation in L-glutamic acid production may probably be as a result of the non-directional effect of the mutagen used. Although Kinoshita *et al*. (1958), Nakayama *et al.* (1961) and Shah *et al*. (2002) reported a similar induction of nutritional mutants from various amino acid-accumulating strains for the production of ornithine, lysine and valine. Pasha *et al*. (2011) tested the UV and chemical mutation on *C. glutamicum* for increase in glutamic acid productivity.

Cell immobilization

Production of L-glutamic acid in mg/mL using wild and selected immobilized mutant *Bacillus* strains on different supporting matrix is presented in Table 3. L-glutamic acid production by immobilized wild and selected immobilized mutant *Bacillus* strains in sodium alginate, PUF and agar–agar matrix ranged from 1.65^{j} to 4.03^{a} mg/mL. The highest L-glutamic acid was observed in UV mutant of *B. megaterium* on sodium alginate (MUSO17) while the least L-glutamic acid production was observed in acridine orange dye mutant of *B. subtilis* (MA2IR4) immobilized on PUF. Entrapment of the selected mutants and wild types in sodium alginate supported the highest production of L-glutamic acid when compared with other supporting matrix used. High L-glutamic acid production in immobilized cells could be attributed to the strength of ionic interaction and also the stability in the storage of beads (Yugandhar *et al*., 2007).

This research finding is in line with the findings of Pasha *et al*. (2011) which reported high yield of L-glutamic acid (43 g/l) with immobilized (alginate)/chemical mutant in a research work on comparative studies of L-glutamic acid production

Conclusion

Mutation and cell immobilization supported L-glutamic acid production by *B. subtilis* and *B. megaterium,* Acridine orange dye treatment on the *B. subtilis-MAIR4* strain mutant led to higher levels of L-glutamic acid than on the wild-type. Furthermore, in this study, sodium alginate was a better support matrix for L-glutamic acid production. Although cells have been adsorbed onto a variety of L-glutamic acid support materials, this article documents agar-agar as yet another effective support. L-glutamic acid will be used in plant as growth promotor, flavor enhancer and also use as food additives in child foods.

CONFLICT OF INTEREST

The authors declare that they have not conflict of interest.

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