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Full Length Research Paper

Mutational studies for hyper producers of L-glutamic acid from *Bacillus* strains UG1 and OG4 from vegetable proteins

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An investigation was carried out to produce mutant isolates from *Bacillus subtilis* UG1 and *Bacillus licheniformis* OG4 isolated from vegetable proteins using a chemical mutagen N-methyl-N-nitrosoguanidine (NTG) at dosage concentration of 250 µg/ml. The mutant isolates obtained from wild strain of *B. subtilis* UG1 produced mutants (M_1 to M_9) and was screened for hyper producing effect. Mutant M_1UG_1 was obtained. The mutant isolates obtained from wild strain *B. licheniformis* OG4, produced 11 mutants (M_1 to M_{11}) and when screened for hyper producing effect mutant M_6OG_4, was obtained. Hyper-producing mutant strains M_1UG_1 and M_6OG_4 produced L-glutamic acid of 23.0 mg/ml respectively. The quantity produced was higher than the glutamate obtained from *Corynebacterium glutamicum* ATCC 13032 (14 mg/ml). The developed mutant strain was better adapted to a shift in temperature range of 55 to 60°C, pH 9.0 and biotin concentration of 10 µg/ml. As part of the programme to increase glutamate yields and to use materials of agro-industrial wastes, the developed mutants M_1UG_1 and M_6OG_4 were found to utilize the tested agro-industrial wastes maximally for growth and glutamic acid production.

Key words: L-glutamic acid, agro-industrial wastes, mutants, N-methyl-N-nitrosoguanidine (NTG), fermented vegetable proteins.

INTRODUCTION

Mutation can be defined as sudden and spontaneous changes in a gene which changes phenotypic characters (Burrus and Walder, 2004). Since mutation is any heritable changes within the genome, 2 major classes can be identified; chromosomal and point mutation. Chromosomal mutations include changes in structure such as inversions, translocation, deletion, and duplications. Point mutations are alterations of a single base in the genome of an organism. Mutation can arise spontaneously within any cell at a given time during the life cycle or it can be induced. The cause of spontaneous mutation is largely unknown. The effect of cosmic rays radiations from naturally occurring minerals or chemicals present in the environment are inefficient to account for the spontaneous mutation rate observed. Although a number of cell constituents and their by-products are mutagenic, the genome is extra-ordinarily protected from the ill effects of its own metabolite by enzymes capable of destroying such products (Burrus and Walder, 2004; Hasting et al., 2009).

A more frequent and likely cause of spontaneous mutation is the accidental mis pairing of bases during DNA replication (Hasting et al., 2009). Agents that induce mutations are known as mutagens. Mutagens have assumed a great importance as their use has permitted the identification of numerous groups in nearly all types of organisms. Four general groups of mutagens are known in terms of their mode of actions namely: base analogues, direct acting chemicals, alkylating agents, acridine dyes. Other mutagens include U.V and ionizing radiations (Burrus and Walder, 2004). The most widely

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spread mutagens used by bacterial geneticists is N-methyl-N-nitrosoguanidine (NTG). This is because it induces a frequency of mutation at doses, which results in little killings and appear to be the most potent chemical mutagen. Its mutagenic power stems from generation to generations of a diazo methane which induces a G-C, A-T transition (Kozmin et al., 2005).

Considering the various industrial requirements, one could isolate various suitable natural mutants or using the techniques of microbial genetics or developing hyper producing strains that could be genetically manipulated to produce enzyme preparations that could meet the criteria of the industry. Mutants occur spontaneously in bacterial populations. The observed frequency of a particular mutant however is usually very low (less than 10^5).

Fortunately, one can increase the proportions of mutants in a bacterial population by using mutagens or biological agents which introduce genetic changes (Kozmin et al., 2005). These mutagenic substances cause various types of mutation, these are transition, transversion, insertions and deletions. N-methyl-N-nitrosoguanidine (NTG) is a potent mutagen and carcinogen, widely used by bacterial geneticist because it induces a high frequency of mutations at doses, which result in little killing. These mutagens induce primary base substitution at the replication point (Cerdaolmedo et al., 1968). This leads to the clustering of induced mutations. Evidence suggests that the replication origin is at least 200 times more susceptible to mutation by NTG than the remainder of the chromosome under condition widely used for NTG mutagenesis. It is estimated that as many as 50 additional changes occur within a map distance of 2 min of NTG induced mutation (Guerola et al., 1971). The high frequency of double mutation is one disadvantage of NTG, but it is possible not to monitor the level of mutagenesis in each experiment (Cerdaolmedo et al., 1968).

NTG acts as powerful mutagens under conditions in which 50% of cells survive, but significant killing occurred only when the cells were allowed to grow and metabolize in the presence of the mutagens (Kozmin et al., 2005). A maximal yield of Val R. mutant *E. coli* was obtained at NTG concentrations of 100 µg/ml, the observed yield of 100 µg/ml NTG was 11.3% at pH 6.0 for 15 to 30 min. One advantage in the use of NTG is the high probability that every cell will be mutated at more than one site (Guerola et al., 1971).

It is therefore on the basis of the ability of mutant strains from wild isolates using NTG for an enhanced glutamic acid yield, withstand high temperature, high pH value and high biotin concentrations and the making of mutants from wild *Bacillus* strain was sought.

MATERIALS AND METHODS

Strains of *Bacillus* sp.

Bacillus subtilis UG1 and *Bacillus licheniformis* OG4 from fermented vegetable proteins of “Ugba” and “Ogiri” had earlier being reported as the best glutamic acid producing isolates (Lawal et al., 2010).

Obtaining mutant strains as hyper producers for L-glutamic acid production

For these test wild strains of glutamate acid producing isolates namely UG1 and OG4 were chosen for mutational experiments as follows:

Mutation process

Mutation process was carried out as described by Haruo and Takashi (1978). N-methyl-N-nitro-N-nitrosoguanidine (NTG) was used for mutagenesis. A culture strain of *B. subtilis* UG1 and *B. licheniformis* OG4 grown up to late logarithmic phase in 5 ml of synthetic medium (as earlier described) was washed and re-suspended in the same volume of 0.1 M phosphate buffer (pH 7.0) and treated with 250 ug/ml of NTG at 30°C for 15 min. The survival fractions were approximately 15%. The treated cells were washed three times with buffer and spread onto the complete plate medium after dilution. Colonies were formed by incubating these plates at 30°C for several days were mutant from the wild strains.

Selection of hyper producing mutant for L-glutamic acid production

Synthetic medium as earlier described was fortified with different concentrations of L-glutamic acid crystals in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10% (w/v) and 1.5 g (agar technical, oxoid). The molten agar was autoclaved at 121°C for 15 min and later dispensed onto sterile plates and left to solidify. The solidified medium was later streaked with various mutants obtained and incubated at 30°C for 24 to 48 h. Surface growth was observed on the plates.

Physiological studies of the test isolates and mutant strains

The effects of the following processing variables were out on the test isolates and mutant strains using synthetic medium and agro-industrial wastes.

Effects of temperature on growth and L-glutamic acid production by test isolates and mutant strains using synthetic medium and hydrolysate of the agro-industrial waste

This was carried out as described by Haruo and Takashi (1978). Synthetic medium corn, cassava peel hydrolysate and pineapple wastes with composition as earlier described were separately dispensed as 100 ml aliquots into 250 ml Erlenmeyer flasks and sterilized by autoclaving at 115°C for 0 min. 24 h cultures of the test isolates and the mutant strains were inoculated into the medium in the flasks as 1 ml (2.6×10^6 cfu/ml). The inoculated flasks were later incubated at 25, 30, 35, 40, 45, 50, 55, 60, 70 and 80°C in a shaker incubator. Growth measurements of the isolates and L-glutamic acid produced were carried out as earlier described.

Effects of pH on the growth and glutamic acid production by the test isolates and mutant strains using synthetic medium and hydrolysate of agro-industrial wastes

The method of Haruo and Takashi (1978) was used. Synthetic medium, hydrolysate of cassava peel, corn pomace and pineapple

Table 1. Effect of biotin on the growth and glutamic acid production by the isolates in synthetic medium.

Biotin (mg/ml)	Isolates				
	***B. subtilis* UG1**	***B. pumilus* DD4**	***B. licheniformis* OG4**	***B. polymyxa* OG7**	***C. glutamicum* ATCC 13032**
0.1	[a]0.34 ±0.01 [b](3.80 ± 0.05)	(0.32 ± 0.02) (3.4 ± 0.01)	(0.31 ± 0.01) (3.3 ± 0.02)	(0.31 ± 0.02) (3.2 ± 0.05)	(0.32 ± 0.02) (3.2 ± 0.02)
0.5	[a]0.52 ± 0.02 [b](4.4 ± 0.05)	(0.54 ± 0.01) (4.2 ± 0.02)	(0.54 ± 0.22) (4.7 ± 0.01)	(0.6 ± 0.01) (4.4 ± 0.02)	(0.50 ± 0.01) (3.5 ± 0.02)
1.0	[a]1.18 ± 0.01 [b](3.0 ± 0.05)	(1.14 ± 0.03) (2.4 ± 0.04)	(1.10 ± 0.02) (2.4 ± 0.04)	(1.38 ± 0.01) (2.8 ± 0.05)	(1.21 ± 0.01) (3.6 ± 0.05)
5.0	[a]1.3 ± 0.01[b] (2.8 ± 0.05)	(1.22 ± 0.01) (2.6 ± 0.04)	(1.32 ± 0.01) (1.80 ± 0.05)	(1.41 ± 0.05) (2.7 ± 0.01)	(1.32 ± 0.01) (3.3 ± 0.03)
10.0	[a]1.52 ± 0.02 [b](2.6 ± 0.05)	(1.38 ± 0.01) (2.1 ± 0.01)	(1.42 ± 0.01) (1.40 ± 0.02)	(1.16 ± 0.01) (1.90 ± 0.005)	(1.43 ± 0.01) (2.8 ± 0.05)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

wastes were separately prepared with composition as earlier described and their pH adjusted to the following values: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12; using 0.2 M Na_2HPO_4 and 0.1 M citric acid buffer. The medium was sterilized at 115°C for 10 min and later inoculated with 1 ml (2.6×10^6 cfu/ml) of the test isolates and the mutant stains in separate flasks. Incubation was at room temperature for 48 h. Growth of the isolates and L- glutamic acid estimation were carried out as earlier described.

Effect of biotin on the growth and glutamic acid production by the test isolates and mutant strains using synthetic medium

The method of Chatopadhayay and Banerjee (1978) was used. Synthetic medium with composition as earlier describe was fortified with different concentration of biotin, 0.1, 0.5, 1.0, 5.0 µg and 10.0 µg/liter. Sterilization was done at 121°C for 15 min. The sterilized medium was later inoculated with 1 ml (2.6×10^6 cfu/ml) of a 24 h old culture of the test isolate and mutant strains in separate flasks. Incubation was carried out at room temperature (28±2°C). Growth measurements were determined using the spectrophotometer (Miltroy spectronic 20D) at 540 nm, while Glutamic acid concentration was estimated using nihydrin reagent as earlier described by Spies (1957).

Growth and glutamic acid production by mutant strains and *Corynebacterium glutamicum* ATCC 13032 on agro-industrial wastes

For this test, the ability of the mutant isolates of M_1UG_1 and M_6OG_4 from wild strains to utilize the agro-industrial waste were compared with the reference strain to *Corynebacterium glutamicum* ATCC 13032. The hydrolysate of cassava peel, corn pomace and pineapple wastes with composition as earlier described was autoclaved at 121°C for 15 min and thereafter inoculated with a 24 h culture as 1 ml (2.6×10^6 cfu/ml) in separate flasks. The cell growth, glutamic acid production was compared with reference strain.

Growth of isolates in production medium

Growth was measured turbidometrically at 540 nm using a spectrometer (Miltroy spectronic 20D) without centrifugation (Chatopadhayay and Banerjee, 1978).

pH determination

The pH of the fermenting medium was determined with the aid of a previously standardized pH meter (Unicam 9450 model). The pH meter was calibrated using pH 4.0 and 7.0 buffer.

RESULTS

Effect of biotin on growth and L-glutamic acid production

The effect of biotin on the growth and glutamic production by the test isolates was studied and presented in Table 1. Growth and glutamic acid were both enhanced at varying concentrations of biotin by the test isolates. However, the highest concentration of biotin that supported growth and glutamic acid in all the test isolates was 0.5 mg/ml. Highest glutamic acid was observed in *B. licheniformsis* with 4.7 mg/ml and lowest was 3.5 mg/ml by *C. glutamicum* ATCC 13032 (Table 1). Concentrations of biotin above 0.5 mg/ml was observed to favour the growth of the isolates but was however accompanied by a fall in the level of glutamic acid with the lowest being 1.40 mg/ml.

Similarly, the biotin concentration on the growth and glutamic acid production by mutants of *B. subtilis* M_1UG_1, *B. licheniformis* M_6OG_4, and the reference strain *C. glutamicum* ATCC 13032 is presented in Table 2. The mutant isolates were able to grow and produced glutamate at all levels of biotin concentrations. However, comparative study with *C. glutamicum* ATCC 13032 was observed to show an enhanced growth but a fall in the level of glutamic acid at concentrations above 0.5 mg/ml (Table 2).

Obtaining mutant isolates of *B. subtilis* UG1 and *B. licheniformis* OG4 is presented in Tables 3 and 4. The mutant isolate from *B. subtilis* were able to grow at varying concentrations of minimal salts fortified with different concentrations of L-glutamic acid (1 to 5%) while only the mutant isolate M_1 was able to grow beyond 5% level of minimal salts

Table 2. Effect of biotin on the growth and glutamic acid production by the mutants strains of *B. subtilis* M_1UG_1, *B. licheniformis* M_6OG_4, *Corynebacterium glutamicum* ATCC 13032 in synthetic medium.

Biotin (mg/ml)	Isolates		
	***B. subtilis* M_1UG_1**	**Mutant of *B. licheniformis* M_6OG_4**	***C. glutamicum* ATCC 13032**
0.1	[a]1.42 ± 0.01 [b](3.8 ± 0.05)	0.50 ± 0.01 (3.3 ± 0.02)	0.32 ± 0.01 (2.7 ± 0.01)
0.5	[a]1.52 ± 0.01 [b](4.6 ± 0.05)	0.54 ± 0.01(4.4 ± 0.05)	1.48 ± 0.01 (3.4 ± 0.02)
1.0	[a]1.86 ± 0.01 [b](5.20 ± 0.05)	0.73 ± 0.01 (5.20 ± 0.05)	1.37 ± 0.02 (3.0 ± 0.01)
5.0	[a]1.44 ± 0.01 [b](6.4 ± 0.05)	1.20 ± 0.01 (6.9 ± 0.02)	1.84 ± 0.01 (3.0 ± 0.02)
10.0	[a]1.68 ± 0.02 [b](6.8 ± 0.01)	1.38 ± 0.02 (7.20 ± 0.05)	1.92 ± 0.02 (2.0 ± 0.01)

Values represent the mean score (n = 3) ± S.D. a = Growth value (Optical density at 540 nm), b = Glutamic acid (mg/ml).

Table 3. Selection of mutant strains *Bacillus subtilis* UG1 as hyper producers of glutamic acid.

Minimal medium fortified with L- glutamic acid (%w/v)	Mutants								
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	-	-	-	-	-	-	-	-
7	+	-	-	-	-	-	-	-	-
8	+	-	-	-	-	-	-	-	-
9	+	-	-	-	-	-	-	-	-
10	+	-	-	-	-	-	-	-	-

+ = Growth on minimal medium, - = No growth on minimal medium.

Table 4. Selection of mutant strains *Bacillus licheniformsis* OG4 as hyper producer of glutamic acid.

Minimal medium fortified with L- glutamic acid (%w/v)	Mutants										
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}
1	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	+	-	-	-	-	-
6	-	-	-	-	-	+	-	-	-	-	-
7	-	-	-	-	-	+	-	-	-	-	-
8	-	-	-	-	-	+	-	-	-	-	-
9	-	-	-	-	-	+	-	-	-	-	-
10	-	-	-	-	-	+	-	-	-	-	-

+ = Growth on minimal medium, + = No growth on minimal medium.

fortification with L-glutamic acid (Table 3). Similarly, mutant isolates from *B. licheniformis* OG_4, grew on minimal salts fortified with 1 to 5% concentrations of L-glutamic acid while only mutant was able to grow in the minimal salts fortified with L-glutamic acid beyond 5% level (Table 4).

Qualitative analysis of L- glutamic acid from mutant isolate

The result of qualitative analysis of mutant isolate M_1UG_1 and M_6OG_4 was presented in plate 3. The result revealed that the mutant isolate M_1UG_1 and M_6OG_4 had an Rf

Table 5. Effect of temperature on growth and glutamic acid production by test isolates and mutant strains in synthetic medium.

Isolates	Temperature (°C)									
	25	30	35	40	45	50	55	60	70	80
B. subtilis UG1	[a]0.23± 0.01 [b](2.00)	0.28± 0.01 (3.20)	0.42± 0.02 (2.80)	0.28± 0.02 (2.20)	0.25± 0.01 (2.00)	0.22± 0.01 (1.80)	0.18± 0.01 (1.50)	0.14± 0.01 (1.30)	0.15± 0.01 (1.10)	0.90± 0.01 (1.00)
B. pumilus DD4	[a]0.25± 0.02 [b] (2.00)	0.33± 0.01 (3.00)	0.53± 0.01 (2.72)	0.32± 0.02 (2.40)	0.30± 0.01 (2.10)	0.26± 0.00 (2.00)	0.21± 0.01 (1.60)	0.91± 0.01 (1.40)	0.81± 0.01 (1.10)	0.13± 0.01 (0.80)
B. licheniformis OG4	[a]0.32± 0.01 [b] (2.10)	0.41± 0.01 (3.60)	0.63± 0.02 (3.10)	0.50± 0.02 (2.92)	0.30± 0.02 (2.64)	0.23± 0.01 (2.40)	0.20± 0.01 (2.00)	0.81± 0.01 (1.70)	0.16± 0.01 (1.40)	0.± 0.01 (1.20)
B. polymyxa OG7	[a]0.34± 0.01 [b](2.00)	0.40± 0.01 (3.20)	0.58± 0.02 (2.90)	0.32± 0.02 (2.86)	0.25± 0.02 (2.60)	0.20± 0.02 (2.40)	0.18± 0.01 (2.10)	0.17± 0.01 (1.80)	0.13± 0.01 (1.70)	0.13± 0.01 (1.20)
C. glutamicum ATCC 13032	[a]0.42± 0.03 [b](2.40)	0.45± 0.02 (4.10)	0.64± 0.01 (3.90)	0.38± 0.02 (3.60)	0.32± 0.02 (3.10)	0.22± 0.01 (3.00)	0.18± 0.01 (2.80)	0.16± 0.01 (2.70)	0.14± 0.01 (2.40)	0.12± 0.01 (2.20)
B.subtiliis Mutant M_1UG_1	[a]0.25± 0.01 [b](2.30)	0.28± 0.01 (3.70)	0.83± 0.02 (4.10)	0.86± 0.02 (4.30)	1.10± 0.02 (4.70)	1.20± 0.02 (4.90)	1.40± 0.01 (5.10)	1.42± 0.01 (4.70)	0.98± 0.01 (3.90)	0.74± 0.01 (3.70)
B. licheniformis Mutant M_6OG_4	[a]0.24± 0.01 [b](2.20)	0.26± 0.01 (3.64)	0.84± 0.03 (4.00)	0.85± 0.02 (4.50)	1.18± 0.01 (4.90)	1.37± 0.02 (5.20)	1.43± 0.02 (5.30)	1.48± 0.01 (4.60)	0.95± 0.02 (4.30)	0.80± 0.01 (4.00)

Values represent the mean scores (n = 3) ± S.D, a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

value of 0.72 and 0.72 respectively. The reference strain *C. glutamicum* ATCC 13032 and standard L-glutamic acid had Rf value of 0.72 and 0.72 respectively. Rf value as obtained from the mutant isolate M_1UG_1 and M_6OG_4 compares favourably with the reference strain and standard L-glutamic acid.

Effects of temperature on growth and glutamic acid by test isolates and mutant strains

The effects of temperature on the growth of the test isolates and production of L- glutamic acid in synthetic medium are shown in Table 5. The entire test isolates had the glutamic acid production at 30°C. However, *B. subtilis* mutant M_1UG_1 and *B. licheniformis* mutant M_6OG_4 had optimum and glutamic acid production at 55°C.

Studies of the effects of different temperature on the growth and glutamic acid production in cassava peel hydrolysates using test isolates and mutant strains is presented in Table 6. The optimum growth temperature for the test isolates was 30°C, while that of mutant strains M_1UG_1 and M_6OG_4 for *B. licheniformis* respectively was 55°C (Table 6). Studies on the effects of different temperature on hydrolysates of corn pomace and pineapple wastes were carried out on all the test isolates, mutant strains and the reference strains. All the test isolates could grow and produce glutamic acid at temperature of 30°C. However, only the mutant strains of M_1UG_1 and M_6OG_4 was able to grow and produced glutamic acid at optimal temperature of 55°C (Tables 7 and 8).

Effect of pH on growth and glutamic acid by test isolates and mutant strains

Effect of pH on growth and glutamic acid production by the test isolates and mutant strains

Table 6. Effect of temperature on the growth and glutamic acid production by test isolates and mutant strains in cassava peel hydrolysates.

Isolates	Temperature (°C)									
	25	30	35	40	45	50	55	60	70	80
B. subtilis UG1	[a]0.20± 0.01 [b](1.80)	0.28± 0.02 (2.34)	0.47± 0.01 (2.00)	0.43± 0.01 (1.40)	0.37± 0.01 (1.32)	0.32± 0.02 (1.27)	0.28± 0.01 (1.20)	0.25± 0.02 (1.40)	0.20± 0.01 (0.90)	0.18± 0.01 (0.80)
B. pumilus DD4	[a]0.23± 0.02 [b](2.00)	0.32± 0.01 (2.40)	0.50± 0.01 (2.10)	0.45± 0.01 (2.00)	0.40± 0.01 (1.80)	0.34± 0.00 (1.62)	0.27± 0.01 (1.37)	0.25± 0.01 (1.22)	0.19± 0.01 (1.00)	0.16± 0.01 (0.90)
B. licheniformis OG4	[a]0.25± 0.01 [b](2.10)	0.30± 0.02 (3.60)	0.44± 0.02 (3.10)	0.40± 0.02 (2.92)	0.35± 0.02 (2.64)	0.30± 0.02 (2.40)	0.22± 0.02 (2.00)	0.20± 0.01 (1.70)	0.18± 0.01 (1.40)	0.17± 0.02 (1.20)
B. polymyxa OG7	[a]0.23± 0.02 [b](2.00)	0.34± 0.01 (2.30)	0.52± 0.02 (2.00)	0.37± 0.01 (1.72)	0.32± 0.01 (1.64)	0.28± 0.01 (1.50)	0.25± 0.01 (1.40)	0.18±0.02 (1.30)	0.16± 0.01 (1.10)	0.13± 0.02 (1.00)
C. glutamicum ATCC 13032	[a]0.30± 0.02 [b](2.40)	0.47± 0.01 (3.20)	0.62± 0.02 (3.8)	0.50± 0.02 (4.00)	0.42± 0.01 (3.20)	0.40± 0.05 (2.80)	0.38± 0.02 (2.10)	0.30± 0.02 (2.00)	0.27± 0.02 (1.80)	0.17± 0.02 (1.70)
B. subtilis mutant M_1UG_1	[a]0.37± 0.01 [b](2.20)	0.42± 0.02 (3.40)	0.50± 0.01 (3.60)	0.68± 0.01 (4.10)	0.82± 0.01 (4.30)	0.93± 0.01 (5.00)	0.93± 0.01 (5.30)	0.73± 0.01 (5.00)	0.60± 0.01 (4.80)	0.38± 0.01 (4.70)
B. licheniformis Mutant M_6OG_4	[a]0.35± 0.01 [b](2.30)	0.42± 0.01 (3.30)	0.52± 0.02 (3.72)	0.66± 0.02 (4.00)	0.76± 0.02 (4.20)	0.80± 0.02 (5.10)	0.90± 0.01 (5.40)	0.70± 0.02 (5.00)	0.57± 0.01 (4.60)	0.35± 0.02 (4.20)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates, b = Glutamic acid yield.

M_1UG_1 and M_6OG_4 was studied in the synthetic medium, cassava peel, corn pomace hydrolysate and pineapple waste. In the synthetic medium, all test isolates were observed to show an enhanced growth and glutamic acid as the pH was increased from 2 to 8. The highest optical density at pH 8.0 was 0.55 with glutamic acid production of 4.30 mg/ml in *C. glutamicum* ATCC 13032 and the lowest optical density of 0.42 and glutamic acid of 3.92 mg/ml in *B. licheniformis* (Table 9).

However, similar observations as shown in Table 9 also revealed that mutant isolates *B. subtilis* M_1UG_1 and *B. licheniformis* M_6OG_4 were able to grow at pH range of 2 to 9 in synthetic medium. The optical density for growth and glutamic acid was 0.70 and 5.00 mg/ml for mutant M_1UG_1. For M_6OG_4, it was 0.68 for growth and 0.43 mg/ml for glutamic acid.

In the cassava peel hydrolysates, an enhanced growth and glutamic acid level at pH range of 2 to 8 was observed with the highest growth being at an optical density of 0.75 and glutamic acid of 4.00 mg/ml in *C. glutamicum* ATCC 13032 and the least with an optical density growth of 0.64 and 3.10 mg/ml glutamic acid in *B. subtilis* UG1 (Table 10). Similarly, pH range of 2 to 9 was observed for mutant isolates *B. subtilis* M_1UG1 with optical density and glutamic acid being 1.420 and 5.71 mg/ml respectively. For M_6OG4, it was 1.540 and 5.84 mg/ml for optical density and glutamic acid respectively (Table 10).

In corn pomace hydrolsate, an enhanced growth and glutamic acid was observed at pH range of 2 to 8, *C. glutamicum* ATCC 13032 gave the highest growth optical density of 0.88 and glutamic acid yield of 3.40 mg/ml. The least optical density

Table 7. Effect of temperature on the growth and glutamic acid production by test isolates and mutant strains in corn pomace hydrolysates.

Isolates	Temperature (°C)									
	25	30	35	40	45	50	55	60	70	80
B. subtilis UG1	[a]0.18± 0.02 [b](2.00)	0.41± 0.01 (2.30)	0.74± 0.02 (2.10)	0.62± 0.02 (1.90)	0.50± 0.01 (1.70)	0.47± 0.05 (1.58)	0.42± 0.02 (1.40)	0.32± 0.02 (1.20)	0.28± 0.01 (1.10)	0.22± 0.01 (1.00)
B. pumilus DD4	[a]0.21± 0.03 [b](2.00)	0.40± 0.02 (2.40)	0.73± 0.01 (2.20)	0.60± 0.02 (2.00)	0.50± 0.04 (1.92)	0.40± 0.03 (1.80)	0.32± 0.02 (1.74)	0.20± 0.02 (1.54)	0.18± 0.02 (1.42)	0.16± 0.02 (1.40)
B. licheniformis OG4	[a]0.19± 0.02 [b](2.10)	0.38± 0.03 (3.00)	0.68± 0.01 (2.70)	0.58± 0.02 (2.20)	0.55± 0.03 (2.00)	0.42± 0.02 (1.84)	0.37± 0.02 (1.72)	0.30± 0.02 (1.64)	0.32± 0.02 (1.50)	0.28± 0.03 (1.40)
B. polymyxa OG7	[a]0.20± 0.02 [b](2.00)	0.37± 0.02 (2.50)	0.72± 0.02 (2.30)	0.49± 0.03 (2.10)	0.39± 0.01 (2.00)	0.35± 0.02 (1.92)	0.32± 0.02 (1.70)	0.28± 0.02 (1.60)	0.22± 0.02 (1.50)	0.20± 0.02 (1.30)
C. glutamicum ATCC 13032	[a]0.22± 0.02 [b](2.30)	0.42± 0.03 (4.00)	0.64± 0.02 (3.10)	0.47± 0.03 (2.92)	0.42± 0.02 (2.60)	0.38± 0.02 (2.40)	0.32± 0.02 (2.00)	0.24± 0.05 (1.90)	0.18± 0.03 (1.70)	0.15± 0.02 (1.42)
B. subtilis mutant M_1UG_1	[a]0.20± 0.05 [b](2.40)	0.47± 0.02 (3.20)	0.54± 0.03 (4.30)	0.72± 0.03 (5.00)	0.84± 0.02 (5.10)	1.12± 0.03 (5.20)	1.30± 0.05 (5.22)	0.94± 0.02 (5.10)	0.87± 0.02 (4.00)	0.74± 0.02 (3.20)
B. licheniformis Mutant M_6OG_4	[a]0.22± 0.02 [b](2.30)	0.46± 0.02 (3.10)	0.52± 0.05 (3.20)	0.65± 0.03 (4.00)	0.80± 0.05 (4.20)	1.00± 0.03 (4.40)	1.10± 0.02 (5.00)	0.90± 0.04 (4.92)	0.88± 0.05 (4.00)	0.60± 0.02 (3.62)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates, b = Glutamic acid yield.

(growth) of 0.75 and glutamic acid yield of 2.70 mg/ml was observed in *B. polymyxa* (Table 11). Similarly, further enhancement in utilization of corn pomace hydrolysate was observed when mutant isolates were used (Table 11). The result revealed that M_1UG_1 had optical density (growth) of 1.42 and glutamate yield of 5.00 mg/ml. For M_6OG_4, it was an optical density (growth) of 1.46 and glutamate yield of 5.40 mg/ml.

In pineapple peel waste, the test isolate *C. glutamicum* ATCC 13032 gave the highest optical density (growth) of 0.78 and glutamic acid yield of 4.30 mg/ml (Table 12). Similarly, M_1UG_1 in pineapple waste gave an optical density (growth) of 1.40 and 5.10 mg/ml glutamic acid yield. For M_6OG_4, the optical density and glutamic acid yield was 1.24 and 5.40 mg/ml respectively (Table 12).

DISCUSSION

A variety of induced mutations has so far been applied to the production and improvement of industrial micro-organisms especially in the fields of amino acids, nucleic acids-related compounds and antibiotics fermentation industries (Terui, 1972; Abe, 1972; Demain, 1973; Haruo and Takashi, 1978). However, the mutations used in these fields have almost been limited to genetic alterations in enzyme activity and in metabolic regulations, whose phenotypes are not affected by change of cultural circumstances, and conditional mutations of any kind remains unutilized in spite of its potential availability for various industrial purposes. Mutations are studied for the following reasons:

Table 8. Effect of temperature on growth and glutamic acid production by test isolates and mutant strains in pineapple waste.

Isolates	Temperature (°C)									
	25	30	35	40	45	50	55	60	70	80
B. subtiilis UG1	[a]0.24± 0.02 [b](1.90)	0.37± 0.01 (2.50)	0.61± 0.02 (2.10)	0.54± 0.02 (2.00)	0.34± 0.02 (1.84)	0.29± 0.04 (1.72)	0.24± 0.02 (1.60)	0.20± 0.01 (1.40)	0.18± 0.05 (1.30)	0.16± 0.02 (1.20)
B. pumilus DD4	[a]0.26± 0.02 [b](1.80)	0.34± 0.02 (2.62)	0.65± 0.02 (2.20)	0.50± 0.01 (2.00)	0.38± 0.03 (1.80)	0.27± 0.04 (1.64)	0.18± 0.02 (1.20)	0.15± 0.04 (0.80)	0.14± 0.05 (0.70)	0.13± 0.02 (0.64)
B. licheniformis OG4	[a]0.26± 0.03 [b](1.92)	0.34± 0.02 (2.70)	0.66± 0.02 (2.40)	0.52± 0.02 (2.25)	0.42± 0.03 (2.00)	0.31± 0.05 (1.80)	0.27± 0.03 (1.72)	0.22± 0.05 (1.30)	0.19± 0.03 (0.90)	0.16± 0.03 (0.70)
B. polymyxa OG7	[a]0.23± 0.02 [b](1.84)	0.33± 0.02 (2.62)	0.65± 0.02 (2.41)	0.50± 0.02 (2.30)	0.42± 0.05 (2.00)	0.29± 0.02 (1.80)	0.23± 0.05 (1.60)	0.19±0.03 (1.10)	0.16± 0.03 (0.90)	0.14± 0.05 (0.87)
C. glutamicum ATCC 13032	[a]0.28± 0.050 [b](1.93)	0.33± 0.03 (2.80)	0.72± 0.02 (2.62)	0.64± 0.02 (2.30)	0.50± 0.02 (2.00)	0.39± 0.01 (1.84)	0.36± 0.03 (1.42)	0.30± 0.04 (0.92)	0.24± 0.04 (0.70)	0.81± 0.03 (0.65)
B. subtilis mutant M_1UG_1	[a]0.26± 0.02 [b](2.10)	0.47± 0.02 (2.70)	0.64± 0.02 (3.40)	0.84± 0.03 (4.10)	0.94± 0.02 (5.00)	1.12± 0.02 (5.20)	1.32± 0.02 (5.40)	1.40± 0.02 (5.20)	0.84± 0.02 (5.10)	0.74± 0.05 (4.20)
B. lichenformis Mutant M_6OG_4	[a]0.27± 0.02 [b](2.20)	0.45± 0.02 (2.80)	0.63± 0.04 (3.20)	0.73± 0.05 (4.00)	0.84± 0.02 (5.10)	1.10± 0.02 (5.30)	1.28± 0.04 (5.50)	1.38± 0.03 (5.10)	0.80± 0.04 (5.00)	0.70± 0.05 (4.20)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

(1) Auxotrophic and regulatory properties of amino acid found in them
(2) Feedback control mechanism is bypassed since they are feedback insensitive
(3) They are not affected by biotin and hence used to ferment high biotin substrate.
(4) Higher yield of glutamate is produced and are not affected by any physiological changes in fermentation.

Mutational studies for an ornithine fermentation has been achieved by an appreciation of an arginine auxotrophic mutants (Kinoshita et al., 1975b). Homoserine auxotrophic mutants for successful lysine fermentation were also devised (Kinoshita et al., 1975b; Nakayama, 1982). However, mutational studies for *Bacillus* for an enhanced amino acid production has never been recorded in literature, particularly, those to utilize agro-industrial wastes. Attempts were therefore made to carry out genetic mutation on two best glutamic acid yielding strains, *B. subtilis* UG1 and *B. licheniformis* OG4. A chemical mutagen, N-methyl-N-nitro-N-nitrosoguadinine was used because of their high frequency and simplicity (Haruo and Takashi, 1978).

Detailed knowledge accumulated so far on the mechanism of L-glutamic acid fermentation by micro-organisms revealed that specific changes in structure and function of the bacterial cell membrane during the culture is one important factor for the accumulation of a large amount of glutamic acid into the medium. Glutamic acid producing strains in general can produce amino acid only when the cells are changed by special Means from physiological normal glutamate non-

Table 9. Effect of pH on growth and glutamic acid production by test isolates and mutant strains in synthetic medium.

Isolates	pH										
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B. subtiilis UG1	[a]0.18±0.02 [b](1.80)	[a]0.24±0.02 [b](2.30)	[a]0.24±0.02 [b](2.40)	[a]0.33±0.02 [b](2.50)	[a]0.35±0.02 [b](2.84)	[a]0.47±0.02 [b](3.10)	[a]0.49±0.02 [b](3.80)	[a]0.33±0.03 [b](3.62)	[a]0.22±0.03 [b](3.00)	[a]0.18±0.02 [b](2.40)	[a]0.16±0.05 [b](2.00)
B. pumilus DD4	[a]0.18±0.02 [b](1.80)	[a]0.24±0.02 [b](2.62)	[a]0.24±0.02 [b](2.20)	[a]0.35±0.04 [b](2.00)	[a]0.37±0.02 [b](1.80)	[a]0.39±0.02 [b](1.64)	[a]0.47±0.04 [b](1.20)	[a]0.40±0.03 [b](0.80)	[a]0.36±0.03 [b](0.70)	[a]0.31±0.03 [b](0.64)	[a]0.30±0.02 [b](0.50)
B. licheniformis OG4	[a]0.24±0.02 [b](1.94)	[a]0.24±0.02 [b](2.84)	[a]0.24±0.02 [b](3.20)	[a]0.28±0.04 [b](3.30)	[a]0.30±0.03 [b](3.60)	[a]0.39±0.02 [b](3.80)	[a]0.42±0.02 [b](3.92)	[a]0.37±0.02 [b](3.62)	[a]0.34±0.02 [b](3.10)	[a]0.30±0.02 [b](3.20)	[a]0.28±0.03 [b](3.10)
B. polymyxa OG7	[a]0.24±0.02 [b](1.70)	[a]0.24±0.02 [b](2.64)	[a]0.24±0.02 [b](3.20)	[a]0.32±0.05 [b](2.94)	[a]0.33±0.03 [b](2.98)	[a]0.45±0.02 [b](3.10)	[a]0.50±0.02 [b](3.20)	[a]0.44±0.02 [b](3.00)	[a]0.35±0.02 [b](2.80)	[a]0.32±0.02 [b](2.64)	[a]0.30±0.02 [b](2.40)
C. glutamicum ATCC 13032	[a]0.24±0.02 [b](1.94)	[a]0.24±0.02 [b](2.62)	[a]0.24±0.02 [b](2.84)	[a]0.45±0.05 [b](3.10)	[a]0.48±0.02 [b](3.50)	[a]0.52±0.02 [b](4.20)	[a]0.55±0.02 [b](4.30)	[a]0.49±0.03 [b](4.00)	[a]0.41±0.02 [b](2.80)	[a]0.37±0.02 [b](2.90)	[a]0.34±0.02 [b](2.00)
B. subtilis Mutant M_1UG_1	[a]0.24±0.02 [b](1.90)	[a]0.24±0.02 [b](2.58)	[a]0.24±0.02 [b](3.00)	[a]0.42±0.05 [b](3.70)	[a]0.50±0.02 [b](3.84)	[a]0.63±0.02 [b](4.00)	[a]0.68±0.05 [b](4.37)	[a]0.70±0.02 [b](5.00)	[a]0.66±0.02 [b](4.90)	[a]0.60±0.02 [b](4.70)	[a]0.40±0.02 [b](4.00)
B. lichenformis Mutant M_6OG_4	[a]0.40±0.02 [b](1.92)	[a]0.24±0.02 [b](2.64)	[a]0.24±0.02 [b](3.10)	[a]0.47±0.04 [b](3.25)	[a]0.48±0.02 [b](3.40)	[a]0.59±0.04 [b](3.72)	[a]0.62±0.02 [b](4.00)	[a]0.68±0.02 [b](4.30)	[a]0.63±0.02 [b](4.00)	[a]0.60±0.04 [b](3.80)	[a]0.42±0.02 [b](3.00)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

excreting state to rather abnormal glutamate excreting state. The special means devised include:

(a) Limitation of biotin in fermentation medium
(b) Additional of surfaced active agents such as between 60 or an antibiotic such as penicillin to biotin rich medium
(c) Addition of a limited amount of substances related to membrane structure such as Oleic acid (Huchenq et al., 1984). On the whole, the test isolates of *B. subtilis* UG1, *Bacillus pumilus* DD4, *B. licheniformis* OG4 and *Bacillus polymyxa* OG7 produced glutamic acid which were lower than the reference strain *C. glutamicum* ATCC 13032. The reason for this might probably be as a result of the nature of the strain used as well as the strain inability to produce phosphoenol pyruvate carboxylase and alpha-ketoglutarate dehydrogenase (Shiio and Ujigawa, 1980). The need to improve production yields for L-glutamic acid through mutations from high producing tests isolates of UG1 and OG4 became very necessary. Mutant isolates M_1UG_1 and M_6OG_4 from test isolates of UG1 and OG4 developed were able to realize a large amount of L-glutamic acid in a biotin rich media, extreme of temperature as well as an increased level of pH in the fermentation medium of the agro-industrial wastes. The present study on the test isolates *B. subtilis* UG1, *B. pumilus* DD4, *B. licheniformis* OG4 and *B. polymyxa* OG7 and the reference strain *C. glutamicum* ATCC 13032 could not ferment the medium for glutamic acid at temperature of 35°C, high biotin concentration, and pH values above 8. Mutant strains M_1UG_1 and M_6OG_4 developed can be described

Table 10. Effect of pH on the growth and glutamic acid production by test isolates and mutant strains in cassava peel hydrolysates.

Isolates	pH										
	2	3	4	5	6	7	8	9	10	11	12
B. subtiilis UG1	[a]0.18±0.02 [b](1.84)	[a]0.20±0.02 [b](1.92)	[a]0.30±0.02 [b](2.10)	[a]0.50±0.02 [b](2.30)	[a]0.58±0.02 [b](2.84)	[a]0.60±0.02 [b](2.90)	[a]0.64±0.02 [b](3.10)	[a]0.54±0.05 [b](3.00)	[a]0.16±0.05 [b](2.00)	[a]0.20±0.02 [b](2.40)	[a]0.16±0.02 [b](2.00)
B. pumilus DD4	[a]0.22±0.05 [b](1.92)	[a]0.30±0.02 [b](2.20)	[a]0.35±0.02 [b](2.40)	[a]0.54±0.02 [b](2.54)	[a]0.63±0.02 [b](2.74)	[a]0.68±0.02 [b](3.00)	[a]0.70±0.02 [b](3.30)	[a]0.54±0.02 [b](3.00)	[a]0.30±0.02 [b](2.00)	[a]0.35±0.02 [b](2.64)	[a]0.30±0.02 [b](2.50)
B. licheniformis OG4	[a]0.21±0.03 [b](1.80)	[a]0.34±0.02 [b](2.20)	[a]0.42±0.02 [b](2.40)	[a]0.54±0.02 [b](2.60)	[a]0.62±0.02 [b](2.72)	[a]0.68±0.03 [b](2.94)	[a]0.72±0.04 [b](3.20)	[a]0.58±0.04 [b](3.00)	[a]0.37±0.02 [b](2.00)	[a]0.42±0.02 [b](2.40)	[a]0.28±0.02 [b](3.00)
B. polymyxa OG7	[a]0.24±0.02 [b](1.74)	[a]0.37±0.02 [b](1.90)	[a]0.43±0.02 [b](2.00)	[a]0.57±0.02 [b](2.40)	[a]0.64±0.02 [b](2.54)	[a]0.70±0.02 [b](2.88)	[a]0.72±0.02 [b](3.40)	[a]0.62±0.02 [b](3.00)	[a]0.36±0.02 [b](2.20)	[a]0.40±0.04 [b](2.40)	[a]0.30±0.02 [b](2.40)
C. glutamicum ATCC 13032	[a]0.25±0.02 [b](1.84)	[a]0.38±0.02 [b](2.20)	[a]0.49±0.02 [b](2.60)	[a]0.60±0.02 [b](3.10)	[a]0.68±0.02 [b](3.40)	[a]0.72±0.02 [b](3.84)	[a]0.75±0.02 [b](4.00)	[a]0.60±0.02 [b](3.60)	[a]0.32±0.02 [b](2.70)	[a]0.43±0.02 [b](3.10)	[a]0.34±0.02 [b](2.00)
B. subtilis Mutant M_1UG_1	[a]0.28±0.02 [b](1.94)	[a]0.58±0.04 [b](2.30)	[a]0.71±0.04 [b](3.10)	[a]0.84±0.04 [b](3.20)	[a]0.92±0.02 [b](4.30)	[a]1.20±0.02 [b](5.20)	[a]1.30±0.02 [b](5.60)	[a]1.42±0.02 [b](5.71)	[a]0.75±0.02 [b](4.00)	[a]0.80±0.02 [b](4.20)	[a]0.40±0.02 [b](4.00)
B. lichenformis Mutant M_6OG_4	[a]0.30±0.02 [b](1.94)	[a]0.64±0.02 [b](2.25)	[a]0.74±0.02 [b](3.30)	[a]0.90±0.02 [b](3.50)	[a]0.92±0.02 [b](4.10)	[a]1.35±0.02 [b](4.80)	[a]1.40±0.02 [b](5.30)	[a]1.54±0.02 [b](5.84)	[a]0.84±0.02 [b](4.10)	[a]0.92±0.02 [b](5.00)	[a]0.42±0.02 [b](3.00)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

as a novel strain of microorganisms which are better adapted to producing a high yield of glutamic acid than the reference strain ATCC, 13032. This observation agrees with the findings of Nampoothiri et al. (2002) that the developed temperature sensitive L-glutamic acid and producing strain *C. glutamicum* with strongest cardiolipin synthetase inactivation. Also, Delaunay et al. (2002), observed a flexibility in the metabolism of *C. glutamicum* 2262, a glutamic acid producer in response to temperature shifts from 33 to 40°C.

In other to obtain high yields of L-glutamic acid, the biotin concentration in the medium must be strictly controlled at a sub-optimal level for maximum cell growth (Hirose et al., 1985; Shioo and Ujigawa, 1862). Biotin concentration of 0.5 µg/ml allowed for the isolates as well as the reference strains for growth and glutamic acid. However, concentrations above 0.5 µg/ml allows for cell growth and decrease in glutamic acid production. The mutant strain M_1UG_1 and M_6OG_4 from the test isolates of UG1 and OG4 were able to grow and produce glutamic acid at biotin concentrations above 5 µg/ml. The developed mutant has merits of the novel fementation process in that;

(1) The use of chemical agent such as surface active agents or antibiotics which are generally expensively used in the fermentation medium rich in biotin is avoided.
(2) The use of expensive additives to natural carbon source of plant origin in which biotin is abundantly distributed is also prevented. Similar observations were obtained by Haruo and Takashi

Table 11. Effect of pH on the growth and glutamic acid production by test isolates and mutant strains in corn pomace hydrolysates.

Isolates	pH										
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B. subtiilis UG1	[a]0.19±0.02 [b](1.70)	[a]0.28±0.02 [b](1.90)	[a]0.37±0.02 [b](1.94)	[a]0.54±0.02 [b](2.10)	[a]0.64±0.02 [b](2.30)	[a]0.72±0.02 [b](2.70)	[a]0.73±0.02 [b](2.82)	[a]0.54±0.02 [b](2.60)	[a]0.50±0.05 [b](2.40)	[a]0.42±0.02 [b](2.10)	[a]0.29±0.02 [b](1.97)
B. pumilus DD4	[a]0.22±0.02 [b](1.75)	[a]0.35±0.02 [b](1.84)	[a]0.57±0.02 [b](1.93)	[a]0.60±0.02 [b](2.54)	[a]0.65±0.02 [b](2.74)	[a]0.73±0.02 [b](3.00)	[a]0.78±0.02 [b](3.30)	[a]0.73±0.02 [b](3.00)	[a]0.62±0.02 [b](2.00)	[a]0.55±0.02 [b](2.64)	[a]0.43±0.02 [b](2.50)
B. licheniformis OG4	[a]0.17±0.04 [b](1.80)	[a]0.30±0.02 [b](1.94)	[a]0.43±0.02 [b](2.20)	[a]0.64±0.02 [b](2.30)	[a]0.79±0.02 [b](2.64)	[a]0.73±0.02 [b](2.82)	[a]0.78±0.04 [b](2.94)	[a]0.65±0.02 [b](3.00)	[a]0.60±0.02 [b](2.00)	[a]0.40±0.02 [b](2.10)	[a]0.32±0.02 [b](2.00)
B. polymyxa OG7	[a]0.20±0.02 [b](1.70)	[a]0.33±0.02 [b](1.92)	[a]0.50±0.02 [b](1.98)	[a]0.50±0.02 [b](2.20)	[a]0.58±0.02 [b](2.54)	[a]0.72±0.02 [b](2.40)	[a]0.75±0.02 [b](2.70)	[a]0.64±0.02 [b](2.60)	[a]0.36±0.02 [b](2.40)	[a]0.54±0.02 [b](2.20)	[a]0.30±0.02 [b](2.40)
C. glutamicum ATCC 13032	[a]0.21±0.02 [b](1.75)	[a]0.37±0.02 [b](1.94)	[a]0.62±0.02 [b](2.20)	[a]0.66±0.02 [b](2.80)	[a]0.68±0.02 [b](3.40)	[a]0.82±0.02 [b](3.30)	[a]0.88±0.02 [b](3.40)	[a]0.64±0.02 [b](3.10)	[a]0.61±0.04 [b](3.00)	[a]0.54±0.04 [b](2.64)	[a]0.34±0.02 [b](2.50)
B. subtilis Mutant M_1UG_1	[a]0.22±0.02 [b](1.80)	[a]0.47±0.02 [b](2.20)	[a]0.64±0.02 [b](3.10)	[a]0.73±0.02 [b](3.30)	[a]0.92±0.02 [b](4.20)	[a]1.00±0.02 [b](4.50)	[a]1.30±0.02 [b](4.80)	[a]1.42±0.02 [b](5.00)	[a]0.75±0.02 [b](4.70)	[a]0.83±0.02 [b](4.30)	[a]0.75±0.02 [b](4.00)
B. lichenformis Mutant M_6OG_4	[a]0.20±0.02 [b](1.92)	[a]0.54±0.02 [b](2.60)	[a]0.70±0.02 [b](2.84)	[a]0.84±0.02 [b](3.40)	[a]0.86±0.02 [b](4.50)	[a]1.00±0.02 [b](4.70)	[a]1.25±0.02 [b](5.00)	[a]1.40±0.02 [b](5.40)	[a]0.87±0.02 [b](4.82)	[a]0.78±0.02 [b](4.72)	[a]0.69±0.02 [b](4.20)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

(1978), which developed mutants of 23, 51 and 88 from wild strains of *Brevibacterium lactofermntum* strain 2256 using chemical mutagens of N-methyl-N-nitro-N-nitrosoguadinine at 250 µg/l. The mutants strain M_1UG_1 and M_6OG_4 were able to grow in high biotin medium for an enhanced growth as well as for high amounts of L-glutamic acid than the reference strain ATCC 13032. The developed mutant isolates could therefore be exploited for practical fermentation process on large-scale production of glutamic acid from substrates of sugar beet and molasses which are high in biotin.

The test isolates (UG1, DD4, OG4, and OG7) and reference strain ATCC 13032 clearly showed that temperatures of 30°C are needed for growth and glutamic acid production. However, temperature above 30°C in any of the agro-industrial wastes utilized as substrates lead to a fall in growth and glutamic acid production.

The mutant isolates M_1UG_1 and M_6OG_4 from test isolates of *B. subtilis* UG1, and *B. licheniformis* OG4 were able to grow above 50°C. The implication of temperature shift is that the expense required for the cooling of the heat generated is cut down since thermo tolerant strains will be used. The mutant strains M_1UG_1 and M_6OG_4 were able to change from non-glutamate excreting cells of the test isolates to an excreting state. The mutant strains were also highly sensitive to increased pH of 9.0 unlike the test isolates and the reference strains that had maximum pH value of 8.0. The mutant isolates produced 18 to 24.5 g/ml of L-glutamic acid while the reference strain ATCC 13032 produced 13 to

Table 12. Effect of pH on the growth and glutamic acid production by test isolates and mutant strains in corn pomace hydrolysates.

Isolates	pH										
	2	3	4	5	6	7	8	9	10	11	12
B. subtiilis UG1	[a]0.22±0.02 [b](2.10)	[a]0.23±0.02 [b](2.40)	[a]0.24±0.02 [b](2.68)	[a]0.32±0.02 [b](3.20)	[a]0.42±0.02 [b](3.70)	[a]0.65±0.02 [b](4.10)	[a]0.67±0.02 [b](4.15)	[a]0.54±0.02 [b](4.00)	[a]0.38±0.02 [b](3.70)	[a]0.24±0.02 [b](3.00)	[a]0.18±0.02 [b](2.80)
B. pumilus DD4	[a]0.20±0.02 [b](2.00)	[a]0.33±0.04 [b](2.30)	[a]0.60±0.02 [b](2.50)	[a]0.68±0.02 [b](2.72)	[a]0.70±0.02 [b](2.94)	[a]0.75±0.02 [b](3.00)	[a]0.80±0.02 [b](3.40)	[a]0.73±0.02 [b](3.00)	[a]0.60±0.02 [b](2.00)	[a]0.55±0.02 [b](2.60)	[a]0.40±0.02 [b](2.30)
B. licheniformis OG4	[a]0.21±0.02 [b](2.12)	[a]0.23±0.02 [b](2.39)	[a]0.25±0.02 [b](2.45)	[a]0.33±0.02 [b](2.60)	[a]0.44±0.02 [b](2.84)	[a]0.64±0.02 [b](3.10)	[a]0.69±0.02 [b](3.85)	[a]0.65±0.02 [b](2.70)	[a]0.36±0.02 [b](3.00)	[a]0.25±0.02 [b](2.72)	[a]0.16±0.02 [b](2.60)
B. polymyxa OG7	[a]0.23±0.02 [b](2.00)	[a]0.33±0.02 [b](2.30)	[a]0.42±0.02 [b](2.62)	[a]0.54±0.02 [b](2.80)	[a]0.58±0.02 [b](2.94)	[a]0.62±0.02 [b](3.10)	[a]0.64±0.02 [b](3.40)	[a]0.64±0.02 [b](2.60)	[a]0.38±0.02 [b](2.80)	[a]0.34±0.02 [b](2.60)	[a]0.29±0.02 [b](2.40)
C. glutamicum ATCC 13032	[a]0.29±0.02 [b](2.05)	[a]0.42±0.02 [b](2.20)	[a]0.53±0.04 [b](2.42)	[a]0.64±0.02 [b](2.60)	[a]0.66±0.02 [b](3.80)	[a]0.70±0.03 [b](3.90)	[a]0.78±0.02 [b](4.30)	[a]0.64±0.02 [b](3.10)	[a]0.58±0.02 [b](3.70)	[a]0.42±0.02 [b](3.40)	[a]0.37±0.02 [b](3.10)
B. subtilis Mutant M_1UG_1	[a]0.38±0.02 [b](2.10)	[a]0.54±0.02 [b](2.30)	[a]0.72±0.02 [b](3.00)	[a]0.78±0.02 [b](3.70)	[a]0.84±0.02 [b](3.94)	[a]0.97±0.02 [b](4.30)	[a]1.10±0.02 [b](4.70)	[a]1.40±0.02 [b](5.10)	[a]0.90±0.02 [b](4.84)	[a]0.82±0.02 [b](4.20)	[a]0.80±0.02 [b](4.00)
B. lichenformis Mutant M_6OG_4	[a]0.36±0.02 [b](2.30)	[a]0.50±0.02 [b](2.50)	[a]0.68±0.02 [b](3.10)	[a]0.70±0.02 [b](3.84)	[a]0.73±0.02 [b](3.98)	[a]0.84±0.02 [b](4.32)	[a]1.00±0.02 [b](4.84)	[a]1.24±0.02 [b](5.30)	[a]0.84±0.02 [b](4.92)	[a]0.70±0.02 [b](4.50)	[a]0.64±0.02 [b](4.00)

Values represent the mean scores (n = 3) ± S.D. a = Growth of the isolates (Optical density), b = Glutamic acid yield (mg/ml).

14 mg/ml of glutamic acid in pineapple waste medium.

In the extra cellular production of desired amino acid or regulatory controls are required, several earlier workers (Kinoshita et al., 1957b; Kinoshita, 1963; Nakayama, 1982) made attempts to induce auxotrotrophic mutants through feedback control mechanisms by limiting the intracellular accumulation of feedback inhibitors or repressors. The purpose of regulatory mutants is to bypass feedback control mechanisms by using feedback insensitive to end product inhibition or to end product repressors. The use of mutant isolates of M_1UG_1 and M_6OG_4 that are insensitive to temperature rise will help to cut down the expenses for cooling the heat generated during fermentation. This novel strain mutant will be of immense benefits and help to expand the span of fermentation industry in the future through approaches to the pilot industrial purposes for L-glutamic acid production.

The present study has exploited the use of agro-industrial wastes of cassava peel, corn pomace and pineapple as potential substrates for L-glutamic acid and hence the pollutional effect produced through their indiscriminate disposal is thus prevented.

The present research is targeted at solving some of those problems associated with our industries involved in the production of industrial amino acids. The strategy is to look for organisms having useful characters in fermentation for L-glutamic acid and hence their use as starter cultures.

Also mutation of high producing test isolates of *B. subtilis* UG1 and *B. licheniformis* OG4, to produce M_1UG_1 and M_6OG_4 which are high yielding strains with useful and desirable character that are insensitive to high biotin, temperature and pH

than the reference strain *C. glutamicum;* ATCC 13032.

In the present study mutant *Bacilli* having character such as insensitivity toward temperature shift pH and high biotin contents have been identified and would go a long way at solving the long standing problem of our food industries.

In future, application of DNA recombination techniques to the improvement of L-glutamic acid producing bacteria is another promising route. Several kinds of plasmids relating to streptomycin resistance could be used as a possible vector system. Construction of a chimera plasmid involving a gene associated with L-glutamic acid biosynthesis could also be performed. The possible clone of gene for starch hydrolysis and for L-glutamic acid could be packaged into a single strain and both functions exhibited at the same time instead of carrying out the functions separately.

Conclusion

This study has established that Agro-industrial wastes of cassava peel, corn pomace and pineapple wastes are suitable for the production of L-glutamic acid using organisms isolated from vegetable proteins. The organisms include *B. subtilis, B. licheniformis, B. polymyxa* and *B. pumilus.* The amount of L-glutamic acid produced by them compared favourably with that produced by the reference strain *C. glutamicum* ATCC 13032.

Mutagenic studies on two of the best test isolates *B. subtilis,* UG1 and *B. licheniformis* OG4 produced mutant strains M_1UG_1 and M_6OG_4 which were able to produce a yield of 18 to 24.5 mg/ml of L-glutamic acid from pineapple wastes. This is considerably more than the highest yield recorded in the literature produced by any organism including *C. glutamicum* ATCC 13032, the reference standard strain. These mutant strains were able to withstand a shift in temperature to 50°C, insensitive to increase pH of 9.0 and a biotin content of above 5 µg/ml unlike the earlier and standard strains that could not perform adequately well under these conditions.

Hence, the use of mutant strains M_1UG_1 and M_6OG_4 for the production of L-glutamic acid from locally available agro-industrial wastes will be of immense benefits to our food industries especially those that depend on flavour enhancing L-glutamic acid in their products. This will save these industries the huge foreign exchange which they are currently spending on the information of this item.

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