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A Study of Naturally-occurring L-glutamic Acid Producing Bacteria From Tropical Soil and Aquatic Environments.

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ABSTRACT

Microbial means of L-glutamic acid production have advantage of directly yielding optical and biological active form of the amino acid. The production of L-glutamic acid was investigated using bacterial isolates obtained from tropical soil and water samples obtained from Lagos, Nigeria. L–glutamic acid producing bacterial strains were isolated and identified using the Analytical Profile Index (API) Test 50CHB/20E kit. The L-Glutamic acid production was tested on Luria Bertani Broth and determined by qualitative and quantitative approaches. Six (6) isolates were L-Glutamate producers of which the best producing isolates were *Paenibacillus alvei* and *Corynebacterium glucuronolyticum – seminale* giving a yield higher than 0.7 g/L. The best L-glutamic acid producing isolates were further tested to determine the effects of a variety of carbon and nitrogen sources as well as varying concentration of biotin on the yield of the amino acid produced. Glucose gave yields of 1.19 g/L and 1.05 g/L of L-glutamic acid for *Paenibacillus alvei* and *Corynebacterium glucuronolyticum–seminale* respectively, while ammonium chloride and optimum biotin concentration at 2µg gave yields of 1.15 g/L and 2.09 g/L as well as 1.59 g/L and 1.75 g/L respectively. These bacterial strains therefore offer tremendous promise as candidates for further manipulation for enhanced production of L-glutamic acid using indigenous bacteria from the tropical environment.

INTRODUCTION

Amino acids have been commercially harnessed as feed supplement, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals (Shyamkumar *et al.*, 2014). Interest in L-glutamic acid as the first amino acid to be produced by fermentation on a large scale was stimulated by the increasing demand for monosodium glutamate as a flavour enhancing agent. It is presently consumed worldwide in the form of monosodium salt as a flavour enhancer in foods (Ikeda, 2003). Microbial means of L-glutamic acid production have the advantage of yielding exclusively optically active and biologically required L-form of glutamic acid directly (Amin and Al- Talhi, 2007). *Corynebacterium* sp., *Brevibacterium* sp., *Microbacterium* sp. are among the patent glutamic acid-producing strains by direct fermentation and are collectively referred to as glutamic acid bacteria (Okamoto and Ikeda, 2000).

Numerous studies have reported the production of glutamic acid by various microorganisms like Lactic acid Bacteria (Zarajen *et al.,* 2012) and *Bacillus* sp. (Lawal *et al.,* 2011). L-glutamic acid producing bacteria have been isolated from the soil, sewage wastewater, vegetables, condiments and aquatic environments (Shakoori *et al.,* 2012, Lawal *et al.,* 2011, Fodou *et al.,* 2002).

Studies have therefore been carried out on factors affecting glutamic acid production as well as the yield (Lawal *et al.,* 2011). Optimum conditions for production of glutamic acid by *Corynebacterium glutamicum* are obtained when the fermentation media contain low amounts of biotin (Vijayalaskim and Sarvamangala, 2011). Large numbers of carbon sources and nitrogen sources have been used in L-glutamic production via microbial fermentation (Gupta *et al.,* 2002).

Recent studies have shown that vast numbers of glutamic acid producing *Bacillus* species are emerging. In the present study, natural glutamic acid producing bacteria were isolated from tropical soil and aquatic environments while the effects of carbon, nitrogen and biotin concentration on growth and glutamic acid yield were investigated and hereby reported.

MATERIALS AND METHODS

Bacterial strains were isolated from soil samples and wastewater sample collected from University of Lagos and Vitabiotics Pharmaceutical firm Ikeja, Lagos respectively on Nutrient agar plates. Each isolate was tested for L-glutamic acid-production on Luria Bertani broth. Initial and final pH of the fermenting medium, quantitative and qualitative determinations of L-glutamic acid and cell growth were carried out.

The isolates were characterized using standard biochemical tests and Analytical Profile Index (API) Test Kit 50CHB/20E, used for the study of carbohydrate metabolism of organisms especially *Bacillus* species.

Qualitative Determination of L-Glutamic Acid

Fermenting culture media of the isolates were centrifuged at 5,000 rpm for 10 minutes, while 20µl portions of the supernatants were screened for L-glutamic acid production using Thin Layer chromatography developed in a solvent mixture of n- butanol, acetic acid and distilled water (4:1:1 v/v) in ascending order for 6 h. The chromatogram was dried, sprayed with 0.2 % (w/v) ninhydrin in ethanol and heated in oven at 110ºC for 3minuties, violet coloured spots in respect to RF value of standard glutamic acid were compared for intensity (Lawal *et al.*,2011, Okamoto and Ikeda, 2000).

Quantitative Determination of L-Glutamic Acid

Fermented culture was centrifuged at 800 rpm are one millilitre of the supernatant was added to 1.0 ml of ninhydr in reagent. The absorbance of the resulting coloured solution was read using spectrophotometer at 570 nm against a blank, according to Spies (1957). The glutamic acid content in the sample was determined by reference to a standard curve of graded concentrations of L –glutamic acid.

Effect of Carbon Sources on L-Glutamic Acid Production

The effect carbon sources were investigated using different carbon sources in the fermenting medium which include glucose, maltose, sucrose, xylose, sorbose and naphthalene at 2% (w/v).

Effect of Nitrogen on L-Glutamic Acid Production

The effect of different Nitrogen sources were investigated using NH_4Cl, $(NH_4)_2SO_4$, KNO_3, $NaNO_3$ and urea at 1% (w/v) concentrations.

Effect of Biotin Concentration on L-Glutamic Acid Production

The effect of biotin concentration were investigated which include 2µg, 5µg, 10µg, 20µg and 30µg respectively and adjusting the pH of the fermenting medium to 7.2.

RESULTS

Selection of L – Glutamic Acid-producing Bacteria

Out of twenty – six isolates obtained from soil (19) and water (7) samples only six isolates produced L-glutamic acid as shown in Table 1. The isolates were identified on the basis of cultural and biochemical characteristics shown in Table 2 as species of *Bacillus, Enterobacter, Corynebacterium* and *Paenibacillus*.

Table 1: Bacterial Isolates obtained from soil and Aquatic environment

Habitat	Total Isolates	Producers	Non producers	% Producers
Soil	19	3	16	15.8
Water	7	3	4	42.9
Total	**26**	**6**	**20**	**23.1**

Table 2: Biochemical and Morphological Characteristics of Microorganisms Isolated from Environmental samples

Biochemical characteristics	Sg 12	W28	W29	W30	Sg10	S2
Colour	Dull cream	Dull cream	Dirty cream	Cream	Cream	Dull cream
Gram reaction	+ rod	+rod	+rod	-rod	+short rod	+short rod
Cellular Morphology	Flat. Smooth, translucent	Smooth, spreading, translucent	Flat. Smooth, translucent	Filamentous translucent	Spreading, flat, opaque	Slimy, smooth spreading
Catalase test	+	+	+	+	+	+
Oxidase test	+	+	+	-	-	+
Indole test	-	-	-	-	-	-
Motility test	+	+	+	+	+	+
Methyl Red	+	+	-	-	-	+
Voges Proskauer	-	-	+	+	+	+
Urease activity	-	-	-	+	-	-
Citrate Utilization	-	-	-	+	-	-
Starch Hydrolysis	+	+	+	+	+	+
Gelatin Hydrolysis	+	+	-	+	-	+
Caesin Hydrolysis	+	+	-	-	+	+
H_2S Production	-	-	-	-	+	-
Spore test	+	+	+	-	+	+
NO_3 reduction	+	+	+	-	+	-
Growth on MacConkey	+	+	+	+	-	+
Glucose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-
Lactose	-	-	-	-	+	-
Xylose	-	-	+	+	+	-
Trehalose	+	+	+	-	+	+
Rhamnose	-	-	-	-	+	+
Inositol	-	+	+	-	-	+
Sorbitol	+	-	-	-	-	-
Maltose	+	+	+	+		+
Mannitol	+	+	+	+	-	-
Raffinose	-	-	-	+	-	-
Fructose	+	+	+	+	+	+
Salicin	-	-	-	+	-	-
Probable Identity	*Bacillus firmus*	*Bacillus firmus*	*Bacillus validus*	*Enterobacter cloacae*	*Corynebacterium glucuronolyticum – seminale*	*Paenibacillus alvei*

Table 3 shows the concentration of L-glutamic acid produced by the six isolates after four (4) days of growth on fermentation medium. It was noted that only two strains yielded L-glutamic acid above 0.70g/L. The two high-yielding strains (*Corynebacterium glucuronolyticum-seminale* and *Paenibacillus alvei*) were selected for further studies

Table 3: Evaluation of L-glutamic acid production by bacteria from soil and water samples

Bacterial Isolates	Cell density (A540nm)	Final pH	L-glutamic acid (g/L)
Bacillus firmus	1.188	4.9	0.22
Bacillus validus	1.215	5.1	0.63
Bacillus firmus	0.820	5.2	0.27
Corynebacterium gluconolyticum – seminale	1.329	6.1	0.77
Paenibacillus alvei	1.943	6.5	0.92
Enterobacter cloacae	0.931	5.0	0.52

Effect of carbon sources on L-glutamic acid production

The effect of carbon sources on the growth of the selected strain for glutamic acid yield is presented in Table 4. Glucose, sucrose and maltose best supported the growth of both isolates. The best utilized carbon source was glucose for L – glutamic acid production with a yield of 1.19 g/L and 1.05 g/L by *Corynebacterium glucuronolyticum -seminale* and *Paenibacillus alvei* respectively as shown in Fig. 1.

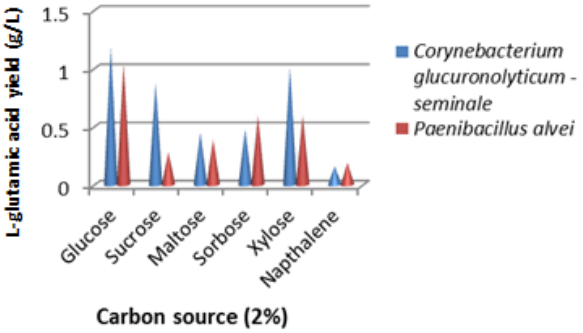

Fig. 1: Comparison of the glutamic yield by isolates *Corynebacterium glucuronolyticum – seminale* and *Paenibacillus alvei* in different carbon sources

Effect of Nitrogen sources on L-glutamic acid production

The effect of Nitrogen sources on growth of the selected strains for glutamic acid is presented in Table 4. Ammonium chloride was the best utilized nitrogen source for L-glutamic acid production while ammonium sulphate was more suitable for cell growth. Potassium nitrate and Sodium nitrate were poorly utilized for L-glutamic acid production by both test isolates as indicated in Fig. 2.

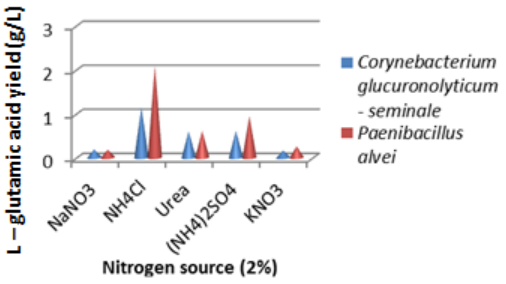

Fig. 2: Comparison of the glutamic yield by isolates *Corynebacterium glucuronolyticum – seminale* and *Paenibacillus alvei* in different Nitrogen source

Effect of biotin concentration on L-glutamic acid production

The effects of biotin concentrations on growth of the selected strain for glutamic acid are also presented in Table 4. At varying concentrations the test isolates utilized the substrate differently despite the same growth conditions. As shown in Table 4, the highest growth observed for *Corynebacterium glucuronolyticum-seminale* at 10µg/L while highest cell growth for *Paenibacillus alvei* occurred at

5µg/L and the highest amount glutamic acid for both strains occurred at a biotin concentration of 2µg/L as presented in Fig. 3.

Table 4: Comparison of Effects of Carbon, Nitrogen and Biotin concentration on Growth and L-glutamic acid yield

		Corynebacterium glucuronolyticum – seminale		*Paenibacillus alvei*	
Substrate @ 96h		**Growth Difference (Final – Initial) OD (540nm)**	**L-glutamic acid Yield (g/L)**	**Growth Difference (Final – Initial) OD (540nm)**	**L-glutamic acid Yield (g/L)**
Carbon sources	**Glucose**	1.24	1.19	1.38	1.05
	Sucrose	1.15	0.88	0.90	0.28
	Maltose	0.94	0.45	1.03	0.39
	Sorbose	0.15	0.48	0.20	0.60
	Xylose	0.80	1.01	0.23	0.60
Nitrogen sources	**Naphthalene**	0.15	0.16	0.16	0.19
	$NaNO_3$	0.49	0.19	0.45	0.18
	NH_4Cl	0.47	1.15	0.43	2.09
	Urea	0.39	0.60	0.43	0.61
	$(NH_4)2SO_4$	0.93	0.61	0.66	0.94
	KNO_3	1.15	0.16	0.44	0.26
Biotin Concentration	**2µg**	1.05	1.59	0.75	1.75
	5 µg	1.12	1.05	1.17	1.08
	10 µg	1.24	0.45	0.73	0.51
	20 µg	1.16	0.15	0.65	0.21
	30 µg	0.95	0.19	0.70	0.18

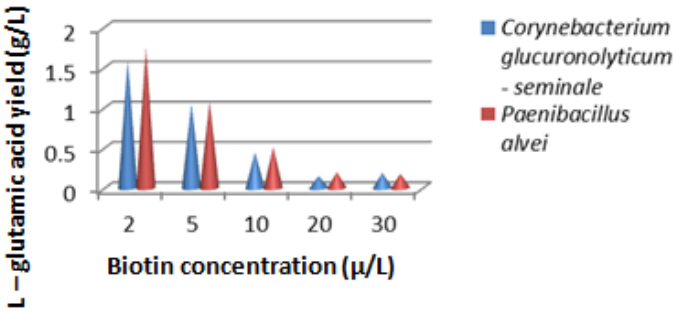

Fig. 3: Comparison of the glutamic yield by isolates *Corynebacterium glucuronolyticum – seminale* and *Paenibacillus alvei* in varying Biotin concentration.

DISCUSSION

The present investigation has indicated the presence of natural glutamate-producing bacteria in tropical environmental samples as reported other workers in literature (Shakoori *et al.*, 2012, Zarajen *et al.*, 2012 and Lawal *et al.*, 2011), which suggest the ubiquitous nature of L-glutamic acid producers. Six bacteria isolates *Paenibacillus alvei, Corynebacterium glucuronolyticum-seminale, Bacillus firmus, and Bacillus validus* were identified to produce L-glutamic acid of which *Paenibacillus alvei and Corynebacterium glucuronolyticum-seminale* were better producers with a yield above 0.7g/L of the amino acid. The identified isolates belongs to the family Corynebacteriaceae, Bacilliaceae and Enterobacteriaceae, which had also been reported by Kinoshita *et al.*, (1999) and Aida, (1986) as significant L-glutamic acid producers. Glucose was the best utilized carbon source for both cell growth and L-glutamic acid production for both selected isolates (Figure 1 and Table 4), and this is similar to the observation of Lawal *et al.*,

(2011). The findings also indicate that the monosaccharide sugars are better utilized compared to disaccharide sugars and hydrocarbons (Fig. 1) because simple sugars are easily hydrolysed in the medium due to short hydrocarbon chain present and are readily metabolised by microorganisms.

Ammonium chloride was the best nitrogen source for both isolates for the amino acid production (Figure 2) followed by ammonium sulphate and urea while potassium and sodium nitrates were poorly utilized by the isolates which support the report by Leuchtenberger *et al*., (2005) and Lawal *et al*., (2011) that ammonium salts are generally most suitable nitrogen sources for glutamic acid fermentation, although Lawal *et al*., (2011) stated that urea was not a suitable nitrogen source for production of the amino acid. In the current findings the yield of 0.60g/L and 0.61g/L was obtained respectively for both isolates which support previous finding by Vijayalaskim and Sarvamangala (2011) which support urea as a good and cheap source of nitrogen for glutamic acid production.

It was noted that L-glutamic acid was produced substantially at a biotin concentration of 5µg/L and best at 2µg/L for both isolates in 96h culture (Figure 3). At higher Biotin concentrations the glutamic acid yield was lower (Figure 3) which agreed with Vijayalakshimi and Sarvamangala (2011) that under excessive biotin growth conditions, synthesis of L-glutamic acid is inhibited by feedback control. The enhanced secretion at suboptimal biotin was due to permeability change prompted by limiting the biotin supply required by the bacterium.

Wild type glutamic acid-producing bacteria used in this study produced a low yield of the amino acid when compared with the L-glutamic yield by *Corynebacterium glutamicum* ATCC 13032 (Lawal *et al.,* 2011) and *Corynebacterium glutamicum* AJ1510 (Ahmed *et al.,* 2013). This could be due to the fact that the natural overproduction of amino acids is rare because the metabolic pathway for the production is tightly controlled and operates economically. Therefore, to achieve an overproduction of L-glutamic acid by the natural bacterial strains, methods have to be devised to eliminate the metabolic regulatory processes.

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