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FERMENTATIVE PRODUCTION OF L-GLUTAMATIC ACID BY THE *BACILLUS* SPECIES ISOLATED FROM SEWAGE CONTAMINATED SOIL

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ABSTRACT
Experiments were conducted to isolate glutamic acid producing bacteria from soils contaminated with sewage water. The bacteria were isolated from sewage contaminated soils in Anantapur city and cultured on agar plates and were screened for glutamic acid production by performing bioautographic technique. The glutamic acid producing strains were designated as INSPDST8, INSPDST9, SKUDBT03 and they were identified as new variants of *B. flexus, B. stratosphericus, B. thuringenesis* respectively by physiological, biochemical characteristics and with 16s rDNA sequencing. All the strains were gram positive, spore forming, and motile, aerobic, alkaliphilic bacteria. The optimum growth pH range was 8-10 and temperature 37 ^{o}C. The amount of glutamic acid produced by *B. flexus, B. stratosphericus, B. thuringenesis* was 76, 72, 78mg/100ml.

KEY WORDS: *Bacillus sps,* glutamic acid, sewage soil, alkaliphilic bacteria.

INTRODUCTION

Bacillus is a Gram-positive, rod-shaped bacteria and a member of the division Firmicutes. *Bacillus* Species can be obligate aerobes or facultative anaerobes, and test positive for the enzyme catalase. [1] *Bacillus* Species continue to be dominant bacterial workhorses in microbial fermentations. *Bacillus subtilis* is the key microbial participant in the ongoing production of the soya-based traditional natto fermentation, and some *Bacillus* Species are on the Food and Drug Administration's GRAS (generally regarded as safe) list. Glutamic acid has been produced from quite a large number of carbon and nitrogen sources by fermentation using different bacterial strains. [2] From literature there is little detailed information on the use of different *Bacillus* Species from sewage contaminated soils for the production of L-glutamic acid. [3] Hence the present research work was focused on the use of *Bacillus* spp. isolated from sewage contaminated soils for the production of L-glutamic acid.

MATERIALS AND METHODS

The bacteria used in this study were isolated from the soils of Anantapur city contaminated with sewage water. 1 gm of soil sample was suspended in 10 ml of sterile distilled water and was subjected to serial dilution. The soil suspension was spread on the Petri plates containing a modified Bouillin medium of the following composition (g/l) separately for each dilution. Peptone, 10.0, Meat extract, 5.0 Yeast extract, 2.0, Sodium chloride, 2.5 and Agar, 20.0 of pH-10. The plates were incubated at 30 ^{o}C for 48-72 hrs [4]. The colonies thus obtained were selected as the source of culture to be evaluated for the production of L-glutamic acid. [5] Colonies were screened by performing replica plating technique.[6] Detection of L- glutamic acid produced on screening medium by individual colonies was done by performing Bioautographic technique with screening organism *Pediacoccus acidilactii* ATCC 8042 which formed halo zone around the glutamic acid producing bacteria.

Maintainance of micro organisms

The positive bacterial cultures were purified by streak plate technique and maintained on modified Bouillin medium slants at 4 ^{o}C.[4]

Identification of microorganisms

Biochemical characterization was done by performing different tests such as Grams staining, catalase test, motility, methyl red, indole concentrations, endospore , starch, citrate, casein hydrolysis, gelatin, nitrate reduction and sugar utilization.[7] [8] The organisms were identified as per Bergey's manual of systemic Bacteriology.[9]

Medium used for production of Glutamic acid

The composition of production medium was similar to that of screening medium used with exception of agar. [5] The medium was filter sterilized and inoculated with 1

ml of test culture and incubated at 30 °C on an orbital shaker incubator at 200 rpm for 72 hrs.

Qualitative and quantitative determination of L-Glutamic acid

Thin layer chromatography was used for qualitative detection of glutamic acid. TLC pates of 0.2 mm thickness were prepared using silica gel. The fermented broth culture was centrifuged and supernatant was collected. The supernatant which served as source of glutamic acid, was spotted on TLC plates along with glutamic acid standard and developed in a solvent system containing a mixture of n-butanol, acetic acid and water (4:1:1:v/v) for about 6 hrs. After development the chromatogram was sprayed with 0.15% ninhydrin ethanol solution and was kept in oven at 110°C for 3 min spots of all amino acids were visualized. R_f value of the samples were compared with the R_f value of glutamic acid standard. [4] The ninhydrin colour reaction was used for quantitative determination of L-glutamic acid where the absorbance was measured at 570 nm. 1ml of freshly prepared ninhydrin reagent was added to the equal volumes of supernatant and heated in water bath for 15 minutes and cooled under running tap water. The absorbance was measured against blank without supernatant. The amount of glutamic acid present in the sample was estimated from the standard curve for L-glutamic acid. [10]

RESULTS AND DISCUSSION

The production of glutamic acid by different *Bacillus* Species have been reported earlier. [11] *Bacillus* Species are predominantly found in soil, in sewage contaminated soils and in vegetable proteins. *Bacillus* Species particularly are important in food industry, because they produce desired flavor during fermentation by enhancing L-glutamic acid synthesis and by the production of enzymes like amylases (α and β amylases) which are having applications in the brewery, textiles and paperindustry.[12]

Table 1: Biochemical characteristics of micro-organisms isolated from sewage contaminated soils

Isolate Code	Colony morphology	Cell characteristic	endospore	catalase	Voges proskauer	Methyl red	starch	mobility	citrate	Nitrate	urease	Anaerobiosis	Indole	Casein	Gelatin	Sugar fermentation									Growth in Nacl		
																LA	A R	M A	G L	X Y	R I	F R	MN	S U	5%	7%	10%
INSP DST0 01	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	-	-	+	+	+ W	-	+	+	+	+	+	+	+	+	+	+
INSP DST0 02	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	-	+	+	+	+ W	+	+	+	+	+	+	-	+	+	+	+
INSP DST0 03	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	-	-	+	+ W	+	+	+	+	+	+	-	+	+	+	+
INSP DST0 04	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	-	+	+	+ W	+	+	+	+	+	+	+	+	+	+	+
INSP DST0 05	Cream Flat 1-2 mm Diameter	+ long Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+	+	+
INSP DST0 06	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	+	-	+	+ W	+	+	+	+	+	+	+	+	+	+	+
INSP DST0 07	Cream entire 2-3 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	-	+	+	+	+

INSP DST0 08	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	-	-	+	+	+ W	-	+	+	+	+	+	+	+	+ + +
INSP DST0 09	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	-	+	+	+	+ W	+	+	+	+	+	+	-	+	+ + +
INSP DST0 10	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	-	-	+	+ W	+	+	+	+	+	+	-	+	+ + +
INSP DST0 11	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	-	+	+	+ W	+	+	+	+	+	+	+	+	+ + +
INSP DST0 12	Cream Flat 1-2 mm Diameter	+ long Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+ + +
SKU DBTI NSP0 13	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	+	-	+	+ W	+	+	+	+	+	+	+	+	+ + +
SKU DBTI NSP0 14	Cream entire 2-3 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	-	+	+ + +
SKU DBTI NSP0 15	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	-	-	+	+	+ W	-	+	+	+	+	+	+	+	+ + +
SKU DBTI NSP0 16	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	-	+	+	+	+ W	+	+	+	+	+	+	-	+	+ + +
SKU DBTI NSP0 17	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	-	+	+	+	+	+	+	+	+ + +
SKU DBTI NSP0 18	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	-	+	+	+	+ W	+	+	+	+	+	+	-	+	+ + +
SKU DBTI NSP0 19	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	-	+	+	+ W	+	+	+	+	+	+	-	+	+ + +
SKU DBTI NSP0 20	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+ + +

SKU DBTI NSP0 21	Cream Flat 1-2 mm Diameter	+ long Rods	E	+	+	+	+	+	+	+	+	+	+	-	+	+ W	+	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 22	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	+	-	-	+ W	+	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 23	Cream entire 2-3 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	-	+	+ W	+	+	+	+	+	+	-	+	+	+	+
SKU DBTI NSP0 24	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	-	+	+	+	+ W	-	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 25	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	-	+	+	+	+
SKU DBTI NSP0 26	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	-	+	+	+	+
SKU DBTI NSP0 27	Cream Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 28	Cream Flat 1-2 mm Diameter	+ long Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 29	Cream Flat 1-2 mm Diameter	+ Rods	E	+	-	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	+	+	+	+	+
SKU DBTI NSP0 30	Cream entire 2-3 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	+	+	+	+	+	+	-	+	+	+	+
SKU DBTI NSP0 31	Cream surface Flat 1-2 mm Diameter	+ Rods	E	+	+	+	+	+	+	+	+	+	+	+	+	+ W	-	+	+	+	+	+	+	+	+	+	+

+ = positive; LA=lactose; FR =fructose; GL = glucose; AR =arabinose; MN = mannose; Xy = xylose; - =negative; MA= maltose; SU =sucrose; RI = ribose; W=weak reaction.

Bacillus strains were isolated from soils contaminated with sewage water. Out of 300 isolates, 31 isolates produced glutamic acid. The isolates were identified based on morphological, biochemical and physiological tests according to Bergeys manual of Systematic Bacteriology. Most of the isolates were *Baciullus* sp. as shown in table 1. Three of the isolates INSPDST8, INSPDST9, SKUDBT03 were identified as *Bacillus flexus, Bcillus stratosphericus, Bacillus thuringenesis* respectively. Amongst these organisms colonies of *B. flexus* were small, dull, light creamy and were of 1-2 mm in diameter. *B. stratosphericus* were creamy, flat and 1-2 mm in diameter. *B. thuringenesis* were creamy and entire with differences in their diameter. During fermentation in appropriate medium these bacterial strains produced L-Glutamic acid, which was assayed by employing TLC

qualitatively. The Rf value of the glutamic acid produced by the isolates were determined and compared with an authenticated glutamic acid standard. Rf value of standard glutamic acid was 0.3 and Rf value of glutamic acid produced by *B.flexus* was 0.3, *B. stratosphericus* was 0.3 and for *B.thuringenesis* it was again 0.3. The Rf value of the test isolates were coinciding with the standard L- glutamic acid which reflects the quality of the glutamic acid produced by the isolates. The glutamic acid was quantitatively estimated spectroscopically using Ninhydrin colour reaction. The amount of glutamic acid produced by *B. flexus* was 76 mg/100ml, *B. stratosphericus* was 72 mg/100ml and with *B. thuringenesis* it was 78 mg/100ml.

CONCLUSION

The glutamic acid producing bacteria were isolated from sewage contaminated soils, three of the strains INSPDST8, INSPDST9, SKUDBT03 were identified as new variants of *B. flexus, B. stratosphericus, B. thuringenesis* by physiological, biochemical characteristics and with 16s rDNA sequencing. All the strains were gram positive, spore forming, and motile, aerobic, alkaliphilic bacteria. The quantity of glutamic acid produced by *B. flexus, B. stratosphericus, B. thuringenesis* was 76, 72, 78mg/100ml respectively.

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