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## Isolation and identification of amino acid-producing *Corynebacterium*

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### Abstract

Three sites were selected in Sidi Bel Abbes province (Algeria's northwest) to isolate *Corynebacterium* species, due to their organic matter content. Four isolates were selected according to their morphology, microscopy and biochemical tests according to Bergey's manual that they are *Corynebacterium*. Then, the isolates were cultivated in two media Nutrient Broth and Brain Heart Infusion at 37°C to determine the natural growth rate. S5 exhibited the highest growth rate  $\mu=0.182h^{-1}$  in NB, and  $\mu=0.224h^{-1}$  in BHI, indicating a 23.1% improvement. BHI's complex nutrient composition (including proteins, and other growth factors) likely favors fast-growing isolates like S4 and S5, which may possess more robust or efficient biosynthetic pathways, compared to the simpler nutrient composition of NB may be a limiting factor for all isolates. Finally, three media were used to produce glutamic acid because it is the most produced and sold worldwide: D1 (glucose as the sole carbon source), D2 (fructose as the sole carbon source), and F1 (both). S4 exhibited the highest glutamic acid production, 7.2 g/l in D1 at pH 7.5 and 30°C. Generally, the lower the initial pH of the medium, the lower the glutamic acid production will be, and the optimum temperature was 30°C compared to 37°C.

**Keywords:** *Corynebacterium*, glutamic acid, fermentation, growth rate.

## 1 Introduction

A milestone event that was the beginning of the further development of the amino acid industry was the discovery of monosodium L-glutamate (MSG) as a food flavor enhancer by Kikunae Ikeda in 1908. He pursued a flavoring component for the seaweed "konbu", a traditional seasoning for cooking in Japan, and successfully identified MSG as the essence of its flavor as the compound imparting the taste of Umami to many foods. Umami (the 5th taste) is distinct from other tastes such as bitter, sour, salty, and sweet (Ikeda, 1908).

Kinoshita and his colleagues at Kyowa Hakko Kogyo Co. in 1957 reported the discovery of a glutamate-producing bacterium, *Corynebacterium glutamicum* (originally described as *Micrococcus glutamicus*), which has a unique ability to produce significant amounts of L-glutamate directly from sugar and cheap ammonia (Kinoshita et al., 1957). The advantage is the use of wild strain of *C. glutamicum* for glutamate overproduction by fermentation under optimal conditions (better than using the methods of protein hydrolysate extraction, chemical synthesis, enzymatic synthesis). The fermentation is carried out in a sterile culture medium with carbon source, nitrogen source, sulfide, phosphorus source, with trace elements, optimum temperature and pH, and aeration in a fermenter (Kimura, 2003).

*Corynebacterium callunae*, closely related to *C. glutamicum*, is an aerobic, non-hemolytic bacterium isolated during screenings for L-glutamic acid-producing microorganisms. It shares the high G+C content characteristic of the genus and contributes to the diversity of industrially relevant *Corynebacteria* (Persicke et al., 2015). *Corynebacterium efficiens* is a thermotolerant species capable of thriving at temperatures up to 45°C, unlike its relatives *C. glutamicum* and *C. callunae*. This trait offers a significant advantage in industrial fermentations by reducing cooling costs during L-glutamate production. Isolated from soil and vegetables (Fudou et al., 2002). The following figure presents Phylogenetic tree of *Corynebacterium* species (Figure 1).

Our study focuses on glutamic acid's production by a wild-type isolated bacteria, because in 2020, the global production volume of glutamate was estimated at 3.2 million metric tons, with Asia-Pacific dominating the market due to high consumption in countries like China, Japan, and South Korea (MarketsandMarkets, 2021). The profitability of the glutamate market is closely tied to its applications in the food industry, where it is used to enhance umami flavor in processed foods, snacks, and ready-to-eat meals. The global MSG market alone was valued at 4.5 billion \$ in 2020 and is expected to grow at a CAGR of 4.8% through 2027 (IMARC Group, 2021).

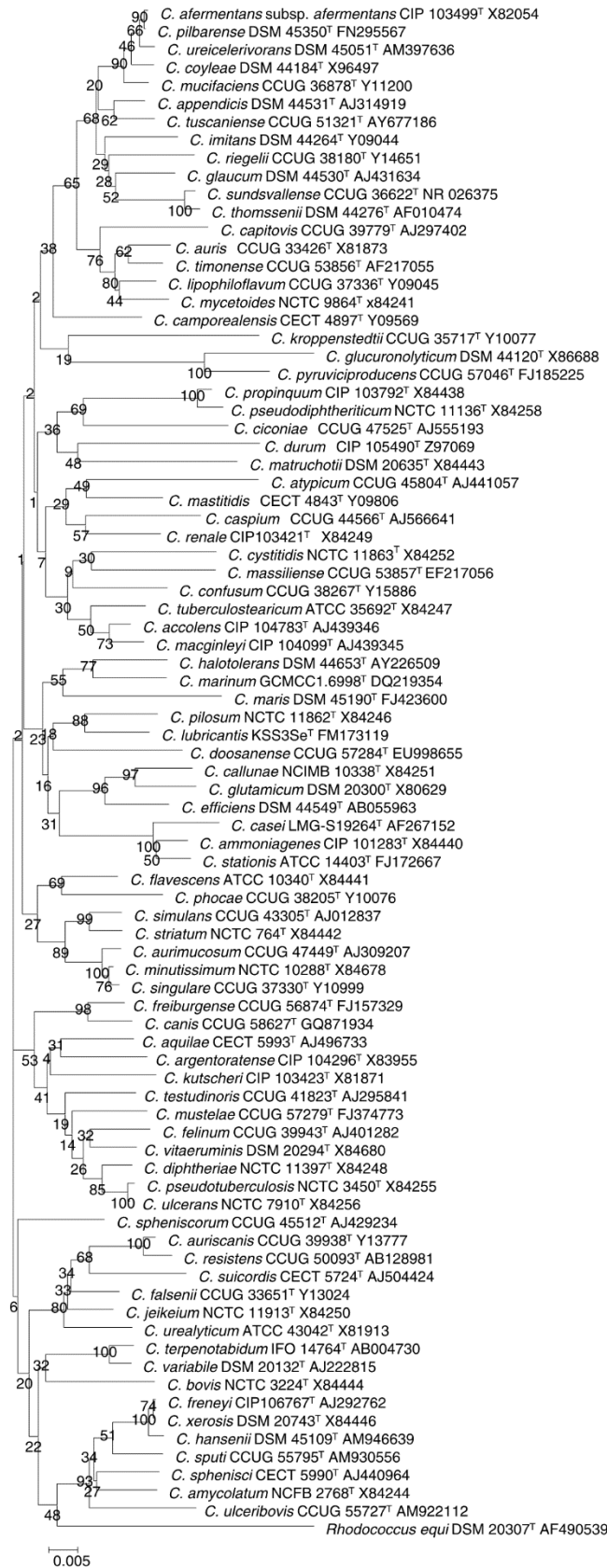


Figure 1. Phylogenetic tree of 16S rRNA gene sequences of *Corynebacterium* species with *Rhodococcus* (Bergey et al., 2012).

## 2 Materials and Methods

Three locations were chosen for sampling in Sidi Bel Abbès Province (Algeria's northwest, Figure 2). Samples are taken (10 cm deep) from the soil in the Faculty of Natural and Life Sciences of the University of Djillali Liabès, from organic soil (rich in organic matter: bird droppings) of a garden next to the Faculty of Medicine, from water of Sidi Mohamed Benali Lake using sterile bags and containers, transported at 4°C to the laboratory.



Figure 2. Geographic location of samples used for *Corynebacteria* isolation.

A weighing of 25 g of the soil is introduced into a volume of 225 ml of sterile physiological water, homogenized using a magnetic stirrer. Decimal dilutions ( $10^{-1}$  to  $10^{-5}$ ) are prepared from the mother solution.

A volume of 1 ml of the lake water is introduced into a volume of 9 ml of sterile physiological water, homogenized using a vortex. Then, serial dilutions ( $10^{-1}$  to  $10^{-5}$ ) are prepared from the stock solution.

Different media were used for *Corynebacteria*'s isolation: TSA, nutrient agar supplemented with an antibiotic (nalidixic acid 35  $\mu\text{g/ml}$ ). The culture media were inoculated in two techniques either using the loop or by spreading of 100 $\mu\text{l}$ , then incubated at 30 °C for 2 to 4 days.

The identification of bacteria was carried out by the following methods: colony morphology, Gram staining, spore staining, Ziehl-Neelson staining, Biochemical tests according to the Bergey's manual (Bergey et al., 2012).

To check the normal growth rate of isolates, they were inoculated in fresh nutrient broth, BHI 50-100 ml of broth in 500ml flasks at 37 °C for 24-72h under shaking (180 rpm), 0.2ml of sample was taken regularly and added to a cuvette containing 1.8ml of blank (decimal dilution) for reading absorbance at 600nm using a spectrophotometer (Specord 200 Plus. Analytik Jena).

Different media were used for fermentation up to 5 days (Table 1). The isolates were firstly inoculated into seed medium containing per liter: 05 g glucose, 10 g casein peptone, 05 g yeast extract, 01 g K<sub>2</sub>HPO<sub>4</sub>, pH was adjusted to 7.5 before autoclaving, 300µg/l biotin was added after cooling of the autoclaved media (syringe-filtration 0.22µm). after 24h of incubation, 05% of inoculum (for fermentation media) was centrifuged and the pellet was washed twice with 0.9% sterile physiological solution, then the fermentation media were inoculated. Each day, 2ml was taken from each Erlenmeyer flask and centrifuged, the supernatant was used for further analysis.

Table 1. Composition of fermentation media.

Components	Media		
	D1	D2	F1
Glucose	18	00	09
Fructose	00	18	09
Urea	7	9	10
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	10	11	10
K <sub>2</sub> HPO <sub>4</sub>	1.2	1.5	1.5
KH <sub>2</sub> PO <sub>4</sub>	0.6	0.7	0.7
MgSO <sub>4</sub>	0.05	0.05	0.05
FeSO <sub>4</sub>	0.005	0.005	0.005
MnCl <sub>2</sub>	0.002	0.002	0.002
MnSO <sub>4</sub>	0.002	0.002	0.002
CaCl <sub>2</sub>	0.01	0.01	0.01
ZnSO <sub>4</sub>	0.001	0.001	0.001
Biotin	80µg	80µg	80µg
Thiamin	100µg	100µg	100µg
pH	7.5	7.5	7.5

Paper chromatography was used to visualize the production of amino acids with the Ninhydrin technique. 20 $\mu$ l of supernatant was spotted in Whatman n1 paper, after drying it was placed inside a tank containing a complex of solvents (Butanol: Acetic acid: Distilled water, 5:1:4), after 6 hours, the paper was dried and then sprayed with 0.25% Ninhydrin, dried and placed in the oven at 105 °C for 10 minutes, calculate Rf value of the spot. To quantify the amino acids produced, the spot was cut and solubilized in 2ml pure methanol over 4 hours, then measured its absorbance at 570nm using a spectrophotometer and compared with the standard curve (Figure 3).

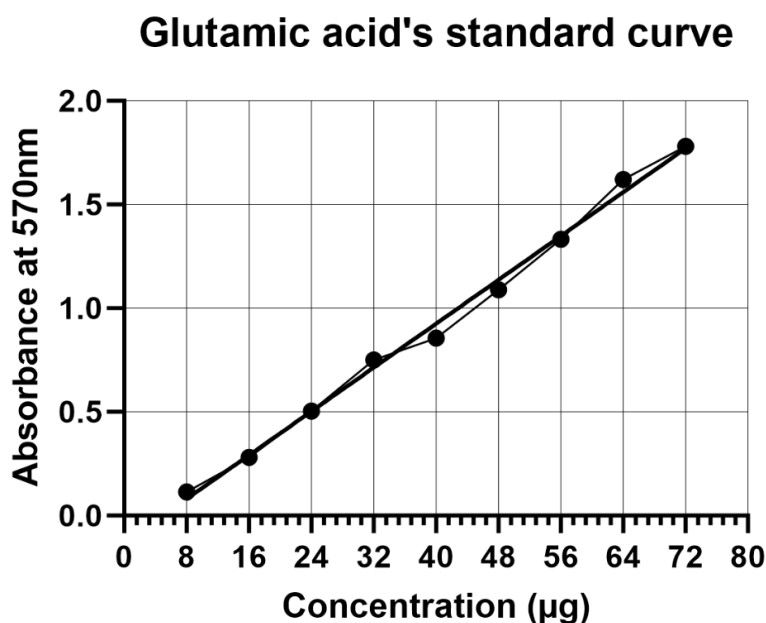


Figure 3. Glutamic acid's standard curve (using 0.08mg/ml stock solution and 0.25% Ninhydrin in acetone).

### 3 Results and Discussions

Four isolates were selected to be *Corynebacterium* after identification based on morphology and cultural characterizations from 62 isolated colonies from the three samples. S2 were isolated from Duck pen soil, S3 and S4 from the Garden Soil, S5 from the Lake as mentioned early.

#### 3.1 Colony morphology

Isolate S2 was yellow, small, smooth, umbonate elevation, round, entire margin. TSA+Tellurite: Grey colony.

Isolate S3 was pale yellow, moderate size, convex, round, entire margin (Figure 4).  
TSA+Tellurite: Black colony.

Isolate S4 was ivory, moderate size, small, convex, round, entire margin. TSA+Tellurite: Grey colony.

Isolate S5 was yellow, moderate size, viscid texture, round, entire margin. TSA+Tellurite: Black mucoid colony.

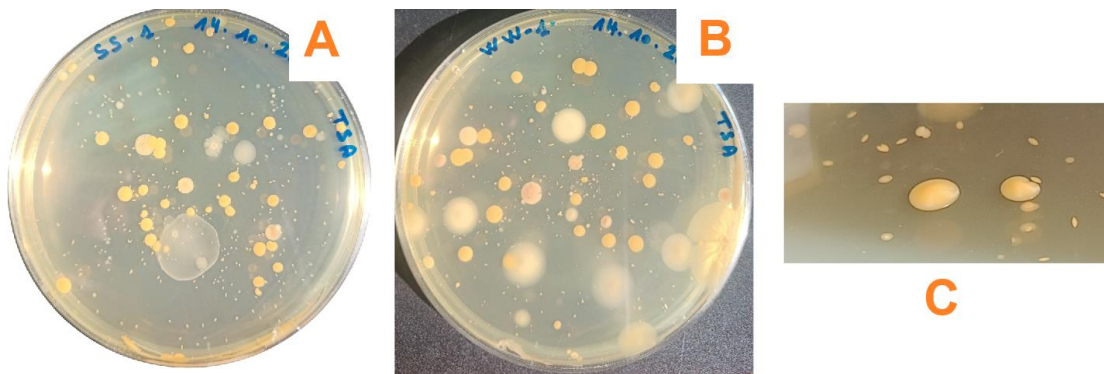


Figure 4. Presentation of inoculated plates of TSA. A: TSA plates inoculated with 100µl of  $10^{-1}$  of soil from the Faculty of Natural and Life Sciences. B: TSA plates inoculated with 100µl of  $10^{-1}$  of Water from West area of Sidi Mohamed Benali Lake. C: Morphology of a selected colony S3.

According to morphology (figure 4), microscopic observation (figure 5), cultural and biochemical tests (table 2 and 3), these isolates were susceptible to be one of biotechnological well-known species like *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Corynebacterium callunae* (Bergey et al, 2012).

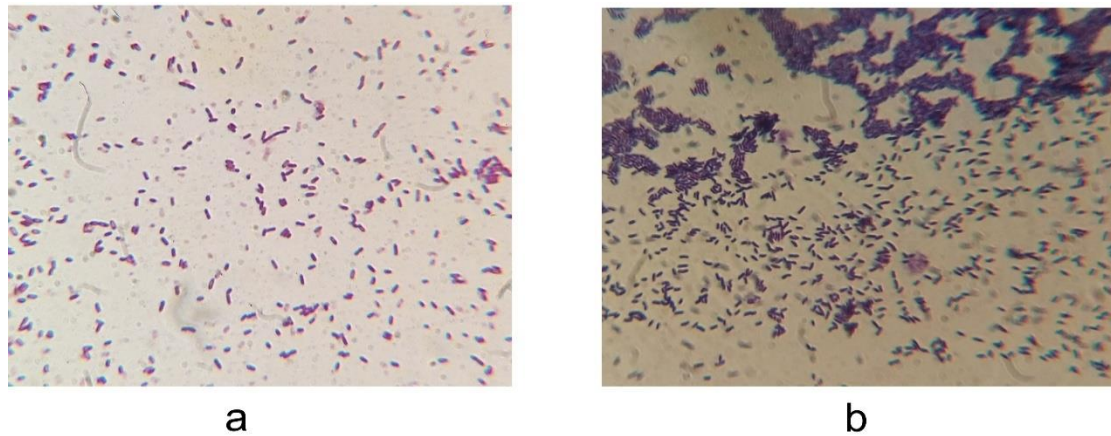


Figure 5. Microscopic observation of two isolates. a: S3. b: S5.

Table 2. Results of some identification tests.

Strain	Gram's staining	catalase	sporulation	Ziehl-Neelson staining	Motility
S2	+ Coryneforms	+	-	-	-
S3	+ V-shaped rods	+	-	-	-
S4	+ Palisade	+	-	-	-
S5	+ V-shaped rods, Palisade	+	-	-	-

Table 3: Results of some biochemical tests.

Strain	Nitrate reductase	Urease	Citrate utilization	TSI	H2S	VP	MR	Mannitol
S2	+	-	+	Acidic /Acidic	-	-	+	-
S3	+	+	-	Alkaline/Acidic	-	+	+	-
S4	-	+	+	Alkaline/Acidic	-	+	-	-
S5	+	+	+	Alkaline/Acidic	-	-	+	-

### 3.2 Growth rate of isolates

The growth rates ( $\mu$ ) of the isolates S2, S3, S4, and S5 were estimated with excel after plotting the curve of OD600 versus time (figure 6 et 7). Isolate S2 exhibited a growth rate of  $\mu=0.169h^{-1}$  in Nutrient Broth (NB) and  $\mu=0.199h^{-1}$  in Brain Heart Infusion (BHI), reflecting a 17.8%

increase in growth rate. Isolate S3 had a growth rate of  $\mu=0.173h^{-1}$  in NB, which increased to  $\mu=0.211h^{-1}$  in BHI, showing a 22.0% enhancement. Similarly, isolate S4 demonstrated a growth rate of  $\mu=0.181h^{-1}$  in NB and  $\mu=0.223h^{-1}$  in BHI, marking the highest increase of 23.2%. Finally, isolate S5 recorded a growth rate of  $\mu=0.182h^{-1}$  in NB, which rose to  $\mu=0.224h^{-1}$  in BHI, indicating a 23.1% improvement. The growth rates in NB show a gradual increase from S2 to S5, with S5 exhibiting the highest growth rate. The lower values of  $\mu$  in this medium suggest that the relatively limited nutritional profile of NB may not fully support the metabolic demands of these isolates, leading to slower growth compared to BHI.

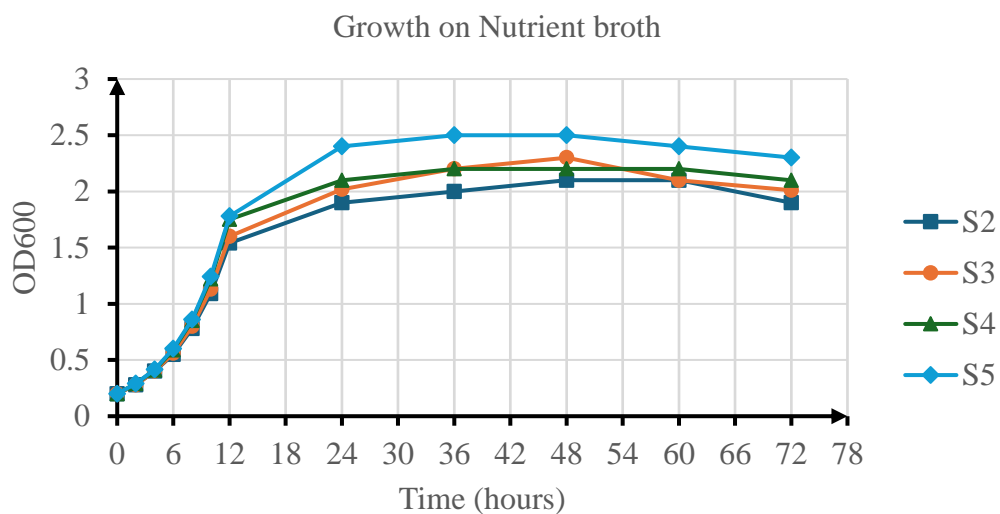


Figure 6. Growth curve of isolates on Nutrient broth.

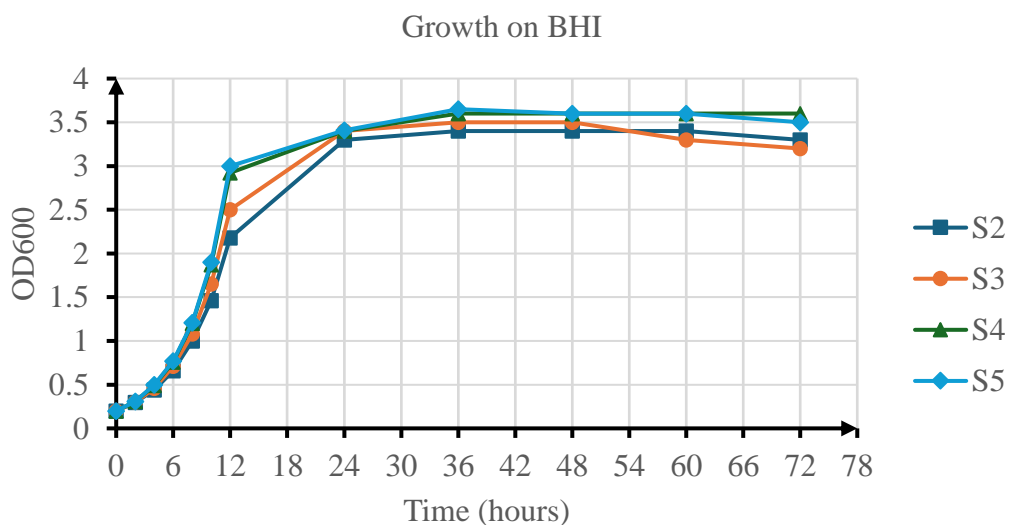


Figure 7. Growth curve of isolates on BHI.

Our results for growth rates in Nutrient Broth and Brain Heart Infusion media are consistent with the findings reported in previous studies. According to Haas et al. (2019), the growth rate of *Corynebacterium glutamicum* was estimated to range between 0.02 and 0.4h<sup>-1</sup>, which aligns with the growth rates observed in our isolates across both media. Additionally, Grünberger et al. (2013) reported a maximum growth rate of  $\mu=0.42\text{ h}^{-1}$  in standard batch cultures. Although our results do not reach the maximum value reported by Grünberger et al., they fall well within the range described by Haas et al., demonstrating that our findings are in agreement with established growth rate benchmarks for *C. glutamicum*.

### 3.3 Glutamic acid's production

The cultivation of isolates S2, S3, S4, and S5 on three different media (table 1) under varying pH conditions (6.8, 7.5, 8.2) and temperatures (30°C and 37°C) reveals significant differences in glutamic acid production (Figure 8 and 9). At 30°C, glutamic acid production varied across media and pH levels, with isolate S4 consistently demonstrating the highest productivity. At pH 6.8, S4 reached its peak production of 3 g/L on F1, while isolates S2, S3, and S5 had lower outputs, particularly on D2 and F1. At pH 7.5, production increased significantly, with S4 achieving 7.2 g/L on D1 and high values also observed for S2 (6.12 g/L) and S3 (5.12 g/L) on F1. At pH 8.2, S4 maintained its lead with 7.1 g/L on D1 and 6.9 g/L on F1, while S2 and S3 showed moderate enhancements, and S5 consistently produced the least glutamic acid across all conditions.

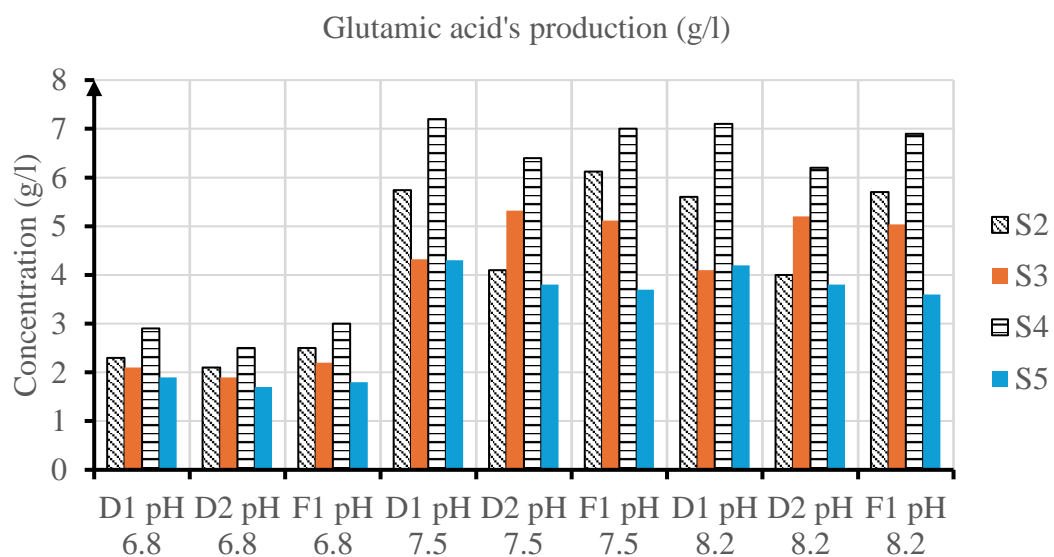


Figure 8. Glutamic acid's production on different media at 30 °C.

At 37°C, glutamic acid production was generally lower than at 30°C, with S4 and S3 consistently outperforming the other isolates. At pH 6.8, S3 had the highest production (2.4 g/L) on F1, closely followed by S4 (2.5 g/L), while S5 produced less than 1.6 g/L across all media. At pH 7.5, production increased for all isolates, with S4 achieving 5.3 g/L on F1 and S3 performing well on D2 (5.1 g/L). At pH 8.2, S4 again led with 5.1 g/L on F1, followed by S3 with 4.6 g/L on D2, while S2 and S5 showed significantly lower production, with S5 producing less than 3 g/L across all conditions. As mentioned in other reference, glutamic acid production using acid-treated and alkali-treated rice husk gave 10.40g/L and 9.08g/L respectively with the wild-type strain (Musa BISHIR *et al.*, 2017).

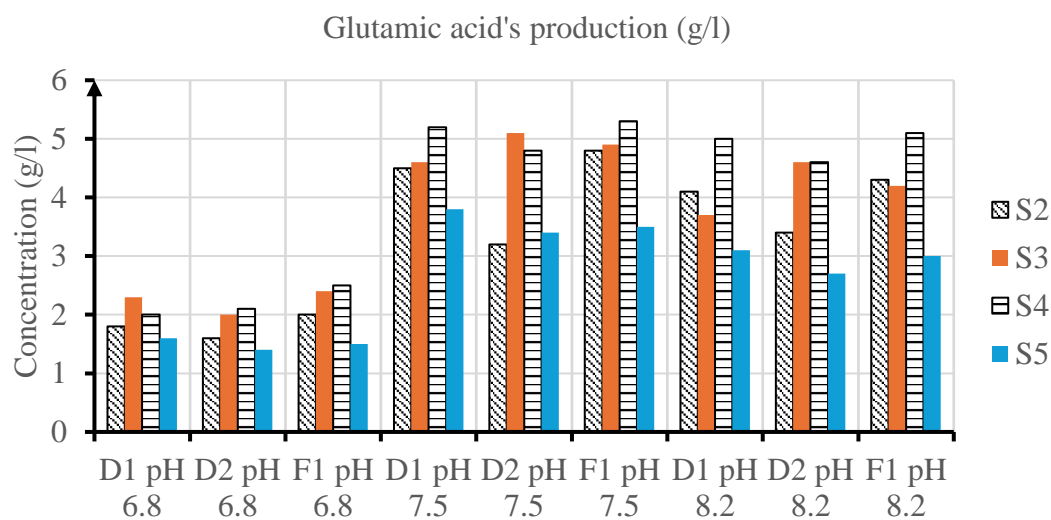


Figure 9. Glutamic acid's production on different media at 37 °C.

#### 4 Conclusion

The growth rates of all isolates improved significantly in the nutrient-rich BHI medium compared to Nutrient Broth, with increases ranging from 17.8% for S2 to 23.2% for S4, highlighting the enhanced growth performance provided by BHI. Glutamic acid production was highest at pH 7.5 across all isolates, media, and temperatures, outperforming production at pH 6.8, where S2 and S5 showed limited activity, and pH 8.2, where S4 and S3 maintained relatively high levels. Temperature significantly influenced production, with higher outputs at 30°C compared to 37°C, indicating more active enzymatic pathways at the lower temperature. Among the isolates, S4 demonstrated the greatest adaptability with minimal production decline between temperatures, while S5 showed a marked reduction at 37°C, reflecting higher sensitivity to temperature changes. The adaptability of isolates to different media highlights the potential for cost reduction by using less expensive nutrient sources without significantly

compromising yield. The relatively consistent production by isolates like S4 at both 30°C and 37°C suggests robustness, making them suitable for large-scale fermentations where precise temperature control may be challenging. The observed variability in productivity between isolates and media suggests potential targets for genetic modification, such as improving nutrient uptake or enhancing tolerance to acidic and alkaline pH levels.

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