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Amino acids seclusion and characterization of amino acid fermenting bacteria in buttermilk

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Abstract

Buttermilk has various applications in kitchen recipes. In this study, buttermilk has been manipulated for the isolation of amino acid fermenting bacteria. In this study, isolation and characterization of bacterial strains were carried out that can be utilized for amino acid fermentation. In buttermilk, on the basis of amino acids production potential, five bacterial isolates B-5-1, B-5-7, B-6-3, B-7-19 and B-7-24 were selected and characterized by biochemical tests, carbohydrates utilization and gram staining, as well as growth curve study. Fermentation conditions were optimized for better amino acid production. Results clearly indicated that different bacterial isolates from buttermilk had a great potential to produce a variety of amino acids, e.g., Isoleucine, methionine, phenylalanine and cysteine. Some other amino acids that appeared in the fermentation broth were not prominent such as alanine, aspartic acid and valine. An isolate B-5-1 produced up to 6.7g/l of glutamic in the medium after 72 hours of fermentation. It is concluded that the isolate B-5-1 was a *Lactobacillus delbruckii* which attained its peak production around 14th hours of incubation.

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Introduction

Buttermilk, based upon its production, was divided into two types. The first type of buttermilk is the liquid produced during the processing of butter and is commonly known as traditional buttermilk (Siva *et al.*, 2019). It is being wasted every day as a by-product of butter-making industries. Frew and Abebe (2020) explained this as traditionally sour and defatted milk. It has a smoother appearance and thinner consistency than sour milk, as well as shorter shelf life (24-48 hrs) as compared to all other traditionally made products (Berhe*et al.*, 2017). The second type is cultured buttermilk that is produced during cow milk fermentation, commonly utilized for manufacturing a variety of dairy-based commodities and its sour taste is produced due to the fermentation process (Szajnar *et al.*, 2021).

The variety of essential amino acids (EAAs) is produced in buttermilk due to the presence of fermenting bacteria during the fermentation process and can be utilized in various products specially formulated for EAAs deficient people (D'Este *et al.*, 2018). Amino acids are being utilized in the food, feed, medicine and cosmetics manufacturing industries (Compeer and De Best, 2018). Amino acids are being produced through various techniques. D'Este *et al.* (2018) explored various fermentation processes to produce the amino acids on an industrial scale by utilizing the microorganism. Their synthesis through microbial activity is found cheaper rather than by chemical synthesis. The different modern fermentation techniques and various strains of amino acids fermenting microbes (Ma *et al.*, 2017) have created opportunities for the industries to produce the glutamate and L-lysine in large quantities (Félix *et al.*, 2019). Amino acids have become a major industrial product of microorganisms. For example, over 800,000 tonnes/year of glutamic acid is produced and used to make the flavor enhancer monosodium glutamate, which is produced every year (biologicaldiscussion.com, 14-Nov-2021).

Bacterial production of amino acids (AAs) has not been exploited in Pakistan. Efforts have been made to produce amino acids exploiting our local resources. The bacterial isolates were obtained from a natural source and studied amino acids production through fermentation. Seclusion and characterization of AAs fermenting bacteria were be carried out from different sources, but the major prospect of the present study was to obtain bacterial isolates from buttermilk (a by-product of butter making industry) and development of fermentation conditions to produce the amino acids. This work was an attempt to investigate another good source of such bacteria that can be employed for amino acid fermentation.

Materials and methods

Sample collection

Buttermilk samples were collected from butter-producing industries.

Isolation of bacterial strains

Bacteria were isolated from buttermilk by making serial dilutions and passing them through sterile Millipore filters of 0.45µm pore size. Nutrient Agar plates were covered by filters and placed for incubation overnight at 37°C (Fig. 1). Afterward, colonies were picked up with the help of a sterilized inoculation needle and streaked twice on other Nutrient agar plates and incubated under the same set of conditions. Finally, pure colonies were slanted cultured on the basis of cell colony isolation technique.

Fermentation studies

For the production of amino acids, the bacterial isolates were fermented in suitable fermentation media for up to 96 hours at 30±1°C and 150rpm, according to the method described by Nadeem *et al.* (1997).

Amino acid analysis

Qualitative estimation

Fermented broths were taken after 24, 48, 72 and 96 hours of incubation examined for amino acid production through paper chromatography (Nadeem *et al.*, 1997) as well as paper electrophoresis (Khan *et al.*, 2006). Sometimes, two spots appearing on the

chromatogram were much closed and could not be separated. Then, Electrophoresis was used for the analysis of fermented amino acids and to verify the results obtained.

Quantitative estimation

The quantitative estimation of amino acids produced was done through spectrophotometry by eluting the spots in 3ml methanol (Nadeem *et al.*, 1997). The optical density (OD) of eluted methanol was measured at a wavelength of 550nm. Then amino acids concentrations were determined through comparison with their respective standard curves.

Characterization study

The bacterial isolates of interest were characterized biochemically by using different tests, i.e., Voges-Proskauer test, Catalase test, Methyl Red test, test through the utilization of citrate, and Indole Production test according to (Stanier *et al.*, 1987). These isolates were also subjected to different sugar tests glucose, sorbose, maltose, starch, lactose, galactose, sucrose, mannitol, xylose and raffinose (Onuoha *et al.*, 1995). 24-hour nutrient agar slant cultures were used for gram staining according to the method proposed by Cappuccino and Welsh (2017).

Growth curve study

The strains of particular interest were full-grown in extracted glucose-yeast media and the growth curves were designed by measuring their dry weights. Glucose-yeast extract media consist of 0.02% glucose, and 0.3% yeast extraction in water (Distilled), (7.0 pH was maintained and sales were autoclaved), 50ml per flask (Erlenmeyer, 250ml capacity), were used for incubation at 30˚C with overnight grown culture. The sample was monitored after every one hour and immediately observing its harvest pH, optical density (OD) and nephalometeric turbidity unit (NTU) were noted (data not shown). The broth was centrifuged and pallet placed in an oven at 70°C for 48 hours. Afterward, the weight of pallets was noted. Following this procedure, other samples were collected from 1-24hrs and weighed accordingly. A graph illustrating the growth pattern of strains was drawn in the end (Nadeem *et al.*, 2002).

Results and discussion

Screening of amino acid fermenting bacteria

The recent studies were conducted in Fermentation Lab, Biological Chemistry Division, B, Faisalabad. Sixty-seven isolates were obtained from the buttermilk.

Table 1. Amino acid (A.A.) producing selected bacterial isolates fermenting in glucose medium, L-6.

Isolates	pH	A.A. Production (hr)	Quantity (g/l)	A.A. found in traces
B-5-1	7.20	Cys(24)	0.3	Ile
	7.14	Phe(24)	0.4	Ala
	7.23	Cys(72)	0.2	
		Glu(72)	6.0	
		Met(96)	0.09	
B-5-7	7.09	Cys(24)	0.5	Glu
		Phe(24)	4.25	Ala
	7.22	Ile(48)	0.23	Val
	7.29	Ile(72)	0.1	
	6.9	Met(96)	0.147	
B-6-3	6.9	Ile(24)	0.5	Cys
		Met(48)	0.4	
	7.2	Ile(48)	0.4	
		Met(72)	2.1	
	7.4	Ile(72)	1.2	
	6.2	Met(96)	1.3	
B-7-19	7.23	Met(24)	0.8	Cys
		Ile(24)	0.6	
	7.40	Ile(48)	1.0	
		Met(72)	1.4	
	6.90	Ile(72)	2.7	
B-7-24	6.7	Ala(24)	0.7	Cys
		Glu(48)	1.2	Met
	7.93	Ala(48)	2.5	Ile
		Glu(72)	0.8	
	7.92	Ala(72)	1.6	
		Glu(96)	1.9	
	8.1	Ala(96)	1.3	

All of these bacteria, while fermented in glucose yeast medium (L-6), revealed production of amino acid production in the fermentation broth. The quantity of the A.A. was though not promising; however it reflected that the isolates had the tendency towards amino acid production.

Table 2. Characterization of selected isolates through biochemical tests as well as by Gram's staining.

Tests	Isolates				
	B-5-1	B-5-7	B-6-3	B-7-19	B-7-24
Grams's staining	+	+	–	+	–
	Coccobacilli	Coccobacilli	Rods	Cocci	Rods
Catalase	–	+	+	–	–
Indole Production (IP)	–	–	–	–	–
Voges-Proskauer (VP)	–	–	–	–	–
Citerate Utilization (CU)	+	+	+	–	+
Methyl Red (MR)	+	+	–	+	–

Isoleucine

Isoleucine appeared to be the most prolific amino acid occurring in the fermentation broth. Out of sixty-seven isolates, forty-six produced isoleucine. An isolate B-5-2 fermented nearly 5.5g/l isoleucine in the fermentation broth after 72 hours of incubation. But this isolate was not selected for final selection because of its inconsistent behavior.

Methionine

Methionine was among those amino acids, which appeared abundantly in the fermentation broth. It is one of those amino acids, which are very rarely produced microbiologically. But in this case, it was produced by 30 isolates. A lot of research on amino acids producing bacteria and bacteria isolation techniques from soil and water have been conducted, but minor data is available on amino acids producing bacteria obtained from the buttermilk. It might probably be the reason that microbial production of methionine has not been very well explored so far. In recent studies, although a large percentage fermented methionine, the overall quantity of methionine produced was not that high. An isolate B-6-3 produced up to 2.7g/l of methionine after 72 hours of fermentation. However, some other isolates also produced nearly 1g/l of methionine in the fermentation broth.

Table 3. Characterization of selected isolates through carbohydrates utilization.

Sugars	Isolates				
	B-5-1	B-5-7	B-6-3	B-7-19	B-7-24
Glucose	+	–	+	+	–
Sarbose	–	–	–	–	–
Maltose	+	–	–	+	–
Starch	+	–	–	+	–
Lactose	–	–	–	+	–
Galactose	–	+	+	+	–
Sucrose	+	–	–	+	–
Mannose	+	+	–	+	–
Mannitol	–	–	–	+	–
Xylose	+	–	+	–	–
Raffinose	–	–	+	+	–

Cysteine

It was followed by cysteine, which was produced by 28 isolates. It was an early rising amino acid and in the majority of cases, it appeared within the first 24 hours in the broth. However, the quantity of cysteine was not very high and only up to 1.6g/l of cysteine was produced by an isolate B-7-13.

Phenylalanine

Phenylalanine was produced by 17 isolates, and their time scale productions were also similar to that of cysteine; however, a quantity was higher.

An isolate B-5-7 fermented 4.8 g/l of phenylalanine after 24-hour fermentation.

Table 4. Growth curve of B-5-1 on the basis of its dry cell mass (DCM) in GYE medium.

Time (Hr.)	pH	DCM (g/l)
0	7.00	0
2	7.33	0.038
4	7.57	0.130
6	7.66	0.215
8	7.81	0.360
10	8.19	0.625
12	8.52	1.022
14	8.65	1.040
16	8.72	1.027
18	8.81	1.026
20	8.77	1.004
22	8.78	0.897
24	8.69	0.670

Glutamic acid

Glutamic acid is among those amino acids, which are normally produced by wild-type isolates along with alanine and valine. In this case, 8 isolates produced glutamic acid in the fermentation broth. An isolate B-5-1 produced up to 6.7g/l of glutamic in the medium after 72 hours of fermentation. Glutamic are generally produced within 48 to 72 hours of incubation. In this case, the trend of glutamic acid production was a bit delayed and peak production by the producers appeared between 72 to 96 hours.

There are some other amino acids, which also appeared in the fermentation broth, but their results were not prominent such as alanine, aspartic acid and valine. On the basis of their amino acid production potential, five isolates, B-5-1, B-5-7, B-7-19 and B-7-24 were selected for characterization studies (Table 1, Fig. 2). Biotechnological techniques used amino acids production through amino acid-producing bacteria bring a boom in the nutrition and medical field. Prior to the start of the production of amino acids by fermentation, almost all industrial microorganisms were discovered by the screening of isolates from natural resources (Wendisch, 2007).

A number of researches were conducted to produce the amino acids from milk by utilizing the various bacterial strains. Muzammil *et al.* (2003) study depicts the presence of Isolucine, Lysine, Alanine, Aspartic acid and Glutamic acid from milk.

The results of the current study indicated that all the above-mentioned amino acids were also present in buttermilk.

Characterization of selected strains

All of the five selected isolates were characterized biochemically through IMViC tests (Table 2). These strains were also studied for Gram's staining (Table 2) and tested against carbohydrate utilization tests (Table 3).

Fig. 1. Streak culturing of bacterial isolates on nutrient agar plates.

Among the five isolates, B-5-1 was selected (on the basis of high glutamic acid production) for a growth curve study. So, it was identified on the basis of results, which are shown in Tables 2 and 3.

It was observed (Table 4) that B-5-1 was Gram-positive (+), Cocobacilli, catalase-negative (–), CU positive (+), MR positive (+), and IP and VP negative (–). Regarding sugar fermentation tests (Table 3), B-5-1 was found Glucose, Maltose, Starch, Sucrose, Mannose and Xylose positive (+) and Sorbose, Lactose, Galactose, Mannitol and Raffinose negative (–). Results indicated that the genus was *Lactobacillus* and sugar results confirmed its species *delbruckii*, it was concluded that the isolate is a *Lactobacillus delbruckii* (De la Torre *et al*., 2019).

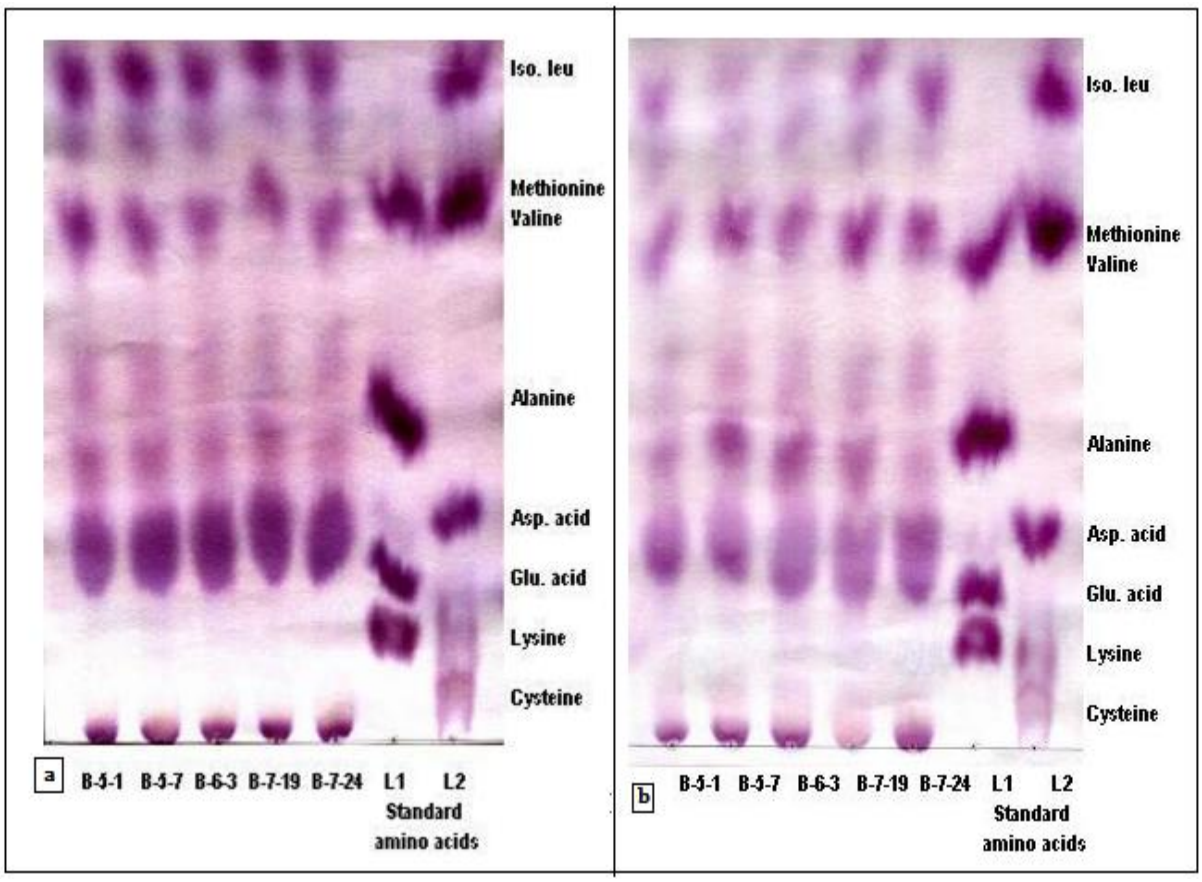

Fig. 2. Chromatograms exhibiting production of amino acids in glucose medium, L-6, after 24 (a) and 48 (b) hour of fermentation.

Growth curve

In order to study the growth pattern of B-5-1, its growth curve was plotted. The isolate was grown in a glucose yeast extract medium for 24 hours and its growth pattern was studied after every two hours. The curve illustrated at the lag phase of the isolate was very short, lasting up to 1-2 hours, then there was a gradual rise reflecting the log phase. This pattern

continued up to 12 hours. However, the peak production was attained till 14th hour of incubation. From here on, the growth remained stable for the next six hours, after which there occurred a downfall in the bacterial population; this was actually the start of the death phase (Table 4, Fig. 3).

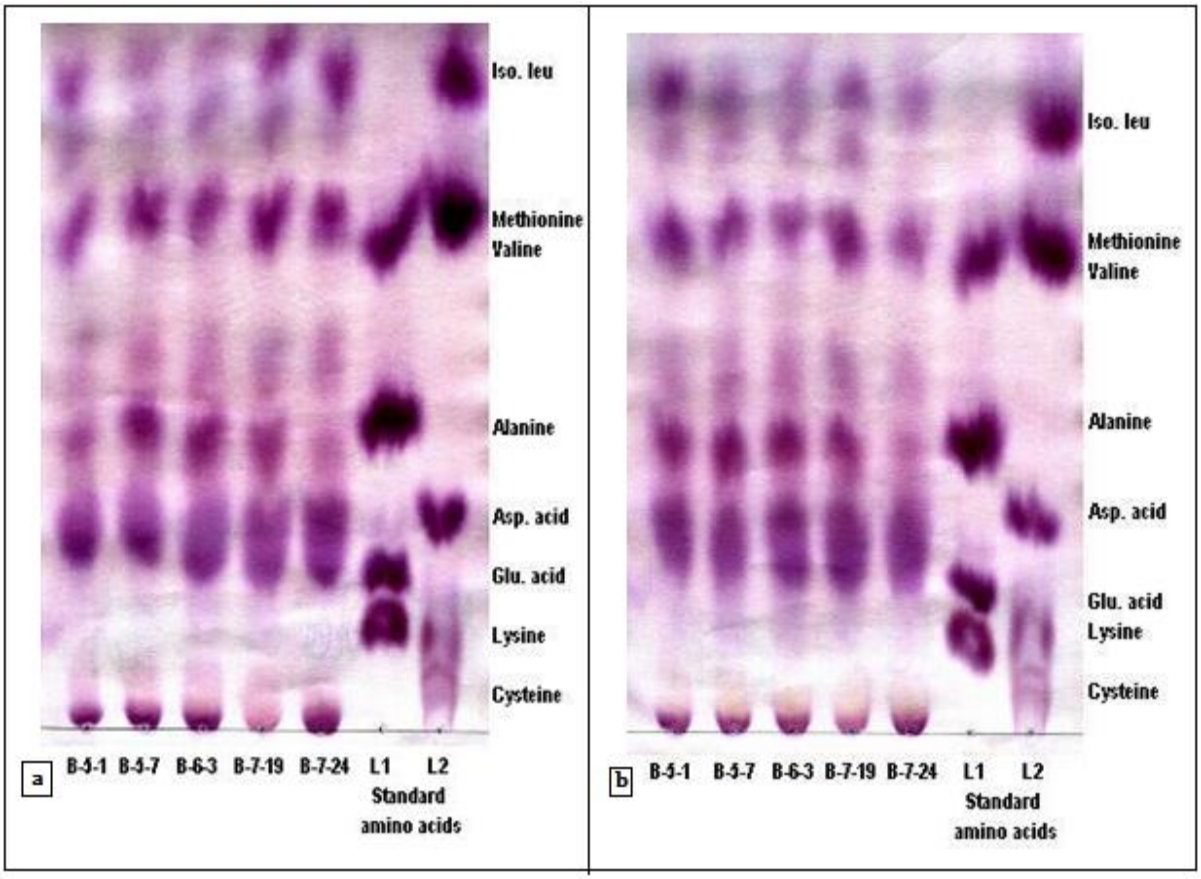

Fig. 3. Chromatograms exhibiting production of amino acids in glucose medium, L-6, after 72(a) and 96(b) hour of fermentation.

Different researchers performed various trials for the optimization of fermentation conditions to produce good quality and quantity of amino acids.

A Study conducted by Bashir (2000) suggested more suitable concentrations of glucose (10%) and $CaCO_3$ (1.5 to 2%) for the production of amino acids. The current study was also carried out by using the same quantity of glucose (10%) and $CaCO_3$ (2%) to obtain the maximum optimized results. Commonly, sugars are used for the fermentation process on an industrial scale to produce amino acids. Carbon from these sugars is one of the most problematic sources of amino acid destruction during fermentation to produce the amino acid, which alters amino acids structures into sugar-like structures available for fermenting bacteria (Barbieri *et al.*, 2020). Awareness of microbial production of amino acids has increased greatly because of the advancement of biotechnological methods.

The isolation of amino acid-producing organisms may be exploited in the design of alternative methods for the production of amino acids. Naturally accruing amino acid-producing bacteria have been isolated from a number of sources such as soil, water, plant material, fecal matter, vegetables, milk, whey, etc. Similarly, in recent studies, buttermilk was exploited for the isolation of amino acid-producing bacteria. Considerable progress has been made in understanding the primary procedures regarding the best use of buttermilk. Several analytical processing techniques have been tested, optimized and developed as a basis for the best use of buttermilk.

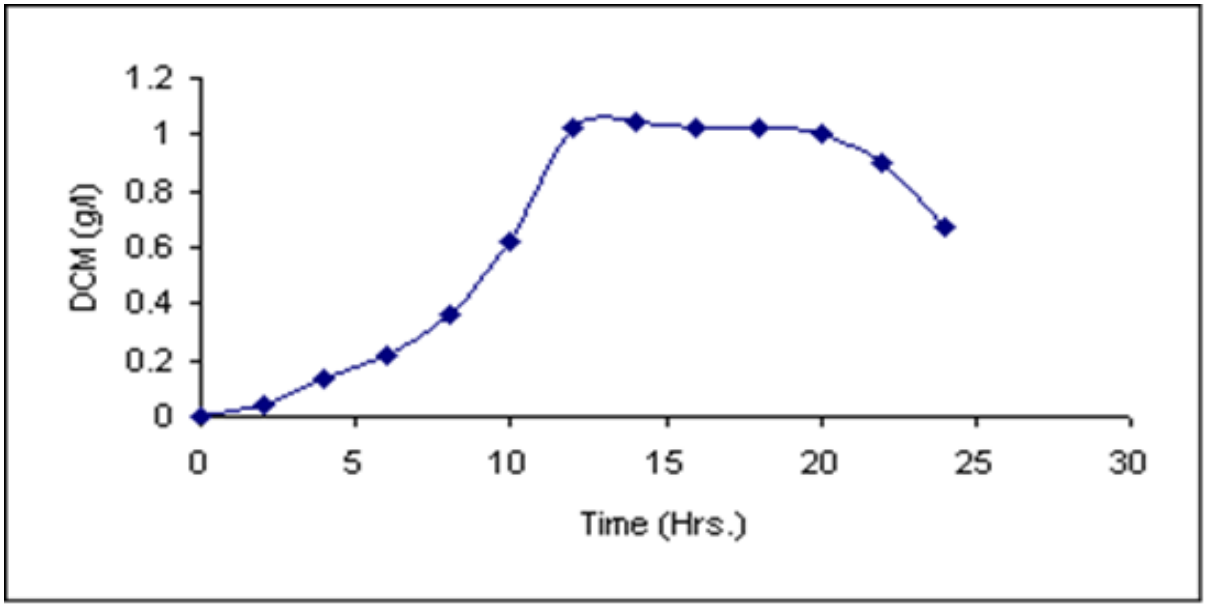

Fig. 4. Growth curve of B-5-1 on the basis of its dry cell mass (DCM) in GYE medium.

The recent work was, however, a different approach since not much work has been done on these aspects of buttermilk exploitation.

Conclusion

Buttermilk has, though, widely been exploited worldwide for a variety of products, but it is amazing that very little attention has been paid to it regarding amino acid production. We have tried to explore buttermilk for the isolation of amino acid fermenting bacteria. Briefly, isoleucine and methionine were the most frequently produced amino acids, whereas glutamic acid was fermented in maximum quantity. The recent study was a humble effort towards this direction, digging out some striking features for the worker involved in amino acid fermentation studies. For instance, the microbial production of methionine by bacteria isolated from buttermilk should be kept in the minds of the scientists while selecting a source for bacteria regarding methionine fermentation.

Conflict of interest

There is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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