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Microbial Production of L-Isoleucine from Different Substrates using Locally Isolated Bacteria

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ABSTRACT

The present study was designed especially to see the microbial production of L-isoleucine from different substrates using locally isolated bacteria. Twenty one bacterial isolates from irrigation water channels and soil were screened for amino acids production using different fermentation media. In FM-1 medium bacterial isolates were grown in which UAF AF12 gave 6.1 g/L of isoleucine, which was followed by strain UAF AF10, which gave 4.9 g/L of alanine. Histidine was another prominent amino acid (4.7 g/L) by UAF AF6. Six isolates were non-producers for isoleucine. Isoleucine was found to be the best produced amino acid, which was produced by an isolate UAF AF12 in a quantity of 6.1 g/L after 24 h of fermentation in FM-1 medium. It was followed by alanine produced by UAF AL10 in an amount of 5.8 g/L after 72 h. Among others, the main amino acids were, valine (5.4 g/L by UAF AL17), glutamic acid (3.9 g/L by UAF AF21), lysine (3.8 g/L by UAF AM1-18), histidine (4.7 g/L by UAF AF6) and aspartic acid (3.9 g/L by UAF AF14). The best fermentation time was between 48-72 h of fermentation but sometimes appreciable amounts of amino acids were also produced after 24 h of fermentation.

Key Words: Agriculture waste products; Bacterial isolates; Amino acids

INTRODUCTION

Proteins are an integral part of human and animal diet as it is of great importance to all living matter (Saima, 1996). Chemically proteins are linear polypeptide chains of amino acids (Raju & Madala, 2005). Amino acids are not only the components of proteins, which makeup our body tissues but niacin (a type of vitamin), epinephrine, nor epinephrine and serotonin (important substances for regulation of neurological function) are also made from amino acids such as tyrosine and tryptophan (Shing, 1998). Amino acids includes in the food industry (approx. 66%), feed additives (31%), medicines and cosmetics (4%) and starting material in many chemical industries (Crueger & Crueger, 1990). The amino acids production methods fall into three classes: extraction from protein hydrolysis, obtained from plant or animal sources, chemical synthesis (Kaneko *et al*., 1974) and by fermentation process (Yamada *et al*., 1972).

The fermentation methods have the advantage of yielding optically active an-d biologically required L-form of amino acids directly (Yamada *et al*., 1972). At present, *corynebacteria* are the main group of microorganisms used in the production of amino acids, although the other genera such as *Escheritia, Serratia* and *Bacillus* (Prokaryotes) and *Hansenule*, *Candida* and *Saccharomyces* (Eukaryote) also have commercial importance (Niederberger, 1989). Amino acid is found cheaper to produce by fermentation processes as compared to chemical synthesis (Nadeem & Ahmad, 1999). Fermentation ensures not only increase shelf life and microbiological safety of a food but also makes some foods more digestible and in the case of cassava fermentation reduces toxicity of a substrate (Caplice & Fitzgerald, 1999). At present sugars are the major sources of carbon (Soda *et al*., 1983). Among carbon sources, carbohydrates, especially glucose, are considered to be the best source of growth media. Amino acid fermentation now considered as one of the key industries supplying natural amino acids on an industrial scale (Meister, 1965).

BCAA's valine, leucine and isoleucine, make up approximately 1/3 of muscle protein (Goto *et al*., 2003). A deficiency in any one of these amino acids will cause muscle loss. Isoleucine like other members of this family (leucine, valine) is an essential amino acid that is not synthesized by mammalian tissues (Metro *et al*., 1999). L-isoleucine is also known as 2-amino-3-methylvaleric acid, alpha-amino-beta-methylvaleric acid and (2S, 3S)-2-amino-3-methylpentanoic acid. It is present in almonds, cashews, chicken, eggs, fish, lentils, liver, meat etc. Its molecular formula is $C_6H_{13}NO_2$ and its molecular weight is 131.17

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Daltons (Suryawan *et al.*, 1998). L-isoleucine, which is both glycogenic and ketogenic, is converted via a number of metabolic steps to alpha-methyl-acetoacetyl-CoA, which in turn is converted to acetyl-CoA (ketogenic) and propionyl-CoA (glycogenic) (Madsen *et al.*, 2002).

Isoleucine is necessary for hemoglobin formation and in stabilizing and regulating blood sugar and energy levels. A deficiency of isoleucine can produce symptoms similar to those of hypoglycemia (Lemon, 1996). The present study was conducted to isolate isoleucine fermenting bacterial strains from irrigation water channel and soil, their screening for isoleucine production and characterization of strains of interest.

MATERIALS AND METHODS

Selection of sample for the study: The present study was conducted in the Food Microbiology and Biotechnology Laboratory, Institute of Food Science and Technology, University of Agriculture Faisalabad district Faisalabad, Punjab, Pakistan. Amino acid producing bacteria were isolated from water of irrigation channels and soil from the vicinity of University of Agriculture Faisalabad (Nadeem *et al.*, 2004).

Isolation of bacterial isolates: Ahmad and Nadeem (1993), while working on the screening of bacterial isolates for amino acid fermentation confirmed that most of the bacterial isolates obtained form soil and water yielded alanine, valine and glutamic acid. The percentage of amino acid producing bacteria obtained from irrigation water channels, garden soils and flower petals was 41.13, 56.45 and 85.71; respectively difference in results is due to different substrates and organisms.

Water samples: Water samples for bacterial isolation were collected from irrigation water channels in clean, sterile 100 mL flasks. Each sample (2 mL) was diluted to 5 mL by the addition of sterile distilled water and passed through a pre-filtration pad to remove the dust particles. After pre-filtration, 2-3 mL was passed through sterilized Millipore filter of 0.45 µm pore size (Whattman Co., USA). The filters that retained bacteria were placed on nutrient agar plates, which were incubated overnight at 37°C. Next day, the colonies appearing were picked up and streaked on Eosin Methylene Blue (EMB) agar plates for identification. The desired colonies were then streak cultured on nutrient agar plates and again incubated overnight at 37°C for pure culture. Well separated colonies from each plate were slant cultured on nutrient agar slants and used for further studies.

For *E. coli* isolation from nature membrane filters were placed on McConkey agar plates and incubated overnight at 37°C. Next day, pink colonies were supposed to be *E. coli*, while picked up and streaked upon EMB agar plates. These plates were incubated at 37°C. Next day metallic green sheet was observed in some isolates. Isolates from these plates were picked on single colony isolation base and slant cultured for further processing.

Soil samples: Similar was the case with bacteria isolated from soil except that in this case different dilutions were made first. A 2.0 g fresh soil was thoroughly mixed in 10 mL sterile water for 10 min in sterilized screw-capped tube. It was followed by a number of serial dilutions, which were passed through Millipore filters (0.45 µm) that retained bacteria. Rest procedure was the same as described earlier.

Fermentation: A loopful bacterial culture from a selected slant was inoculated to flasks containing 50 mL of the fermentation medium. The flasks were incubated at 28±1°C in a gyratory shaker at 150 rpm for a maximum of 72 h, during which, 3 mL sample was collected and evaluated after every 24 h. The supernatant was filtered through Millipore filters (0.45 µm pore size) in order to have fermented broth completely pure and devoid of any cell particle. The filtrate was then examined qualitatively for amino acid production through paper chromatography as well as paper electrophoresis. The quantitative estimation of amino acids produced was done through spectrophotometry (Alberts *et al.*, 1998). Amino acid containing fermentation broths were analyzed for the qualitative as well as quantitative estimation of amino acids produced in the broth.

Fermentation medium: For the production of amino acids, the bacterial isolates were grown in FM-I medium, L-6 medium were used. FM-1 medium was composed of molasses (10%), peptone (1%), meat extract (0.5%), NaCl (0.25%) while L-6 medium was composed of glucose (10%), KH_2PO_4 (0.07%), K_2HPO_4 (0.04%), $MgSO_4.7H_2O$ (0.03%), $(NH4)_2SO_4$ (3%), Trypitcase (0.75%) and $CaCO_3$ (2%) (Sassi *et al.*, 1996; Sultan, 2005). The pH of media was kept within the range of 7.0-7.2 and $CaCO_3$ was added for neutralization. Sterilization was done through autoclaving at 121°C and 15 lbs for 15 min. The collected data was analyzed regarding medium selection and selection of best bacterial isolates. The data of each parameter was subjected to statistical analysis in order to determine the level of significance (Steel *et al.*, 1997).

RESULTS AND DISCUSSION

In the present study 21 bacterial isolates were obtained from local natural habitat (water & soil) of which eleven from soil and ten from water and checked their ability to produce isoleucine. Table I depicts the distribution and percentage of isoleucine production by bacteria. As a whole out of twenty-one, fifteen isolates produced, isoleucine with a percentage of 71.0, while six appeared to be non-producers for isoleucine.

Bacterial production of isoleucine and other amino acids in FM-1 medium: The results pertaining to production of amino acids from bacterial strain in FM-1 mediums have been presented in Table II. All of the isolates were tested for the production of amino acids. However the main emphasis was given to isoleucine. In FM-1 medium twenty one bacterial isolates were checked for isoleucine production.

Table I: Bacterial isolates from local natural habitats for isoleucine production

Habitat	Total isolates	Producers	Non-producers	(%) age producers
Soil	11	8	3	72.0%
Irrigation water channel	10	7	3	72.0%
Total	21	15	6	71.0%

Table II: Bacterial production of prominent amino acids in FM-1 medium

Amino acids	Frequency	Maximum quantity in g/L (**)	Strain
Isoleucine	15	6.1(24)	UAF AF12
		5.6(24)	UAF AF21
Cysteine	9	3.7(48)	UAF AF19
		2.9(72)	UAF AF17
Lysine	8	3.8(24)	UAF AF16
		2.9(24)	UAF AF17
Histidine	10	4.7(48)	UAF AF6
		4.6(48)	UAF AF7
Aspartic acid	12	3.9(48)	UAF AF14
		3.9(72)	UAF AF10
Glutamic acid	14	3.9(24)	UAF AF21
		3.6(48)	UAF AF10
Alanine	19	4.9(72)	UAF AF10
		4.3(72)	UAF AF9
Valine	10	2.9(72)	UAF AF8
		1.9(24)	UAF AF7
Methionine	5	1.7(24)	UAF AF8
		1.2(24)	UAF AF10
Phenylalanine	9	2.5(24)	UAF AF21
		1.4(24)	UAF AF8

Table III: Bacterial production of prominent amino acids in L-6 medium

Amino acid	Frequency	Maximum quantity in g/L (**)	Strain
Isoleucine	14	2.8(48)	UAF AL10
		1.7(24)	UAF AL9
Cysteine	3	0.5(72)	UAF AL8
		0.5(24)	UAF AL6
Aspartic acid	1	3.5(72)	UAF AL10
Glutamic acid	2	1.0(48)	UAF AL7
		1.0 (72)	
Alanine	14	5.8(72)	UAF AL10
		4.3(72)	UAF AL9
Valine	14	5.4(72)	UAF AL17
		4.3(24)	UAF AL10
Phenylalanine	6	1.9(24)	UAF AL12
		1.0(24)	UAF AL17

(**) = hours

Strain UAF AF12 gave maximum production of isoleucine as 6.1 g/L, after 24 h of fermentation followed by 5.6 g/L of isoleucine produced by UAF AF21 after same fermentation interval. Among other amino acids, cysteine, aspartic acid, alanine and valine were prominent but lysine, methionine and phenylalanine were in minute quantities, as shown in the above mentioned table. UAF AF10 produced aspartic, alanine, glutamic acid and methionine. Among these alanine was the most prominent one. The quantity of alanine was 4.9 g/L, glutamic acid (3.6 g/L) and aspartic acid (3.9 g/L) after 24 to 72 h of incubation. Out of twenty one bacterial isolates, frequencies of isoleucine, cysteine, lysine, histidine, aspartic acid, glutamic acid, alanine, valine, methionine and phenylalanine production was found in 15, 9, 8, 10, 12, 14, 19, 10, 5 and 9 bacterial isolates, respectively as shown in the Table II.

Bacterial production of isoleucine in L-6 medium: In the L6 medium as a whole, alanine and valine were the most frequently produced amino acids. Out of fifteen, fourteen isolates produced both alanine as well as valine. An isolate, UAF AL10, produced 5.8 g/L alanine. Maximum quantity of valine was produced by UAF AL17 5.4 g/L in the fermentation broth. Among fourteen isoleucine producers, the maximum production was shown by UAF AL10 (2.8 g/L) in 72 h of fermentation followed by UAF AL9 (1.7 g/L) in 24 h. Among others glutamic acid and cysteine, proline and phenylalanine were also produced by some isolates in the Table III. Hassan *et al.* (2004) worked on the isolation and screening of bacteria from milk obtained from cow and buffalo milk. Fermentation media used were L-6 medium and molasses media and by testing 28 strains, they reported that aspartic acid, alanine, glutamic acid, lysine and isoleucine were the major amino acids produced from the two sources. Bacteria from both sources produced aspartic acid and alanine in molasses media (M-I, M-II). In L-6 medium, lysine (maximum 1.8 g/l) was produced by all the isolates of both the sources. The other amino acid, which was produced in L-6 medium by majority of strains, was isoleucine (maximum 2.1 g/L). Out of fifteen bacterial isolates, frequencies of isoleucine, cysteine, aspartic acid, glutamic acid alanine, valine and phenylalanine production was found in 14, 3, 1, 3, 14, 14 and 6 bacterial isolates, respectively as shown in Table III. Twenty one bacterial isolates when grown in FM-1 medium, data obtained for isoleucine production was statistically analyzed and it was observed through ANOVA that bacterial isolates and time scale both affected isoleucine production significantly. Mean comparison test (DMRt) revealed that best isolates UAF AF-10 gave maximum production of isoleucine (2.20 g/L) and worst one UAF AF-15. Time scale was also studied and it was observed that maximum isoleucine production was observed after 24 h of fermentation time and least production was observed after 72 h of fermentation.

Fifteen bacterial isolates when grown in L-6 medium, data obtained for isoleucine production was statistically analyzed, and it was observed through ANOVA that bacterial isolates and time scale both affected isoleucine production significantly. Mean comparison DMR test revealed that best isolates UAF AL-10 gave maximum production of isoleucine (1.17 g/L) and worst one UAF AL-5. Time scale was also studied and it was observed that maximum isoleucine production was observed after 72 h of fermentation time and least production was observed after 48 h of fermentation.

Fermentation technology has played crucial roles in this progress and currently the fermented amino acids

Fig. 1: Time scale production of L-Isoleucine from selected bacterial isolates in FM-1 medium

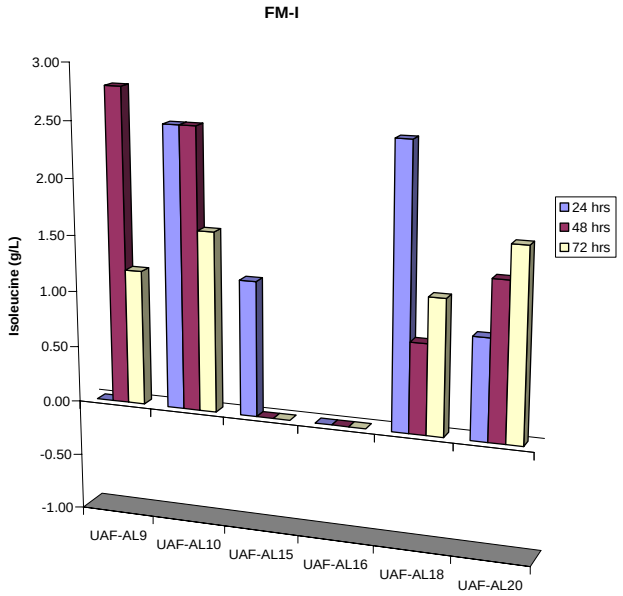
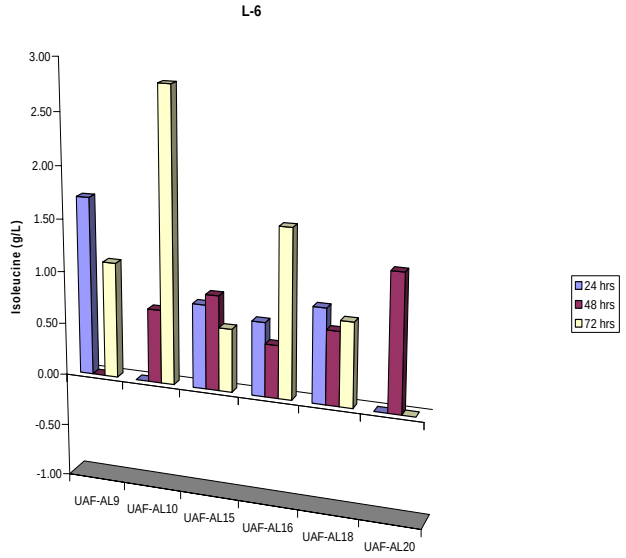


Fig. 2: Time scale production of L-Isoleucine from selected bacterial isolates in L-6 medium



represent the chief products of biotechnology in both volume and value. This area is highly competitive in the world market and process economics are of primary importance. For cost-effective production, many technologies have been developed to established high productive fermentation and recovery process. The producer organisms used in large scale, well established process has been developed to high level production efficiency (Ikeda, 2003).

The first strategy to isolate strains for the production of amino acids is based on screening of natural isolates, which already produce appreciable amount of these metabolites (Malumbers *et al.*, 1995). An organism produce sufficient amount of different amino acids during its normal growth, to meet its needs for proteins synthesis. If in certain cases a particular amino acid is required, the production of other amino acids being produced at the same time ceases trough a complex regulatory control (Clarie, 1998).

Karpouszas *et al.* (2000) worked on isolation, identification and type carbofuran-degrading bacteria from two geographically distant soils. Restriction fragment length from two geographically distant soils. Restriction Fragment Length Polymorphism (RFLP) patterns of the 16S rRNA gene and partial 16s rRNA sequence analysis were used to classify the 23 isolated obtained. Kisumi *et al.* (1977) isolated strain GIHVLAr2795, which produced L-isoleucine as 12 g/L In the present study, maximum isoleucine produced was 6.1 g/L by UAF AF12 in FM medium after 24 h of incubation. Contrary to our results it may be inferred that different microorganisms do have different attitude towards the different fermentation conditions. Moreover they may respond according to the changing carbon source.

CONCLUSION

Bacterial isolates produce higher amounts of amino acids. Although not in handsome amount, UAF-AF 12 produced maximum amount of isoleucine (6.1 g/L). Such attempts to screen bacterial isolates for better isoleucine production can be valuable in the commercial production of essential amino acids. The production of other amino acids along with isoleucine in good quantities was a plus point.

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