Comparative Production of Different Amino Acids by *Pseudomonas Boreopolis* MD-4

Rudakiya Darshan[1], Bande Priya[2]

[1]Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, New Vallabh Vidyanagar, Anand, Gujarat, India

[2]MITCON Biopharma centre, Agriculture University of Pune, Pune, Maharashtra, India

Abstract: ***Amino acids are critical to life and it is important for nutritive, medicinal and cosmetic purpose. Fermentation technology played crucial role in production of different amino acids as well as different products. Strains of Pseudomonas have major role in degradation of natural as well as complex compounds. Different strains were isolated from Pune Agriculture University in which Pseudomonas boreopolis MD-4 was selected for amino acid production. Different media and fermentation technique were used for amino acids production. Pseudomonas boreopolis MD-4 gave more amount of specific amino acid production in mostly natural condition i.e. high amount of alanine obtained in solid state fermentation, high amount of different amino acids were obtained in semi-synthetic media and synthetic media produced more variability of amino acids but little amount of amino acids. This organism produced amino acids in more amounts in solid state fermentation and high variability in agitated batch fermentation.***

Keywords: Pseudomonas boreopolis MD-4, solid state fermentation, submerged fermentation, agitated batch fermentation, amino acid production

1. Introduction

Amino acids are critical to life; they are the building blocks of protein and important as nutrients (food and feed), as seasoning, flavorings and starting material for pharmaceuticals, cosmetics and other chemicals. Fermentation technology has played crucial roles in this progress and currently the fermented amino acids represent chief products of biotechnology in both volume and value [1]. Since microbial production of L-glutamic acid was started in 1957 in Japan, various amino acids production with microorganisms has been developed and almost all protein-constitutive amino acids become able to be produced by microbial biotechnology, fermentation, or enzymatic method [2]. Solid state fermentation has several advantage for industry and economic purpose in which vital feature is the growth of micro-organisms on a pre-dominantly insoluble substrate without a free liquid phase.

Extraction of amino acids from protein hydrolysate as a method of obtaining L-amino acids is now of only limited importance; although still relevant for production of L-serine, L-proline, L-hydroxy-proline, and L-tyrosine. Different amino acids like alanine and cysteine were produced by *Pseudomonas* species [3, 4, 5, 6]. L-alanine production by fermentation is difficult because bacteria have an alanine racemase to racemize the product [3,4]. Production and metabolic pathway of L-cysteine was also studied in *Pseudomonas thiazolinohilum.* Strong aspartase activity was found in cells of *Brevibacterium* genus and it produced L-aspartic acid with the help of intact cells [7]. For the efficient production of L-alanine from ammonium fumarate using the aspartase activity of immobilized *Escherichia coli* cells and L-aspartate β-decarboxylase activity of immobilized *Pseudomonas dacunhae* cells [8].

Strains of *Pseudomonas* are used for degradation of natural compounds as well as complex organic compounds so they have major application in bioremediation. *Pseudomonas boreopolis* is a gram-negative rod, 0.5 to 3.0 microns, occurring singly and in pairs, motile with one to five polar flagella. It grows at 35°C to 37°C [12].

Initially aim of study to investigate production of different amino acids by using natural, semisynthetic and synthetic media. To produce different amino acids solid state fermentation, submerged fermentation and agitated batch fermentation were used. Comparison between fermentation technique and media was also done. This study revealed a better production of amino acids by using a fermentation technique and media.

2. Materials and methods

2.1 Media and Sample Collection

Nutrient broth, nutrient agar, EMB agar, gelatin, Pseudomonas agar, Pseudomonas F agar, Mac Conkey agar, blood agar were purchased from Hi-media Pvt. Ltd. Glucose, casein hydrolysate, K_2HPO_4, $MgSO_4$, urea, sucrose, Na_2SO_4, KH_2PO_4, $MgSO_4$ and ammonium sulphate were purchased from standard Indian chemical suppliers. All chemicals were used in the analysis are of analytical graded. Sample was collected from the soil of Pune Agriculture University, Pune.

2.2 Isolation and Confirmation of *Pseudomonas boreopolis* MD-4

Organisms were isolated from soil of farm area; four strains were isolated and further continued for the physiological and biochemical parameters. Strain of *Pseudomonas* was used for the production of different amino acids. Strain was confirmed by using Bergey's Manual and performed different test on liquid media and solidified medium.

2.3 Media preparation for fermentation

Three types of media were prepared for the fermentation procedure in which natural media was prepared by using corn seeds. Corn seeds were weighted (400.0 g), grinded and sterilized. Semi-synthetic media was prepared by using ingredients and corn seeds (10.g/L) in which glucose (10.0 g/L), casein hydrolysate (0.25 g/L), K_2HPO_4 (0.1 g/L), $MgSO_4$ (0.25 g/L) and urea (0.5 g/L) were used. All ingredients were mixed in water and autoclaved. Synthetic media was prepared by using Sucrose (5.0 g/L), Na_2SO_4 (1.2 g/L), KH_2PO_4 (3.0 g/L), K_2HPO_4 (7.0 g/L), $MgSO_4$ (0.1 g/L), ammonium sulphate (1 g/L) and vitamins. Final pH was kept as 6.93 ± 0.02 for media preparation.

2.4 Fermentation Process and Parameters

Fermentation of different amino acids was done by using *pseudomonas boreopolis* MD-4. Natural media, semi-synthetic media and synthetic media were used for solid state fermentation, submerged fermentation and agitated batch fermentation respectively. Limited amount of distilled water (15 ml) was added along with inoculum in the natural media and flasks were incubated for 7-8 days at 37°C on static condition for solid state fermentation. Prepared semi-synthetic media was sterilized and pure inoculum was added in to flasks. Flasks were incubated for 7-8 days at 37°C on 120 rpm rotary shaker for submerged fermentation. Prepared synthetic media was added into agitation tank. Fermentation media was incubated for 7-8 at 37°C on agitation of 120 rpm for agitated batch fermentation.

2.5 Extraction and Detection of Amino Acids

Protein and media was diluted by addition of water and extracted by two different method i.e. addition of ammonium sulphate and acetone. Crude amino acids were extracted by using centrifugation at 10,000 rpm for 15 minutes and measured by using different methods Bradford, biuret and ninhydrin test. Crude amino acids were also observed on TLC plates for extraction purpose.

3. Result and Discussion

3.1 Isolation and Confirmation of *Pseudomonas boreopolis* MD-4

Different strains were isolated from the soil sample and four strains were carried out for biochemical tests and morphological characters. These strains were identified as *Bacillus* sp., *Streptococcus* sp., *Escherichia* sp. and *Pseudomonas* sp. Fourth strain was selected for amino acid production and further confirmation was needed to identify the organism. This species was confirmed further on Pseudomonas agar, Pseudomonas F agar and milk agar. According to Bergey's Manual, fourth strain was identified as a *Pseudomonas boreopolis*.

3.2 Media preparation for fermentation

Different media was prepared for amino acid production. Natural, semi-synthetic and synthetic media was prepared for solid state fermentation, submerged fermentation and agitated batch fermentation as shown in figure 1 (A, B and C respectively).

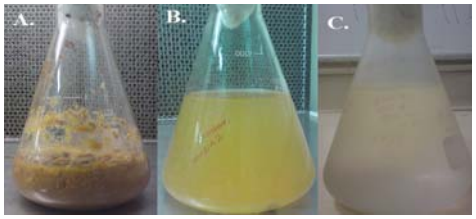

Figure 1: Natural, semi-synthetic and synthetic media for solid state fermentation, submerged and agitated batch fermentation

3.3 Fermentation Process and Parameters

Flasks were kept in static incubator in solid state fermentation and maintained temperature and condition for fermentation. Samples were taken in agitated batch fermentation and directly observed for protein precipitation and protein estimation. Ratio of estimated protein was increased after 3rd day of fermentation and was at peak on 8th day of fermentation. Fermentation was done after 9th day and proceeds for extraction of protein and purification of amino acids.

Figure 2: Agitated batch fermentation was done using laboratory fermenter (Napro Pvt. Ltd.)

3.4 Extraction of Crude Amino Acids

Precipitation of crude protein was done using ammonium sulphate and acetone. Efficient protein precipitated by ammonium sulphate. White precipitate was observed and weighted for purification of different amino acids. 500 ml of distilled water was added for dilution of produced amino acids in solid state fermentation. 15.65 gm. protein precipitate obtained from solid state fermentation.

Table 1: Biochemical characterization and identification of isolates

Characters	*MD-1*	*MD-2*	*MD-3*	*MD-4*
Gram's Staining	*Gram positive*	*Gram positive*	*Gram negative*	*Gram negative*
Shape and arrangement	Rods in chain	Cocci in cluster	Short rods	Rods
Indole production test	-	-	+	-
Nitrate reduction test	+	+	+	+
Ammonia production test	-	-	-	+
Motility test	+	-	+	+

Catalase test	-	-	+	+
Oxidase test	-	-	-	+
Starch hydrolysing test	-	+	-	-
Citrate utilization test	-	-	+	+
Dehydrogenase test	+	+	-	+
Methyl red test	-	-	-	-
V-P test	+	+	+	+
O-F test	+	+	+	-
Sugar Fermentation Test				
Glucose	-	+	+	-
Sucrose	-	+	+	+
Maltose	+	+	-	+
Lactose	+	+	+	-
Identified organism	*Bacillus sp.*	*Streptococcus* sp.	*Escherichia* sp.	*Pseudomonas* sp.

No dilution needed in semi-synthetic and synthetic fermentation for protein precipitation. High amount of protein precipitates were obtained in semi-synthetic media which was 22.32 gm. Results of TLC plate from different fermentation observed that different amino acids were present in precipitated sample. Different tests were done to confirm different amount of amino acids. Different amount of amino acids were observed in figure 3.

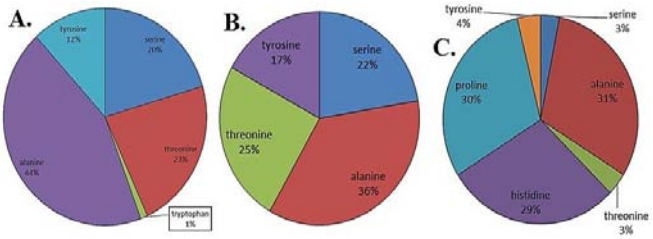

Figure 3: Total amino acid production by A. solid state fermentation B. submerged fermentation C. agitated batch fermentation

4. Conclusion

Different strains were isolated and four strains were carried out for biochemical tests and morphological characters in which *Pseudomonas boreopolis* MD-4 was selected for amino acid production. This strain was confirmed by using Bergey's Manual. Different media and fermentation technique were used for amino acids production. *Pseudomonas boreopolis* MD-4 gave more amount of specific amino acid production in mostly natural condition i.e. high amount of alanine obtained in solid state fermentation. High amount of different amino acids were obtained in semi-synthetic media because optimum condition like optimum temperature and no more aeration obtained in submerged fermentation. Synthetic media produced more variability of amino acids but little amount of amino acids. Thus, this organism produced amino acids in more amounts in natural conditions and high variability in synthetic medium.

5. Acknowledgement

Authors acknowledge Charutar Vidya Mandal, Vallabh Vidyanagar, Anand for giving permission to work MITCON biopharma centre, pune. Author also wants to acknowledge to MITCON biopharma for providing the infrastructure, laboratory, chemicals, computational and other necessary facilities for the successful completion of this work.

References

[1] Masato Ikeda, Microbial Production of l-Amino Acids Advances in Biochemical Engineering/Biotechnology. Amino Acid Production Processes, 79, pp. 1-35, 2003.
[2] Hidehiko Kumagai. Amino Acid Production the Prokaryotes 3rd edition, pp. 169-177, 2013.
[3] Chibata, I., T. Kakimoto, and J. Kato. Enzymatic production of L-alanine by Pseudomonas dachnae Appl. Microbiol. 13, pp. 638–645, 1965.
[4] Sano K, Mitsugi K, Enzymatic production of L-cysteine from DL-2-amino-Δ2-thiazoline-4-carboxylic acid by Pseudomonas thiazolinohilum: optimal conditions for the enzyme formation and enzymatic reaction. Agric Biol Chem (42), pp. 2315–2321, 1978.
[5] Sano K, Eguchi NY, Mitsugi K Metabolic pathway of L-cysteine formation from DL-2-amino-Δ2-thiazoline-4-carboxylic acid by Pseudomonas. Agric Biol Chem 43:2373–2374, (1979).
[6] Terasawa M, Yukawa H, Takayama Y, Production of L-aspartic acid from Brevibacterium by the cell re-using process. Process Biochem 20:124–128, (1985).
[7] Takamatsu S, Umemura I, Yamamoto K, Sato T, Chibata I, Production of L-alanine from ammonium fumarate using two immobilized microorganisms. Eur J Appl Microbiol Biotechnol 15:147–152, (1982).
[8] Srinivasan VR, Summers RJ, Continuous culture in the fermentation industry. In: Calcott PH (ed) Continuous cultures of cells. CRC Press, Florida, 97, (1981).
[9] Kinoshita S, Taxonomic position of glutamic acid producing bacteria. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Wiley, 1330, (1999).
[10] Kinoshita S, Tanaka K, Glutamic acid. In: Yamada K (ed) The microbial production of amino acids. Wiley, 263, (1972)
[11] Y Anzai, H Kim, J Y Park, H Wakabayashi and H Oyaizu, Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. IJSEM, 50 (4): 1563-1589, (2000).
[12] Kimura, E., C. Yagoshi, Y. Kawahara, T. Ohsumi, T. Nakamatsu, and H. Tokuda, Glutamate overproduction in Corynebacterium glutamicum triggered by a decrease in the level of a complex comprising dtsR and a biotin-containing subunit Biosci. Biotechnol. Biochem. 63: 1274–1278, (1999).
[13] Yamada, H., and H. Kumagai, Synthesis of L-tyrosine related amino acids by β-tyrosinase In: D. Perlman (Ed.) Advances in Applied Microbiology Academic Press New York NY 19: 249–288, (1975).
[14] Katsumata R, Mizukami T, Kikuchi Y, Kino K, Threonine production by the lysine producing strain of Corynebacterium glutamicum with amplified threonine biosynthetic operon. Genetics of industrial microorganisms, 21(1): 217, (1986).

Author Profile

Darshan Rudakiya received his B.Sc. and M.Sc. (Biotechnology) degree from Sardar Patel University. He published two research papers on steroid bioconversion and heavy metal degradation.