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EFFICACY OF BACTERIAL ISOLATES FROM SOIL FOR *L*-PHENYLALANINE (L-*PHE*) PRODUCTION IN DIFFERENT FERMENTATION MEDIA

M. Hassan, S. Nadeem*, K. Khaliq and I. Perveen

Deptt. of Zoology, Deptt. of Bio-Informatics*, Govt. College University, Faisalabad
Corresponding author e-mail: snadeem63@yahoo.com

ABSTRACT

Majority of the microbes have a natural ability to convert complex organic compounds into aromatic amino acids. Fifty-three isolates were obtained from soil on Nutrient agar medium through standard selection procedures. These isolates were grown on four different fermentation media containing either cane molasses, corn steep liquor (CSL), glucose or meat extract, and studied for phenylalanine production in the fermentation broths. Nineteen among them produced phenylalanine in CSL, whereas seven in meat extract medium (FM-1). Out of forty-nine percent of the producers, NIAB NFS-3 appeared to be the most prominent with 5.8 g phenylalanine/l.

Key words: Soil, phenylalanine production, fermentation media. ibt

INTRODUCTION

Consequent upon the exploitation of new applications and constantly expanding markets, amino acid production, especially through fermentation, has made a huge progress during the last fifty years. The main advantage of fermentation process is to utilize the agricultural waste/byproducts such as molasses, corn steep liquor, and to upgrade them decreasing the environmental pollution (Nadeem and Ahmad, 1999). At present, the fermented amino acids contribute a major share through biotechnology, both by volume as well as value (Ikeda, 2003). The major applications of amino acids include food industry (60%), feed additives (31%) and in the chemical, medicines and cosmetic industry as starting material (4%).

The production of L-phenylalanine, $C_9H_{11}NO_2$, (*Phe*) is of particular interest being the essential raw materials in the manufacture of the low caloric sweetener, aspartame, which is estimated to be at least 150 times sweeter than the sucrose, and grabs a hefty market worldwide (Steiner, 1977). Chemically, *Phe* is manufactured from glucose (Frost and Draths, 1995; Hodgson, 1994) and broadly used for the preparation and characterization of poly-(gamma-glutamic acid) based biodegradable nanoparticles which play a useful role in pharmaceutical and biomedical fields (Akagi *et al.,* 2005*). Phe* also plays a significant role in the production of many commercially important natural products, like the alkaloids (morphine), tannins, as well as the flavouring of cinnamon oil, cloves, nutmeg, cayenne, vanilla and pepper (Nelson and Cox 2005).

Bacterial production of amino acids has very little been exploited in Pakistan. A research programme was initiated in which bacterial isolates were obtained from soil, and tested for L- *Phe* production.

MATERIALS AND METHODS

Isolation of bacteria: Soil samples, 2g by weight, from different grassy lawns as well as irrigated cotton field were collected, suspended in 10ml of sterile distilled water and shaken vigorously for a few minutes in sterilized screw-capped tubes. A 2ml volume of each sample was further diluted to 5ml. In order to remove the soil particles completely, the dilution was passed through a Millipore pre-filtration pad. The filtrate, 2-3 ml, was then passed through sterile Millipore filter (0.45µm) that retained bacteria. These filters were placed on Nutrient agar plates, which were incubated overnight at 37°C. Thereafter, a well-separated colony from each plate was picked on single colony isolation basis, streaked twice in agar plates and finally slant cultured in Nutrient agar slant for further use.

Fermentation: A comparative study using 5 different fermentation media (Table I) was done for better *Phe* production at laboratory scale. A loopful of desired bacterial culture was inoculated to 50ml medium and incubated at 29±1°C in a gyratory shaker at 150rpm for 96 hours, during which time a 3ml sample from the broth was separated and monitored at regular intervals. Each fraction of the fermented broth was made cell-free through centrifugation and the supernatant was assessed through paper chromatography and paper electrophoresis. The quantitative estimation of amino acids was done through spectrophotometry (Nadeem *et al.,* 2001).

Characterization studies: The bacterial isolates of interest were characterized biochemically through Methyl Red, Voges-Proskauer, citrate utilization and indole production test (Stainer *et al*., 1987) as well as physically through Gram's staining. The population density was also checked using MacConkey and EMB agars.

Statistical analysis: Statistical analysis was done using Tukey-Kramer test in GraphPad Instat 3.0 (free downloadable from internet) software. Significance of difference has been presented in the form of *P* values. Values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Fifty-three bacterial isolates were evaluated for the amino acid production, while 26 of them were found producing *Phe*. Performance of the isolates varied regarding the fermentation media. Results hence obtained are discussed below:

Characterization of selected isolates: All the *Phe* producers were biochemically characterized through IMViC tests. Eighteen of the isolates showed MR test positive (+) and were significantly (*P*=0.0046) higher than negative number. Twelve were IP positive (+) and were significantly (*P*=0.0058) higher than those exhibiting negative IP test. Five VP positive, and 4 CU positive (+) were significantly (*P*=0.0051 and 0.0066 respectively) lower in number than those which depicted negative tests. Regarding Gram's staining, 16 appeared as Gram positive and were significantly (*P*=0021) higher than the rest of them (3) which were Gram negative. On the basis of these results it was concluded that 11 of the 19 isolates were *E. coli* (Table II).

Glucose-trypticase Medium, L-6: None of the isolates produced *Phe* in L-6 medium. However, an isolate NLS-3 produced around 3.6g/l of valine followed by 2.9 g/l of alanine by NLS-2, in the fermentation broth. Cysteine, glutamic acid, tryptophan and isoleucine were also found in traces (Table III).

Molasses Medium, M-I: None of the isolates produced *Phe* in M-I, however, an isolate QMS-1 fermented 6.1g/l of alanine after 72hr of fermentation. Some other amino acids like glutamic acid, methionine and valine were also found in traces in the fermentation broth (Table III).

Molasses medium, M-II: None of the isolates produced significant amount of *Phe* in M-II. Slight production of methionine alongwith aspartic acid, valine, glutamic acid and alanine was observed (Table III).

Corn Steep Liquor Medium, CSL: Nineteen isolates produced *Phe* in CSL medium; QNS-22 and QNS-7 being the best producers fermented 4.3 and 3.7g/l of *Phe*, respectively. Among others, the quantity of *Phe* remained between 2-3g/l. In addition, some other amino acids were also found, alanine being the most prominent with a quantity of 3.9g/l by QNS-14. Some other amino acids, such as histidine (2.9 g/l), glutamic acid (2.5 g/l), lysine (2.3 g/l) and valine (2.2 g/l) were also produced in good quantities, whereas cysteine, aspartic acid, proline and tryptophan were also observed in traces (Table III).

FM-1 Medium: Seven isolates produced *Phe* in this medium, of which NFS-3 and NFS-14 fermented 5.8 and 3.8g/l of *Phe*, respectively. Among the rest, the quantity of *Phe* produced remained between 1.2-2.4g/l. In addition, glutamic acid, alanine, valine and isoleucine were also produced in small quantities (Table III).

Production of *Phe* by QNS-32, QNS-7 from CSL medium and NFS-3 and NFS-14 from FM-1 medium was analyzed statistically. Production of *Phe* by NFS-3 on FM-1 medium was significantly (*P*=0.0002) different from all other strains (Table III). Thus FM-1 medium and NFS-3 strain were selected for all further studies.

Bacteria emerge as the nature's most dynamic, abundant, versatile and useful creature (Canby, 1993). Like all living cells, they do need nutritional supplementation for their metabolism, growth and reproduction in addition to the production of certain metabolites, majority of which is released into the medium through certain transport mechanism (Krammer, 1996). The present study was aimed at the isolation of bacteria from soil that produce/release one of such metabolites, *L-Phe*, in the fermentation broth. Soil was screened after Nakamichi *et al*. (1984), who isolated bacteria from soils of different areas and screened them particularly for *Phe* production.

The main attention regarding the choice of raw material for fermentation is paid towards the selection of carbon source as it not only provides structural framework for amino acids and energy source for fermentable microorganisms, but also plays a vital role in the regulation of transport mechanism of bacterial amino acids. In bacteria the range of carbon compounds to be used in media is wide enough and some species, as for example *Pseudomonas*, may utilize more than 90 organic compounds as a single source of energy (Taylor, 1982). Among carbon sources carbohydrates are supposed to be the best source of energy, however, it may vary according to the nutritional needs of the microbes (Dunn, 1985). Although, sugars extracted from plants (cane, beet) and starches (corn, cassava, sweet potato, sago palm) are the major source, acetic acid, ethanol, methanol, normal paraffine, benzoic acid, propylene glycol etc. have also been reported in this context (Nadeem and Ahmad, 1999). A lot of work for the selection of pertinent raw material in amino acid fermentation has been done, and quite a contrast has been observed among the findings of different workers, as for example glucose is considered to be the most supportive source of energy for amino acid fermentation (Dunn, 1985), but when, Misra *et al.* (1980) compared the efficiency of glucose in contrast to molasses for the same purpose, they found molasses medium better with alanine appearing as the most

abundantly produced amino acid. The similar results were also reported by Nakayama, (1986) and Ahmad and Nadeem (1993). Hassan *et al.* (2003) were also of the view that bacteria from soil gave better amino acid production in molasses media. Same trend was observed in the recent study where more amino acids were produced with molasses than with glucose. Another example is that of trypticase plus glucose (L-6 medium) where soil bacteria gave better amino acid production than that in simple glucose medium.

Phenylalanine is one of those amino acids that have not been reported to be produced in very high quantities as compared to some others such as glutamic acid and lysine. Similar results were obtained from this study where *Phe* was not produced by any of the isolates in noticeable quantity in either of the fermentation media irrespective of molasses or corn steep liquor. In order to meet this challenge, some alterations in the media were made. A molasses medium (FM-I) was devised with the addition of meat extract and peptone; the improved medium was found supportive to *phe* production while two isolates, NFS-3 and NFS-14 produced 5.8g/l and 3.8g/l of *phe*, respectively. However, this quantity (5.8g/l) was not very different from the one reported by Bulot and Cooney (1985). Some other media were also tried. In CSL medium, two locally isolated strains of *Brevibacterium*, QNS-22 and QNS-7 fermented 4.3g/l and 3.7g/l of *Phe*, respectively. In addition, lysine was also observed at a level of around 2.9g/l.

Fermentative process is nonlinear and time dependent. A lot of work has been done on the microbial behaviour regarding the production of amino acids as well as influence of results produced in the earlier part of fermentation upon the product formed in the later part (Zhang *et al*., 1998). In this study it was observed that normally there was no *phe* production during the first 36 hours. The optimum period for the amino acid production was found to be between 48-72 hrs of incubation after which the amount of *phe* in the fermentation broth gradually decreased.

In order to synthesize the cellular material microbes require a variety of elements (Michael *et al.*, 1986). These may include different organic and inorganic nitrogenous fertilizers as well as certain amino acids especially in case of industrially used microbes (Nakayama, 1972; Tortora *et al.,* 1998). We observed that in addition to carbon supply, adding up of nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium phosphate, aqueous ammonium and urea) in the medium improved the efficacy of fermentation processes. It was also observed that in spite of being the necessary part of nutrition, the excessive amount of NH_4^+ inhibits the microbial growth as well as the production of the amino acids. So, enough attention was paid to optimize not only to the selection of suitable nitrogen source but also towards its concentration in the medium. However, there have been reported some strains which showed no response either to nitrogen or carbon sources while grown at different concentrations or even without nitrogen. Even surprisingly, the strains did not exhibit any qualitative or quantitative change in the amount of amino acids produced (Revillas, 2005). But it should be kept in mind that it does not happen very often during the fermentation process.

Phenylalanine has a great demand as a raw material for sweetener, L-α-aspartyl-L-phenylalanine methylester as well as used for amino acid infusion in medical treatments. In order to get it through direct fermentation process, bacteria belonging to different genera such as *Brevibacterium, Corynebacterium, E. coli, Bacillus subtilis* and *Arthrobacter globiformis* have been manipulated. But these wild type bacteria do not produce *L- phe* in abundance as they do possess a regulatory mechanism that protects the overproduction of the amino acids including *L- phe*. So it becomes essential to create such conditions where regulatory mechanism become practically inactive or at least does not hinder the overproduction of the requisite amino acids like *L- phe.* A lot of work has been done in this context. Maiti and Chatterjee (2004, 1994) in two different attempts isolated mutants of *A. globiformis* that could produce 8.7g/l and 9.6g/l of *L-phe*, respectively. They not only mutated the isolates against NTG but also controlled the fermentation conditions through media optimization and pH control. Xu *et al.* (2003) made a similar attempt for efficient production of *L-Phe* using two different strains of *E. coli*. Similarly, Park *et al*. (1984) succeeded in accumulating 11.4g/l of *L-Phe* through regulatory isolates of *E. coli*.

This study upheld the findings of different workers providing the solid evidence regarding the potential of soil bacteria for *L-phe* production (Hummel *et al*., 1984). In general, glucose is supposed to be the best carbon source regarding amino acid production and a lot of work done is supportive to this fact (Nampoothiri and Panday, 1995; Sen *et al.,* 1992). But in the present study, *L-phe* fermenting strain did not produce the amino acid unless provided with sodium (either Na-acetate or NaCl). It also showed that *L-phe* could be produced by using CSL and molasses instead of glucose as a sole source of carbon. Naturally, bacteria exist in a great versatility and hence behave differently; similarly their response also varies towards various media.

The increasing demand for amino acids has lead to great efforts for improvement in amino acid production. Consumers' preference for the products with a natural origin has led to the exploitation of microbial resources producing natural aroma compounds (Krings *et al.,* 1996; Walsh *et al*., 1989). Advancement in the fermentation technology over the last few decades has replaced the chemical technology for the production of amino acids. It will be not long when all of the amino acids would be produced through fermentation technology.

Table I.-Fermentation media used for the microbial production of *Phe.*

Ingredients (%)	Fermentation media				
	Molasses Medium (M-I)	Molasses Medium (M-II)	Glucose-trypticase (L-6)	Corn Steep Liquor Medium (CSL)	FM-1 Medium
Glucose	-	-	10.0	2.0	-
Trypticase	-	-	0.75	-	-
Molasses	10.0	10.0	-	-	10.0
$CaCO_3$	2.0	2.0	2.0	-	-
KH_2PO_4	0.05	0.05	0.07	0.2	0.025
K_2HPO_4	0.05	0.05	0.4	-	0.025
$MgSO_4.7H_2O$	0.025	0.025	0.03	0.02	-
$(NH_4)_2SO_4$	2.0	-	3.0	-	-
NH_4NO_3	-	2.0	-	-	-
NaCl	-	-	-	-	0.25
Na acetate	-	-	-	0.5	-
Biotin ($\mu g/l$)	-	-	60	-	-
Corn Steep Liquor	-	-	-	4.0	-
Meat Extract	-	-	-	-	0.5
Peptone	-	-	-	-	1.0
Thiamine HCL	-	-	5.0 mg/l	-	-
pH	7.2	7.2	7.0	6.0	7.0

Table II Characterization of all the selected isolates through methyl red (MR), indole production (IP), Voges-Proskauer (VP), citrate utilization (CU) tests and Gram's staining.

Test	+ive	-ive	*P* value *
MR	18	01	0.0046
IP	12	07	0.0058
VP	05	14	0.0051
CU	04	15	0.0066
Gram +/-	16	03	0.0021

Tukey-Kramer test using Graph Pad Instat 3.0 software was used to determine statistical significance. Higher numbers in rows were significantly different from lower numbers.

Table III.- Efficacy of different fermentation media regarding amino acids production.

Medium	Isolate	*Phe* (g/l) produced / Fermentation period (hrs)	Amino acids other than *Phe* (g/l)
L-6	NLS-3	1.2^{d} /48	Val (3.6)
	NLS-2	1.8^{c} /48	Ala (2.9)
M-I	QMS-1	2.1^{c}/72	Ala (6.1)
M-II	-	2.4^{c}/72	-
CSL	QNS-22	4.3^{b}/ 48	Ala (3.9), His (2.9), Glu (2.5)
	QNS-7	3.7^{b}/ 72	Lys (2.3), Val (2.2)
FM-1	NFS-3	5.8^{a}/ 72	
	NFS-14	3.8^{b}/72	-
	P value	0.0001	

Phe, Phenylalanine; Val, Valine; Ala, Alanine; His, Histidine; Lys, lysine; Glu, Glutamic acid; Ile, Isoleucine. Means in column 3 were statistically analyzed using Tukey-Kramer test using GraphPad Instat 3.0 software. Mean followed by different letter differ significantly at $P \leq 0.05$.

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