Extracellular Methionine Production by Submerged Fermentation in Shake Flasks using Bacteria Isolated From the Soil

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Abstract

Extracellular methionine production by submerged fermentation using bacteria isolated from the soil was studied. Soil samples were collected from a depth of 5cm at different locations within Ihiala town in Ihiala LGA Anambra State. Each sample suspension was inoculated into plates of nutrient agar and tryptone soy agar using standard procedure. The bacteria isolated were screened for methionine production on solid minimal medium already inoculated with methionine auxotroph Escherichia coli. Qualitative observation of the plates for halo growth of the E.coli revealed the active methionine producers. The best producers were selected and employed in methionine production by submerged fermentation. A total of 87 isolates were screened for extracellular methionine production and eighteen of the isolates were found to be methionine producers, with isolates FZ13, ZM10 and ZM22 selected as the best producers. The selected isolates were characterize and identified as Bacillus species. Submerged methionine production for FZ13 and ZM22 increased with time and was highest at 72h (1.90 and 1.26 mg/ml).The growth of both isolates was maximum also at the same period , while the residual sugar gradually decreased throughout the fermentation period. For isolate ZM10 maximum methionine production was observed after 96h (1.63mg/ml), while the highest growth was recorded at 72h.The residual sugar decreased till the end of fermentation. The result showed that methionine producing bacteria abound in the soil.

Keywords: Extracellular, Methionine, Submerged Fermentation, Bacteria

INTRODUCTION

Methionine is an essential amino acid is required in the diet of humans and livestock. It cannot be synthesized internally but must be added to food and feed materials to improve the protein quality (Pharm, Galvzez, and Padolina, 1992). The impact of L-methionine on animal nutrition and the consequences of its absence as nutritive feed additive have been investigated very well. It has been observed for poultry that the stability of egg shells decreases just as the milk production in cow does (Noftsger*et al*., 2003; Keshavarz, 2003). Plant proteins are often deficient in essential amino acids such as L-lysine, L-methionine, L-threonine or L-tryptophan (Hegsted, 1956; Dutre, Oliveira, Edith, Salata, and Campos, 1973; Mertz, 1974) and consequently an exclusively vegetable diet may fail too meet nutritional requirements (Pharm, Galvzez, and Padolina, 1992). Methionine is a principle supplier of sulphur which prevents disorders of the hair, skin and nails; it helps lower cholesterol levels by increasing the liver's production of lecithin; it reduces liver fat and protects the kidneys (Borton, 1978; Gelenberg, 1980; Kelly, Neaga, Schwahn, Rosen, and Trasler, 2005). Methionine is used as an antidote in paracetamol poisoning to prevent liver damage and also in treatment of Parkinson's disease (Smythies and Halsey, 1984). Methionine is normally being produced chemical synthesis, but these processes are expensive and produce racemic mixture that has to be resolved (Fong *et al*., 1981). Fermentation optically active and more readily utilizable amino acid (Kinoshita, Udaka and and Akata, 1957). The objective of this study is to isolate *Bacillus* species form the soil and screening them for methionine production.

MATERIALS AND METHODS

Isolation of soil sample

Soil sample were collected from different sites within Anambra State University Uli Campus. Soil samples were collected from a depth of 4 – 8cm and taken to the laboratory for analysis. One gram of soil sample was aseptically put into 9ml of sterile normal saline in a test tube and shaken.Thereafter, a five fold dilution was done and aliquots of 0.1ml were inoculated on plates of nutrient agar and tryptone soy agar. The plates were incubated at 30^0C for24h. Colonies that developed were subcultured and pure cultures preserved at 4^0C on sterile nutrient agar slants.

Screening of bacteria for methionine production

The isolates were screened for methionine production using a minimal medium consisting of glucose,4.0g; $(NH_4)_2SO_4$, K_2HPO_4, 0.5g; KH_2PO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.4H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.001g; $CaCO_3$, 2.0g; agar, 15.0g; deionized water, 1liter; pH 7.0. The medium which was seeded with a 24h broth culture of the methionine auxotroph, was inoculated with the isolate by spread plate technique. An uninoculated agar plate served as control. After 72h incubation at 30^0C, the plates were examined for growth of the auxotroph. Halo growth of the *E.coli* indicates methionine production by the isolates. The methionine producers were stored for further studies. A total of 137 bacteria isolates were screened and 37 of them were selected as lysine producers.

Methionine production in submerged culture

The concentration of the bacteria suspension (24h) was standardized using the Mcfarland nephelometer. Two milliliters of the standardized bacteria suspension (4.3×10^6) were inoculated into 250ml Erlenmeyer flasks containing 50ml of fermentation medium already sterilized at 121^0C for 15min. The medium was composed of: K_2HPO_4, 0.5g; KH_2PO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.001g; $CaCO_3$, 2.0; deionized water, 1liter; pH 7.0. The inoculated medium was shaken on a rotary shaker at 160rpm at 30 ^{0}C for 144h. At interval of 24h, 5ml broth was taken aseptically for the determination of methionine, bacteria growth and residual sugar.

Determination of bacteria growth

The growth was determined turbid metrically from the culture broth in spectrophotometer at 660nm.

Determination of methionine

Quantitative determination of L-methionine in the culture broth was carried out by the modified calorimetric method of Greenstem and Winitz (1961).

RESULT AND DISCUSSION

Table 1 shows the colonial morphology of the bacteria isolates. The colonial morphology ranged from yellow to milky white to orange and grey colonies. Eight of the isolates were Gram positive cocci, 7 were Gram positive rods and 3 Gram negative rods. A total of 87 bacteria isolates were screened and 18 were selected as methionine producers (Table 2). The degree of halo growth of the bacteria isolates of FZ13, ZM 10 and ZM22 were observed to be highest methionine producers and hence were chosen for further studies. Eighteen *Bacillus* species isolated from the soil were found to be methionine producers. The occurrence of methionine producers in the soil is corroborated by the finding of Ekwealor *et al*. (2012) who isolated 6 methionine producers from the soil. Similarly, Dike and Ekwealor(2012) screened 500 bacterial isolates from the soil for methionine production and obtained 3 isolates. Yamada *et al*, (1982) examined 400 methanol-utilizing microorganisms isolated from the soil and selected strain OM33 which excreted methionine. Nwachukwu and Ekwealor (2009) reported the isolation of actinomycetes from Nigerian soil and obtained isolate SP 05 as a methionine producer. Result of the screening showed that out of 137 bacteria isolated from the soil 18 of them were capable of producing methionine. This is similar to the report of Ekwealor *et al.*(2012) who recovered 24 bacteria as methionine producers out of 200 isolated from the soil. In the study some of the isolates recorded low yield during screening. Ekwealor *et al* (2012), opined that it resulted because wild strains were employed. Rowbury and Woods (1961) also noted that wild type strains are not usually capable of producing significant amount of methionine because their biosynthesis are highly regulated. Using morphological and biochemical characteristics these isolates were identified as *Bacillus* species. The result of methionine production profile of *Bacillus* species FZ13 is shown in Table 3. As observed methionine production reached a maximum at 72h (1.90 mg/ml) and subsequently decreased till 144h. The bacteria growth also increased and reached its peak at 72h and then subsequently decreased till 120h.The residual sugar decreased continuously till the end of the fermentation period at 144h. The result of methionine production profile of *Bacillus* species ZM22 is shown in Table 4. It revealed that methionine production reached the highest at 72h (1.26 mg/ml) and then subsequently decreased till 120h.The bacteria growth also increased and reached the peak at 72h and became constant till 96h and then subsequently decreased till 120h.The residual sugar decreased till the end of the fermentation period at 120h. The result of methionine production profile of *Bacillus* species ZM10 is shown in Table 5. It showed that methionine production reached the highest level at 96h (1.63 mg/ml) and then subsequently decreased till 144h. The bacteria growth increased and reached its peak at 72h and then decreased till the end of the

fermentation period. The residual sugar kept decreasing till the end of the fermentation period. The production profile of methionine showed that *Bacillus* species FZ13 and ZM22 produced maximum methionine level(1.90 and 1.26 mg/ml) after 72h. This is consistent with the report of Anike and Okafor (2008) who observed that *Bacillus* species produced 1.35mg/ml of methionine after 72h incubation. In the study it was observed that *Bacillus* species ZM10 recorded highest methionine production after 96h. This is corroborated by the report of, Dike and Ekwealor, (2012) that *Bacillus cereus* DS13 and RS16 produced methionine after 96h of fermentation. Conversly, Banik and Majumdar (1974) reported 4.5g/l methionine yield after 76h.

Table 1: Partial Characterization of *Bacillus* species isolated from the soil

Bacteria isolate	Colonial and cell morphology	Gram stain	Endospore test
FZ9	Yellow colonies	+ cocci	-
ZM27	" "	+ cocci	-
FZ13	Milky white	+rods	+
FZ4	Whitish colonies	+ rods	+
JQ11	Milky White	+ rods	-
FT16	Milky White	+cocci	-
ZM8	Milky White	-rods	-
ZM13	Yellow colonies	+cocci	-
ZM10	White colonies	+rods	+
FT2	Orange colonies	-rods	-
FT15	Orange colonies	+ cocci	-
JQ5	Grayish green	+cocci	-
FZ15	Yellow colonies	-rods	-
ZM22	Milky White	+rods	+
ZM18	Milky White	+rods	-
FZ6	Whitish colonies	+rods	+
JQ7	Whitish colonies	+cocci	-
FZ	Yellow colonies	+cocci	-

Table 2: Screening for methionine production by bacteria on solid agar medium

Bacteria isolate	Degree of halo growth
FZ9	+
ZM27	+
FZ13	++++
FZ4	++
JQ11	++
FT16	++
ZM8	+
ZM13	+
ZM10	+++
FT2	+
FT15	++
JQ5	++
FZ15	+
ZM22	+++
ZM18	+
FZ6	+
JQ7	++
FZ	+

Key: ++++ High methionine producer; +++ moderate methionine producer; ++ Low lysine producer; + very low lysine producer

Table 3: Methionine production profile of *Bacillus* species FZ13

Time (h)	Methionine(Mg/ml)	Bacteria growth (OD 660nm)	Residual sugar(Mg/ml)
0	0	1.81	1.27
24	1.28	1.89	1.12
48	1.45	1.96	0.83
72	1.90	2.08	0.66
96	1.77	1.95	0.60
120	1.62	1.91	0.55
144	1.56	1.88	0.52

Table 4: Methionine production profile of *Bacillus* species ZM22

Time (h)	Methionine(Mg/ml)	Bacteria growth (OD 660nm)	Residual sugar(Mg/ml)
0	0	1.58	1.17
24	0.61	1.63	0.94
48	1.04	1.66	0.81
72	1.26	1.68	0.73
96	1.17	1.68	0.
120	0.95	1.62	0.62
144	0.87	1.60	0.60

Table 5: Methionine production profile of *Bacillus* species ZM10

Time (h)	Methionine(Mg/ml)	Bacteria growth (OD 660nm)	Residual sugar(Mg/ml)
0	0	1.70	1.10
24	0.92	1.76	0.81
48	1.17	1.82	0.73
72	1.44	1.93	0.60
96	1.63	1.87	0.51
120	1.58	1.81	0.42
144	1.41	1.70	0.40

CONCLUSION

In the study, methionine producing bacteria were isolated from the soil. This suggests that the soil is replete with useful bacteria that can be harnessed for the production of methionine. Further research which will include optimization studies is needed to see the possibility of enhancing enzyme production.

RECOMMENDATIONS

Methionine is an essential amino acid that is required in the diet of humans and livestock. It is only available through importation and this makes it costly because foreign exchange is involved. In the study, methionine was produced by bacteria isolated from the soil, so it is an indication that methionine can be made available locally and in Nigeria. It is recommended that government should set the ball rolling by encouraging local large scale production of the amino acid and banning the importation of the product. Government should also make interest free loans available to people who interested in investing in this area. Adequate funds should be given to research institutes for the purpose of carrying research in this field with the view to producing methionine economically. Government should also remove import duty on some costly equipments that would be used in the production.

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