Advanced Journal of Microbiology Research Volume 2014
Available online at http://advancedresearchjournals.org/AJMR
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Full Length Research Paper

Methionine-producing *Streptomyces* species isolated from Southern Nigeria soil

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Accepted 24 July, 2013

Actinomycetes isolated from soil were investigated for methionine production, and an isolate, designated SP- 05, was recovered. Taxonomic studies showed that the strain has good growth, sporulation and black pigment production on most media, utilized various sugars, reduced nitrate and degraded tyrosine. The aerial mycelia have spore chains with open loops, and therefore belong to retinaculum apertum group. Presence of LL-diaminopimelic acid on the cell wall of isolate SP-05 also suggested that the strain belongs to the genus *Streptomyces.* The organism was found to produce 3.72 mg/ml methionine in culture broth. Sucrose and ammonium chloride at concentrations of 8.0 and 6.0% (w/v) respectively, in the culture medium, enhanced methionine production.

Key words: Culture medium, methionine production, soil, *Streptomyces*.

INTRODUCTION

Methionine, alpha-L-amino- gamma-methylthio- n-butyric acid, is nutritionally essential for mammals and fowls. It cannot be synthesized internally, but may be added to food and feed materials to improve the protein quality (Pham et al., 1992). Methionine, in medicine and pharmaceuticals, is used as an antidote in paracetamol poisoning to prevent liver damage and also in treatment of Parkinson's disease (Neuvonen et al., 1985, Smythies and Halsey, 1984). In agriculture, it is useful in the stimulation of etiolated pea seedlings and cotton (Mahmood et al., 2008).

Plant proteins are often deficient in essential amino acids such as L-lysine, L-methionine, L-threonine or L-tryptophan (Hegsted, 1956; Dutre et al., 1973; Mertz, 1974) . In view of the scarcity of nutritious food in third world countries, there will be an ever-increasing need to supplement plant proteins with those essential amino acids which occur at suboptimal level (Cruegar and Cruegar, 1990).

Much research activity has been focused on the production of amino acids by fermentation methods, and many processes to produce various amino acids have seem to be the most economical and practicable means of producing optically active and more readily utilizable amino acids (Kinoshita et al., 1957).

The requirement of Nigerians for this amino acid is met only through importation. However, methionine can be made available and at a cheaper cost too, if produced locally by microbiological methods.

The aim of this study, therefore, was to screen actinomycetes isolated from Nigerian soil for methionine production.

MATERIALS AND METHODS

Isolation of actinomycetes

The medium used for isolation of actinomycetes was Starch-Casein-Nitrate agar (Starch, 10.0 g; Casein, 0.003 g; $KN0_3$, 0.02 g; NaCl, 0.02 g; $MgS0_4$. $7H_2O$, 0.5 mg; $CaC0_3$, 0.2 mg; $FeS0_4$.$7H_2O$, 0.1 mg; Agar, 12.0 g; H_2O, 11; pH 7.0) (Kuster and Williams, 1964). Each soil sample (1.0 g) was suspended in 9 ml of sterile water, and 1 ml of the suspension was serially diluted ten-fold in sterile dis-tilled water. One milliliter of the 10^{-5} dilution was introduced into the agar medium by the pour plate technique and after inoculation for 7 days at 30°C, the plates were observed for growth. White discrete and leathery colonies were subcultured and pure cultures of the isolates were streaked on Starch-Casein-Nitrate agar slants and stored at 4°C.

Screening of actinomycetes for methionine production

Fermentation medium: A modified basal medium (K_2HP0_4, 0.3 g;

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KH_2P0_4, 0.7 g; Na_2C0_3, 1.0 g; $CaCl_2$, 5.0 mg; $MgS0_4.7H_2O$, 0.3 g; $FeS0_4.7H_2O$, 1.0 mg; H_2O, 1l) containing sucrose (20.0 g) and NH_4Cl (10.0 g) was used for fermentation (Pham et al., 1992). The pH of the medium was 7.2.

Cultural conditions: Two loopfuls of a 7 day old culture of the actinomycete were inoculated into a 250 ml Erlenmeyer flask containing 30 ml of the fermentation medium.

Methionine production was assayed after incubation of the flask for 5 days on a rotary shaker (160 rpm) at 30°C. Duplicate flasks were prepared and uninoculated flasks served as control in all experiments.

Methionine assay: The presence of methionine in the culture broths of the isolates was examined by paper chromatography following a modified method of Khanna and Nag (1973). The broth culture was centrifuged at 5000 x g for 20 min and 2 µl of the supernatant was applied 1.5 cm above one edge of Whatman No.1 filter paper, with dimensions of 18 cm x 22 cm. A l µl volume of a standard methionine solution (0.1 mg/ml) was applied alongside with the supernatant, and the chromatogram was developed in a solvent mixture of n-butanol, acetic acid and water (4: 1: 1) for 18 h. The chromatogram was air-dried at room temperature, sprayed with 0.15% ninhydrin solution in butanol and dried again before heating at 60°C for 5 min in an oven. The R_f value of the ninhydrin-positive spot (bluish-violet) of the supernatant that corresponded with the R_f value of the standard methionine solution was taken to indicate presence of methionine in the broth culture.

Estimation of methionine concentration: The concentration of methionine produced in the broth culture of isolate SP-05 was estimated as follows. The ninhydrin-positive spot of the supernatant of isolate SP-05 on the chromatogram was eluted in 10% ethanol and the spectrophotometric reading of the eluate at 520 nm recorded. The methionine concentration in the supernatant was determined from a standard curve. A plot of the values of optical densities against varying concentrations (0.1 to 0.9 mg/ml) of a methionine solution served as the standard methionine curve.

Taxonomic studies of isolate SP-05

Cultural characteristics of isolate SP-05 on plates of Inorganic Salt Starch agar, Czapek Dox agar, Glycerol-Asparagine agar, Tyrosine agar, Dextrose Yeast agar, Tryptone Soy agar and Starch-Casein-Nitrate agar were examined. The micromorphological feature of the aerial mycelium on Starch-Casein-Nitrate agar was also examined. The ability of the isolate to hydrolyze starch, degrade tyrosine, reduce "nitrate and utilize glucose, dulcitol, xylose, rhamnose, sucrose, raffinose, maltose, mannitol, galactose and cellulose was examined. Production of hydrogen sulphide and melanin pigment, and resistance of isolate SP- 05 to varying concentrations of NaCl were also studied. Taxonomic studies were performed, following the methods recommended by Shirling and Gottlieb (1966, 1968a, b), Starr et al. (1981), Huang et al. (2004) and He et al. (2005). Presence of LL-diaminopimelic acid on the cell wall of isolate SP-05 was determined following the method described by Becker et al. (1964).

Effect of sucrose concentration on methionine production

The effect of varying concentrations (10.0, 20.0, 40.0, 60.0, 80.0, 100. 0 g/l) of sucrose on methionine production by isolate SP-05 was examined. A 250 ml flask containing 30 ml of the basal medium, NH_4Cl (10.0 g/l) and sucrose at the desired concentrations was inoculated with two loopfuls of a 7 day old culture of isolate SP-05. After incubation of the flask for 5 days on a rotary shaker (180 rpm) at 30°C, methionine accumulation in the culture broth was assayed as described above.

Effect of ammonium chloride concentration on methionine production

The effect of varying concentrations (10.0, 20.0, 40.0, 60.0, 80.0 g/l) of ammonium chloride on methionine production by isolate SP-05 was also examined. An Erlenmeyer flask (250 ml) containing 30 ml of the basal medium, sucrose (80.0 g/l) and NH_4Cl at the desired concentrations was inoculated with two loopfuls of a 7 day old culture of isolate SP- 05. Incubation of the culture and methionine accumulation in the culture broth was assayed as described previously.

RESULTS AND DISCUSSION

A total of 245 actinomycetes, isolated from soil in Anambra State, Nigeria, were screened for methionine production, and an isolate designated SP- 05 was recovered. Good growth, sporulation and black pigment production by isolate SP-05 were observed on various media, and the colours of the aerial mycelium varied from white on Tryptone Soy agar to brownish-white on Glycerol-Asparagine agar and grayish-brown on other media. This colour variation is supported by the International *Streptomyces* Project (ISP) colour groups of Shirling and Gottlieb (1966), and may be a result of the different compositions of the media used. The microscopic appearance of the spore chains on the aerial mycelium are of the large atypical type described by Pridham et al. (1958) as belonging to retinaculum apertum group (Figure I).

Isolate SP-05 hydrolyzed starch, degraded tyrosine, reduced nitrate and also produced hydrogen sulphide. All sugars, except dulcitol and cellulose, were utilized. The isolate tolerated NaCl concentrations higher than 4% but less than 7%. The cell wall contains LL-diaminopimelic acid and, according to Lechevalier and Lechevalier (1970), isolate SP- 05 has a type 1 cell wall. Based on the morphological and physiological characteristics 'of isolate SP-05, it was therefore assigned to the genus *Streptomyces.*

Streptomyces strain SP-05 was observed to produce methionine in a sucrose-ammonium chloride medium. The excretion of amino acids by species of *Streptomyces* has been reported by several authors (Perlman and O'Brien, 1958; Yoshida and Nagasawa, 2003; Hirohara et al., 2006). In addition to *Streptomyces,* methionine-yielding strains are also found among species of *Micrococcus* and *Corynebacterium* (Banik and Majumdar, 1974; Kase and Nakayama, 1975; Trotschel et al., 2005). Kinoshita et al. (1957) reported that the type of amino acid produced is dependent on medium composition, cultural conditions and the species of organism used.

The effects of varying concentrations of carbon and nitrogen sources (Tables 1 and 2) on methionine production by *Streptomyces* strain SP-05 were investigated. The

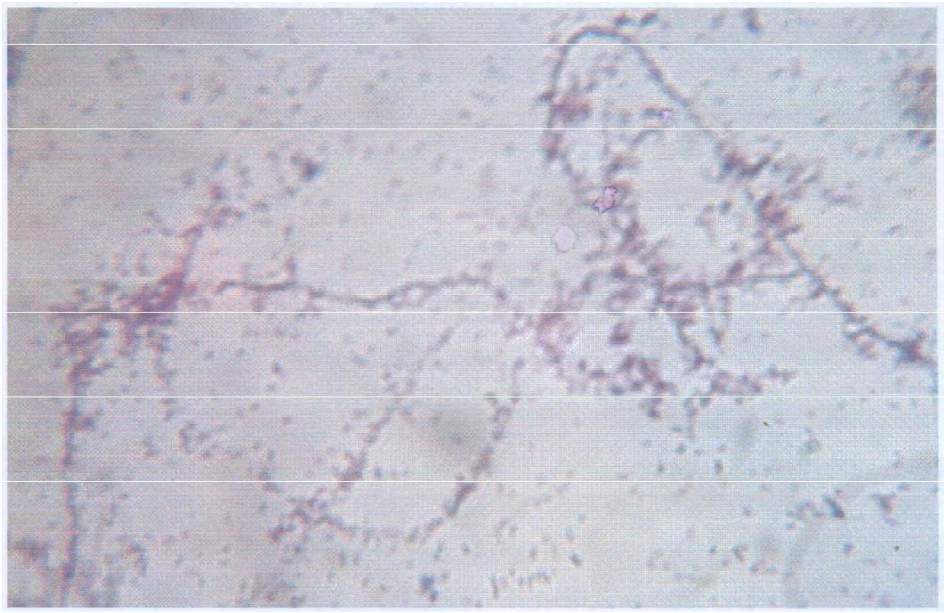

Figure 1. Micromorphological characteristics of SP-05 (Magnification x 1000).

Table 1. Effect of sucrose on methionine production.

Sucrose (g/l)	Methionine (mg/ml)
10.0	1.62
20.0	1.86
40.0	2.20
60.0	2.86
80.0	3.30
100.0	2.65

Nitrogen source: NH_4Cl (10.0 g/l).

Table 2. Effect of ammonium chloride on methionine production.

Ammonium Chloride (g/l)	Methionine (mg/ml)
10.0	3.27
20.0	3.38
40.0	3.56
60.0	3.72
80.0	3.06

Carbon source: Sucrose (80 g/l).

results showed that 3.72 mg/ml methionine accumulated in the culture broth when 8% (w/v) sucrose and 6% (w/v) ammonium chloride were added to the fermentation medium. The influence of carbon and nitrogen sources on carbon and nitrogen sources on methionine production by *Streptomyces* strain SP-05 is likely to be a function of the initial substrate present in the medium. The view is supported by the experimental observations of Perlman and O'Brien (1958), Neijssel et al. (1993) and Ekwealor and Obeta (2005). They noted that the degree of reduction of substrate in a fermentation process is usually equal to the degree of product formation.

Beyond 6% nitrogen concentration, methionine production decreased. This decrease, as suggested by Pham et al. (1992), may be attributed to osmotic pressure exerted by high nitrogen concentration having an adverse effect on the organism, affecting growth and production of methionine. A similar effect may have been responsible for the decrease in methionine production at 100 g/l sucrose concentration.

This preliminary study has shown that methionine-producing *Streptomyces* species can be isolated from Nigerian soil. High methionine yield of 3.72 produced by the *Streptomyces* species is comparable to the report of many workers (Banik and Majumdar, 1974; Kase and Nakayama, 1975; Kumar et al., 2005) even when the production is of bacterial origin involving such organisms as *Micrococcus glutamicus, Corynebacterium glutamicus* and *Brevibacterium flavum.*

This microbiological process of methionine production if well developed will reduce the importation of this product into the country.

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