Screening of Methionine-producing *Bacillus* Species from Nigerian Fermented Food Condiments and Effects of Some Cultural Parameters on Methionine Accumulation

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Abstract  Fermented food condiments are sources of proteins and vitamins and are added as spices or sauces to food for flavour or taste enhancers. The major fermenting microorganisms, *Bacillus* species, produce proteolytic enzymes that hydrolyze proteins and amino acids and peptides. Methionine is the most essential amino acid for chicken feeds and can be produced by *Bacillus* species. The screening of methionine-producing microorganisms from Nigerian fermented food condiments and the effect of some cultural parameters on methionine accumulation were conducted. Bacterial organisms isolated from fermented food condiments, ogiri and okpeye, were screened for methionine producers on minimal solid agar medium seeded with auxotrophic *Escherichia coli* and in submerged medium. The effects of medium to fermenter volume ratio, inoculum size, carbon and nitrogen sources on methionine accumulation by the isolates were investigated. Of the five methionine producers recovered, two of the active isolates identified as *Bacillus pumilus* and *Bacillus amyloliquefaciens* were used for methionine production. A 20.0ml fermentation medium and 5.0% inoculum size gave the highest methionine accumulation by the *Bacillus* species. At 8.0% glucose and 4.0% ammonium sulphate concentrations, methionine yields of 5.22mg/ml and 5.50mg/ml were accumulated in the broth cultures of *B. pumilus* and *B. amyloliquefaciens* respectively. *B. pumilus* and *B. amyloliquefaciens* recovered from Nigerian fermented food condiments were observed to produce methionine and the optimization of some cultural parameters enhanced methionine accumulation by the *Bacillus* species.

Keywords: food condiments, fermentation, bacillus, methionine, optimization

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1. Introduction

Fermented foods constitute a significant component of African diets, and fermented food condiments are spices, sauces or food preparations added to food to impact a particular flavour or enhance its taste [1,2]. These food condiments are known to be good sources of proteins and vitamins [3,4].

The substrates for the fermentation of these condiments harbour diverse microorganisms from the environment [5,6,7]. These microorganisms use the nutritional constituents of the raw materials during fermentation, converting them into products that contribute to the chemical composition and taste of the final products [8,9].

The major fermenting microorganisms involved in the fermentation process of most vegetable proteins (fermented condiments) have been identified as proteolytic *Bacillus* species, example *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus circulans* [10,11,12]. These *Bacillus* species produce proteolytic enzymes which hydrolyze proteins and amino acids and peptides [13,14,15] and are ubiquitous in nature [16].

The discovery of microbial production of glutamic acid [17] triggered the search for other amino acid-producing microorganisms [18]. Since then, there have been many reports about the efforts made by several researchers to develop high yielding strains for producing methionine by submerged fermentation [19,20,21].

Methionine is the most important essential amino acid for chicken and other poultry, and is also required in the diet of humans for rapid growth and health [22,23,24].
The demand for methionine has increased due to the rapid growth of feed additive market, driven by the probably increasing consumption of meat and milk products, as a source of protein or other nutrients [25].

As an important amino acid, methionine is widely used in feed, pharmaceutical and food industries [26,27,28]. Methionine can be produced by chemical routes or biological routes. However, chemical routes not only produce racemic mixtures of D- and L- forms but also result in a series of environmental pollution [27,29]. Therefore, a low cost environmentally-friendly production of pure L-methionine with microorganism based on natural renewable resources is becoming more attractive [25].

Media composition is known to have a profound effect on the physiology of microorganisms, and maximum product formation is associated with particular physiological forms [30]. Since bacterial cells can change patterns of enzyme synthesis, in order to adapt themselves to their specific environments, the media must be designed to provide a favourable environment for particular product formation [22].

The screening of methionine-producing microorganisms from Nigerian fermented food condiments and the effects of some cultural parameters on methionine accumulation were studied.

2. Materials and Methods

2.1. Collection and Treatment of Samples

Wraps of locally fermented fluted pumpkin (Telfairia occidentalis), known as ogiri and fermented cotyledon of African Mesquite (Prosopsis africana), known as okpeye or ukpehe, purchased from the local markets and sun dried for 72h, were used for the isolation of bacterial organisms.

2.2. Isolation of Bacteria

The sun dried mashed sample (10.0g) was placed in 100ml of distilled water in a 250ml Erlenmeyer flask and shaken vigorously for 15min. The suspension was serially diluted 10 folds and 0.1ml of 10^-6 dilution spread inoculated on Nutrient agar (Oxoid) plates. Duplicate plates were prepared and uninoculated flask served as control. Methionine accumulation was determined from the broth culture.

2.4. Screening of Isolates for Methionine Production

2.4.1. Seed Culture

The medium for seed culture consists of yeast extract, 10.0g; NaCl, 5.0g; peptone, 10.0g; H2O, 1L; pH 7.2, and was dispensed in 5ml volume in test tubes. After sterilization at 121°C for 15min, the tubes were each inoculated with a loopful of the methionine-producing isolates and incubated at 30°C for 24h in a water bathing constant temperature vibrator (CHA-C shaker, Bran Sci.Comp.Uk) at 120rpm.

2.4.2. Fermentation Medium

The basal medium (KH2PO4, 0.05g; K2HPO4, 0.05g; MgSO4.7H2O, 0.1g; FeSO4.7H2O, 0.001g; MnSO4.4H2O, 0.001g; CaCO3, 20.0g; water, 1L), glucose, 20.0g; (NH4)2SO4, 10.0g; pH adjusted to 7.2 with NaOH. A 20.0ml volume of the fermentation medium in a 100ml Erlenmeyer flask was inoculated 2.0ml (ca.6.27 × 10^8 cells/ml) of the seed culture and the flask incubated for 72h on a rotary shaker at 160rpm and 30°C. Duplicate flasks were prepared and uninoculated flask served as control. Methionine accumulation was determined from the broth culture.

2.4.3. Methionine Assay

Methionine was assayed for following the method described by [32]. The broth culture of the isolate was centrifuged at 5,000 × g for 20min. To 5ml of the supernatant in a test tube was added 1ml of 5N NaOH and 0.1ml of 10% Sodium nitroprusside. The tube was shaken properly, allowed to stand for 10min before adding 2ml of glycine over a period of 10min, shaking frequently. After another 10min interval, 2ml of concentrated orthophosphoric acid was added drop wise and the test tube properly shaken. Colour development was allowed to proceed for 5min and the colour intensity measured at 540nm in a spectrophotometer (Jenway 640S uv/vis). The blank was similarly prepared without the supernatant. Methionine yield was interpolated from a standard methionine curve.

2.5. Identification of Isolates

Five of the methionine producers recovered were subjected to preliminary identifications. The test carried out include Gram staining, starch hydrolysis, catalase, motility, nitrate reduction, citrate tests, methyl red, Voges-Proskauer tests and spore test. Two of the very active methionine-producing isolates were further identified using 16S RNA sequencing and were subsequently used for further studies.
2.6. Influence of Medium Composition on Methionine Accumulation

2.6.1. Effect of Medium Volume and Inoculum Size

The effects of medium volumes (15.0, 20.0, 25.0, 30.0ml) and inoculum size (1.0, 2.0ml) on methionine accumulation by the isolates were examined. A 100ml Erlenmeyer flasks with varying volumes of the fermentation medium were each inoculated with 1.0ml (ca. 3.24 × 10⁸ cells/ml) of a 24h seed culture and incubated as previously described and methionine determined from the broth culture. A 2.0ml (ca.6.53x 10⁷cells/ml) volume of the 24h seed culture was also similarly treated as previously described. The medium volume and inoculum size with good methionine production were used for subsequent studies.

2.6.2. Effects of Carbon Sources

Carbon sources (glucose, fructose, ribose, sucrose, glycerol) were studied for their effects on methionine production by the isolates. A 20.0ml volume of the fermentation medium (basal medium, carbon source, 20.0g; ammonium sulphate, 10.0g; pH, 7.2) was inoculated with 1.0ml of the seed culture and methionine determined from the broth culture after incubating for 72h at 160rpm and 30°C. The carbon of choice was used for further studies.

The varying concentrations (20.0, 40.0, 60.0, 80.0, 100g/L) of glucose on methionine accumulation by the isolates were determined. Fermentation medium with glucose concentrations under study were inoculated and the fermentation processes carried out as previously described. Glucose concentration of choice was used for further studies.

2.6.3. Effect of Nitrogen Sources

The effects of nitrogen sources (ammonium sulphate, ammonium chloride, urea, potassium nitrate) on methionine production by the isolates were investigated. A 20.0ml of the fermentation medium (basal medium, glucose 80.0g; nitrogen source, 10.0g; pH 7.2) was inoculated with 1.0ml of the seed culture and methionine determined from the broth culture after incubating for 72h at 160rpm and 30°C. The carbon of choice was selected for further experiments.

Varying concentrations (20.0, 40.0, 60.0, 80.0, 100g/L) of ammonium sulphate were examined for methionine accumulation by the isolates. The fermentation medium containing the concentration of ammonium sulphate under study was inoculated and the fermentation procedure was as previously described.

3. Results and Discussion

The fermented condiment samples were sun dried to allow for the development of more aromatic and less ammoniacal flavour [2] and also for the isolation of mostly endospore-forming bacteria. Table 1 shows the screening of the recovered isolates from fermented food condiments, ogiri and okpeye, on solid and submerged medium. It can be observed (Table 1) that methionine accumulation in the broth cultures of the isolates are directly proportional to the halo growths on solid agar medium. This result is similar to the reports [31] and [33], who worked on methionine-producing bacteria and Lysine-producing yeasts respectively. Five of the methionine producers recovered from the fermented food condiments were Bacillus species (Table 1).

Table 1. Screening of isolates for methionine production on solid and submerged medium

| Isolate code | Organism | Methionine production |
|--------------|----------|------------------------|
|              |          | Solid medium (mg/ml) | Submerged medium (mg/ml) |
| OK2          | Bacillus amyloliquefaciens | +++ | 1.98 |
| OK24         | Bacillus sp | ++ | 1.25 |
| OK31         | Bacillus sp | ++ | 1.13 |
| OG4          | Bacillus pumilus | +++ | 1.76 |
| OG10         | Bacillus sp | ++ | 1.18 |

OK- Okpeye, OG-Ogiri.

However, two of the very active isolates OG4 and Ok2, were identified as Bacillus pumilus and Bacillus amyloliquefaciens, and methionine levels of 1.76mg/ml and 1.98mg/ml respectively, were produced by the Bacillus species in submerged medium. The importance of Bacillus species in fermented food condiments have been reported by many researchers [4,10,12]. They observed that the Bacillus species are proteolytic in nature and their metabolic and enzymatic hydrolytic activities serve to breakdown the proteins into amino acids [34].

The involvement of Bacillus species in the accumulation of methionine in the fermentation medium have been reported by many workers [21,35,36], and they have been found also to be ubiquitous [16]. The ability of B. pumilus and B. amyloliquefaciens recovered from fermented food condiments to produce methionine supports the works of [37,38], however, their methionine yields are not as high as those of Corynebacterium and Brevibacterium species [18,20,36].

In order to enhance the L-methionine fermentation titre, the fermentation conditions need to be additionally optimized. The media used must contain all components in appropriate concentrations required for growth and product formation [39]. The influence of medium/fermenter volume ratio was therefore evaluated.

As shown in Figure 1, a medium volume of 20.0ml was observed to be the best for methionine accumulation by the Bacillus species. This finding is in line with the work of [38] and [40], who reported a medium volume of 20.0% for methionine accumulation by Bacillus thuringiensis EC1 and Corynebacterium glutamicum ATCC21608 respectively. Contrary to the result obtained, [37] and [41], reported a 30.0% fermentation broth as optimum for maximum methionine production by Bacillus cereus and Corynebacterium glutamicum MTCC2745 respectively. A 30% fermentation medium was also reported in the work of [42], who noted a maximum L-glutamic acid production by Micrococcus glutamicus. The medium to fermenter volume ratio is dependent on the strain of organism used for metabolite production in submerged fermentation.
Medium composition: **KH₂PO₄, 0.05g; K₂HPO₄, 0.05g; MgSO₄.7H₂O, 0.1g; FeSO₄.7H₂O, 0.001g; MnSO₄.4H₂O, 0.001g; CaCO₃, 20.0g, glucose, 20.0g; (NH₄)₂SO₄, 10.0g; H₂O, 1L; pH 7.2**

**Figure 1.** Effect of medium/fermenter volume ratio and inoculum size on methionine accumulation by *B. amyloliquefaciens* and *B. pumilus*

The size of inoculum in a fermentation process influences the metabolic production [37,43]. The impact of inoculum size on methionine production by the *Bacillus* species was examined. Results (Figure 1), showed that a 5.0% inoculum size was optimal for methionine yields of 2.04mg/ml and 2.40mg/ml produced by *B. pumilus* and *B. amyloliquefaciens* respectively. This observation agrees with the work of [38]. They reported a 5.0% inoculum size in the production of methionine by *Bacillus thuringiensis* EC1 but a 1.0% inoculum size in methionine accumulated by *Bacillus cereus* S8 [44]. [41,45,47], reported a 3.0% inoculum size for optimal growth and metabolite production.

However, it has been noted that the inoculum size to be used is usually dependent on the cell mass and composition of the seed medium to be transferred [37]. Therefore at a suitable inoculum size, the nutrient and oxygen levels support sufficient growth of the organism and then enhance metabolite production [43].

Carbon sources, nitrogen sources and their ratio in the fermentation media, play significant role in the production of particular metabolites [38,46,47]. The effects of different carbon sources: glucose, fructose, ribose, sucrose and glycerol on methionine production by the *Bacillus* species were investigated. As observed in Figure 2a, different carbon sources variedly influenced the production of methionine. Glucose was the best carbon source for methionine accumulation in the broth cultures of *B. pumilus* and *B. amyloliquefaciens*, and methionine yields of 1.83mg/ml and 2.14mg/ml respectively were obtained.

Medium composition: **KH₂PO₄, 0.05g; K₂HPO₄, 0.05g; MgSO₄.7H₂O, 0.1g; FeSO₄.7H₂O, 0.001g; MnSO₄.4H₂O, 0.001g; CaCO₃, 20.0g, carbon source, 20.0g; (NH₄)₂SO₄, 10.0g; H₂O, 1L; pH 7.2**

**Figure 2a.** Effect of carbon sources on methionine production by *Bacillus amyloliquefaciens* and *Bacillus pumilus*
Glucose has been reported as the mostly widely used carbon for methionine production [18,48] but [19,38] observed maltose as the carbon of choice for methionine production. Several other researchers used methanol and n-alkanes as the main carbon sources for methionine production [49,50]. At 8.0% glucose concentration, methionine yields of 5.22mg/ml and 5.50mg/ml were produced by *B. pumilus* and *B. amyloliquefaciens* respectively (Figure 2b). This result is contrary to the findings of [44], who reported a 6.0% glucose concentration for maximum methionine production by *Bacillus cereus* S8. This difference in glucose concentration may be attributed to the difference in strain of *Bacillus* species and media composition used.

Various organic and inorganic nitrogen sources including urea, amino acid and protein have been used as nitrogen sources for the production of methionine [21,46,49]. The influence of ammonium sulphate, ammonium chloride, potassium nitrate and urea on methionine accumulation by *B. pumilus* and *B. amyloliquefaciens* were examined. As shown in Figure 3a, ammonium sulphate was the nitrogen of choice for methionine production. Methionine yields of 2.17mg/ml and 2.50mg/ml accumulated in the broth cultures of *B. pumilus* and *B. amyloliquefaciens* respectively when 1.0% ammonium sulphate was added to the fermentation medium. This finding corroborates with the works of [16,18,20,38,44]. They reported that ammonium sulphate was the best nitrogen source for methionine production.
The effects of varying concentrations of ammonium sulphate on methionine production by the \textit{Bacillus} species (Figure 3b), show that maximum methionine accumulation was obtained at 4.0% ammonium sulphate concentration. Methionine levels of 5.65mg/ml and 5.77mg/ml were accumulated by \textit{B. pumilus} and \textit{B. amyloliquefaciens} respectively. In contrast to our findings \cite{38} and \cite{44} observed that ammonium sulphate at 1.0% concentration gave the best methionine production by \textit{B. thuringiensis} EC1 and \textit{Bacillus cereus} S8 respectively.

The methionine producers identified as \textit{Bacillus pumilus} and \textit{B. amyloliquefaciens} were successfully isolated from Nigerian fermented food condiments, ogiri and okpeye respectively. Optimization of some cultural parameters in submerged fermentation enhanced methionine accumulation by the \textit{Bacillus} species. Further studies on strain improvement of the \textit{Bacillus} species may likely stimulate the output of the target product during fermentation process.

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**Conflict of Interest**

The authors wish to state that there is no competing interest.

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