

Original Research Article

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## Screening of Fermentative Microorganisms with Amylolytic Potentials Involved in the Fermentation of Traditional Fermented Products in Togo

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### ABSTRACT

#### Keywords

Screening, Amylase activity, Lactic acid bacteria, Yeast, Fermented foods, Togo.

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The transformation of cereals and tubers with amylolytic fermentation allows the conservation of foods and increase their energetic and nutritional values. The aim of this work was to search fermentative strains with amylolytic potentials. Isolation of microbial flora on 45 samples of fermented foods (agbelima, eblima and epoma) was performed. The search of microorganisms producing amylases was realized on appropriate culture media combined with 1 % of soluble starch. The combination of morphologic and biochemical characters (API gallery) allowed to identify efficient isolates. Results showed that isolates were amylolytic with respectively 46.67 %, 31.04 % and 32 % of lactic acid bacteria, yeast and mould compared to the total number of isolates. There were six strains having high amylolytic activity. These strains were identified as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Candida guilliermondii*, *Candida krusei*, *Saccharomyces cerevisiae* and *Candida tropicalis*. The development of starter culture from these microorganisms is important for the potential production of standardized fermented products at a commercial, small industrial scale, and for the improvement of their acceptability, microbiological stability and hygienic safety.

### Introduction

The traditional cereal-based foods that are consumed in West Africa are processed by the natural fermentation of cereals and tubers and are particularly important as weaning foods for infants and as dietary staples for adults (Yao *et al.*, 2009). The number of traditional fermented foods in the world is estimated to 3500 (Soomro *et al.*, 2012). Lactic acid fermentation has been shown to improve flavor, shelf life, nutritional value or digestibility of fermented foods and alter food

composition in such a way that it will require minimal energy both in cooking and preservation process (Nout, 1994). Lactic acid fermentation of amylaceous vegetables is very current southern countries (Oyewole, 1997). There exists a large variety of these lactic acid fermentation-based products. Among them, mawè/eblima, ogi, kenkey, uji, kisra, togwa, attiéké, gari, agbelima (Hounhouigan *et al.*, 1993; Amoa-Awua *et al.*, 1996; Yao *et al.*, 2009; Kouame *et al.*, 2013) can be cited.

However, the traditional fermented based materials which are starchy raw slurry form are nutritionally inadequate because of their low energy density and their imbalance in macro and micronutrients. The healthy nutrition in developing countries is essential for infants and young children to have access to complementary foods with appropriate nutritional, bacteriological and organoleptic properties; culturally and economically acceptable, and promoting the way they can be feed (Lutter, 2003; Dewey and Brown, 2003). Previous studies (Agati *et al.*, 1998; Djoulde *et al.*, 2005; Songre-Ouattara *et al.*, 2009) on these fermented foods allowed to isolates microorganisms known as amyolytic fermentative strains. Amyolytic lactic acid fermentation brings an improvement in digestibility of sugars, particularly starch (Haydersah, 2010). It also permits to improve the energetical and nutritional values of amylaceous porridges and reduces their stickiness by hydrolysis of sugars starch like glucose, fructose, saccharose, maltose and dextrans (Nguyen *et al.*, 2007; Songre-Ouattara *et al.*, 2009). During the fermentation of some amylaceous products, many amyolytic bacteria were isolated. Among them, *Lactobacillus plantanum* A6 (homolactic strain) from cassava retting (Giraud *et al.*, 1991) and *Lactobacillus fermentum* OgiE1 (heterolactic strain) isolated from fermentation of bitter starch (Agati *et al.*, 1998) can be cited. Agbelima, eblima and epoma, are fermented foods which are widely consumed in Togo (Ola, 2010). They are produced by traditional uncontrolled fermentation of *Manihot esculenta* Crantz, *Zea mays* and *Sorghum bicolor* respectively. Presently, there is a pressing need to improve the informal traditional food processing sector as a whole in the advent of fast foods which could out compete the indigenous foods. It is in this regard that a lot of other indigenous African fermented foods have become subjects of

intense scientific studies. Such investigations provide a basis for injection of suitable scientific and technological know-how to upgrade the traditional operations and the quality of the indigenous foods. With regards to the presence of fermentatives strains particularly amyolytic in fermentation, there is very little information in the literature on them.

This work was carried out to screen fermentative microorganisms with amyolytic potentials involved in agbelima, eblima and epoma fermentation which could eventually lead to the development of a starter culture for its controlled fermentation during industrial production.

## **Materials and Methods**

### **Collection of fermented products samples**

The biological material used in this study was constituted by three different types of fermented products all drawn in five markets of Lomé, the capital of Togo, namely Adidogomé, Agoè-Assiyéyé, Akodésséwa, Gbossimè and Hanoukopé for searching micro-organisms having amyolytic potentials. Fifteen samples of 200 g of each product, three samples per market and summing up to 45 samples were drawn randomly at sale display. They were put in stomacher bags and quickly take away to the laboratory for analysis.

### **Isolation of fermentative microorganisms**

Ten (10) g of sample were diluted with 90 ml of Tryptone Salt before homogenization in a stomacher lab-blender for 3 min. Decimal dilutions of the mixture were done up to  $10^{-7}$  and then some appropriate dilutions were sown on appropriate agar culture media. Two categories of germ were isolated: yeasts and moulds on Sabouraud dextrose agar

supplemented with 250mg/100ml chloramphenicol and lactic acid flora on De Man Rogosa Sharpe agar. Yeasts and moulds searching were done following NF ISO 7954 standard (1988). Isolation of the lactic acid flora was realized according to the method previously described by Hounhouigan *et al.*, (1993). After incubation, for each box of kneaded, characteristics colonies was purified by successive picking outs and preserved for further steps.

### **Selection of amylolytic strains**

Amylolytic strains were isolated on MRS agar culture media combined with soluble starch at 1% for lactic acid flora and Potatoes Dextrose Agar culture media combined with soluble starch at 1% for yeasts and moulds (Djoulde *et al.*, 2005). A mixture was obtained by putting a well individualized colony in 9 ml of Müller Hinton broth, and then decimal dilutions up to  $10^{-3}$  were carried out. A volume of 0.1 ml of each dilution was spread on selection media. After incubation at 30°C during 24 to 72h, the surface of boxes was flooded with a Lugol's iodine and halo colonies were booked and considered as amylase producers (Olukoya, 1995). The appeared diameter of halo and the diameter of the corresponded colony were measured. The selection of strains was done according to the ratio of halo diameter around the colony (D1) by the diameter of the colony (D2) (Ameyapoh *et al.*, 2006). The ratio D1/D2 is interpreted as follow: null activity: absence of halo around colonies, low activity: ratio D1/D2 in [1.01-1.50], average activity: ratio D1/D2 in [1.50-1.80] and high activity: ratio  $D1/D2 \geq 1, 80$ .

### **Effect of starch concentration on microbial growth and amylase synthesis**

In order to study the effect of starch concentration on amylolytic development and potential of selected microorganisms, five

starch concentrations were tested: 1%, 2%, 4%, 6% and 8%. Culture from 16 to 18 hours is realized from pure colonies of each microorganism. These colonies are hanged in distilled water containing sodium chloride at 9 per thousand leading to have a turbidity similar to McFerland reference (0.5) whose microbial concentration correspond to  $10^8$  UFC/ml. Aliquots of this abeyance (0.1 ml) are sowed on MRS agar and PDA agar with growing soluble starch concentration. The counting, the measure of halo diameter appeared after vaporization of Lugol's iodine and the one of the corresponding colony is done after incubation.

### **Phenotypic characters and identification of strains**

Gram staining, oxidase activity, catalase activity, production of acetoin and sporulation were biochemical tests performed. Cell morphology was determined with cells grown in MRS or PDA at 37°C for 16 hours by using optical microscopy with objectives X40 and X100 to see cell form, cellular arrangement and their mobility. CO<sub>2</sub> and H<sub>2</sub> production from glucose were performed using Kliger Iron Agar. Carbohydrate fermentation patterns were determined by the API 50 CH and API 20C AUX as described by the manufacturer and the identification was done by a computerized database programme provided by the same manufacturer.

## **Results and Discussion**

### **Isolation of microbial flora**

A total of 281 strains of different groups of microorganisms were isolated from the 45 samples of agbelima, eblima and epoma and were divided as follow: 165 isolates of lactic acid flora, 91 isolates of yeasts and 25 isolates of moulds. These strains were tested for their capacity to produce amylases.

### **Selection of strains with amylolytic activity**

The description of the amylase activity of the 281 isolates revealed zones of lysis of various dimensions. Thus 161 isolates didn't present zones of lysis (null amylolytic activity), 75 isolates had ratio D1/D2 between 1.01 and 1.50 (low amylolytic activity), 39 isolates had ratio D1/D2 between 1.50 and 1.80 (average amylolytic activity) and 6 had a ratio  $D1/D2 \geq 1.80$  (high amylolytic activity). Table 1 showed the intensity of amylolytic activity of fermentatives isolates. Among amylase producer microorganisms, some presented interesting enzymatic potentialities. There was notably two (2) isolates on De Man Rogosa Sharpe agar coded LAA and LAB and four (4) isolates on Potatoes Dextrose Agar coded LEA, LEB, LEC and LED as showed in figure 1. The strains LAA and LEA presented the smallest ratio D1/D2 (1.82) and LED, the biggest ratio (3.18). In general, during these experiences, the best amylase synthesis was globally observed with yeasts than lactic acid strains.

### **Effect of starch concentration on microbial growth**

The effect of starch concentration on the growth of strains that have high amylolytic activity was showed by figure 2. The microbial growth decreased when starch concentration increased for lactic isolates LAA and LAB and for yeast isolates LEA, LEB and LED. Regarding the LEC isolate, its charge increased when the starch concentration varied from 1% to 2% before decreasing progressively with increase of the starch concentration. The yeast strain coded LED has the greatest microbial loads at the concentrations of starch 1% to 6% and absence of culture to the concentration 8% while the other isolates presented a culture at 8%.

### **Amylase activity according to starch concentration**

Amylase activities (ratio D1/D2) of the 5 strains isolated from fermented products (LAA, LAB, LEA, LEB and LED) decreased when the starch concentration increased as illustrated in figure 3. Regarding the LEC strain, its amylolytic activity increased when the starch concentration varied from 1% to 2% before decreasing progressively with increase of the concentration. The isolate coded LED, LEC and LAA have respectively the best amylolytic activities with the starch concentration at 1%, at 2% and 4% and at 4% and 6%. The yeast isolate LEB didn't present amylolytic activity at 8% concentration of starch.

### **Phenotypic characters and identification of outstanding isolates**

#### **Identification MRS agar isolates**

Morphological, physiological and biochemical characteristics of lactic isolates were listed in table 2. The physiological characteristics of bacteria (LAA, LAB) showed that both strains are Gram positive, catalase negative and oxidase negative. Examination of their shape and arrangement using a microscope at  $\times 40$  showed that the strains (LAA, LAB) cells were rod-shaped arranged in small chain.

After studying morphological, physiological and biochemical characteristics, identification was completed using ferment properties of isolates on API 50CH gallery, containing 49 sugars. Based on their assimilation of carbon compounds, the lactic acid isolates LAA and LAB were respectively identified as *Lactobacillus fermentum* and *Lactobacillus plantarum*.

**Table.1** Number of isolates of each germ by amyolytic activity intensity

| Microorganisms    | Number of isolates with amyolytic activity |     |         |      |
|-------------------|--|-----|---------|------|
|                   | Null                                       | Low | Average | High |
| Lactic acid flora | 88   | 44  | 31      | 02   |
| Yeasts            | 56   | 23  | 08      | 04   |
| Moulds            | 17   | 08  | 00      | 00   |
| Total             | 161  | 75  | 39      | 06   |

**Table.2** Morphological and biochemical Characteristics of lactic isolates

| Characters         | Strains                          |                                  |
|--------------------|----------------------------------|----------------------------------|
|                    | LAA                              | LAB                              |
| Aspect             | White and smooth colonies/Type S | White and smooth colonies/Type S |
| Shape              | Rod                              | Rod                              |
| Grouping           | Small chain                      | Small chain                      |
| Gram               | Gram +                           | Gram +                           |
| Catalase           | -                                | -                                |
| Oxidase            | -                                | -                                |
| Sporulation        | -                                | -                                |
| Urea               | -                                | -                                |
| VP                 | -                                | +                                |
| Indole             | -                                | -                                |
| Citrate            | -                                | +                                |
| Grow that 10°C     | +                                | +                                |
| Grow that 30°C     | +                                | +                                |
| Grow that 45°C     | +                                | +                                |
| Grow that pH 4.5   | +                                | +                                |
| Grow that pH 6.5   | +                                | +                                |
| Grow that 2% Na    | +                                | +                                |
| Grow that 4% Na    | -                                | -                                |
| Resistance at 60°C | +                                | +                                |
| Esculine           | +                                | +                                |
| Mannitol           | +                                | +                                |
| Glucose            | +                                | +                                |
| Lactose            | -                                | -                                |
| Gas                | -                                | -                                |
| H <sub>2</sub> S   | -                                | -                                |
| Suspected genus    | <i>Lactobacillus sp.</i>         | <i>Lactobacillus sp.</i>         |

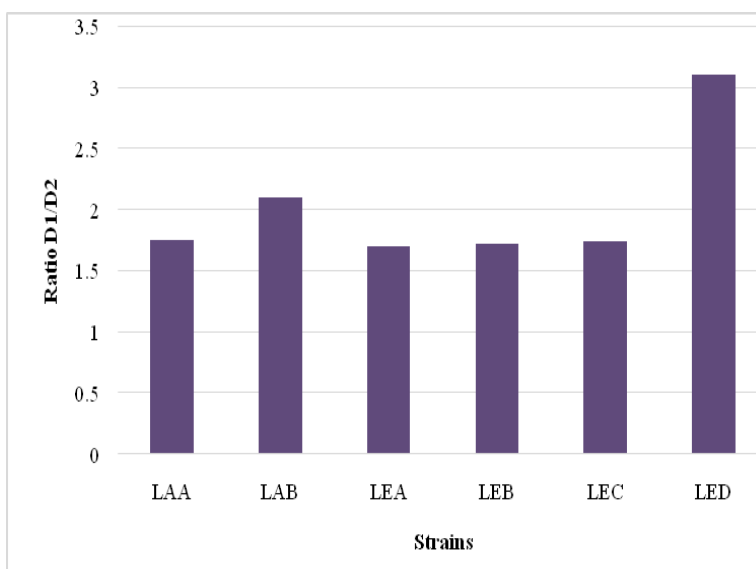
**Legend:**(+) positive reaction; (-) negative reaction; VP: reaction of Vogues-Proskauer

**Table.3** Morphological and biochemical characteristics

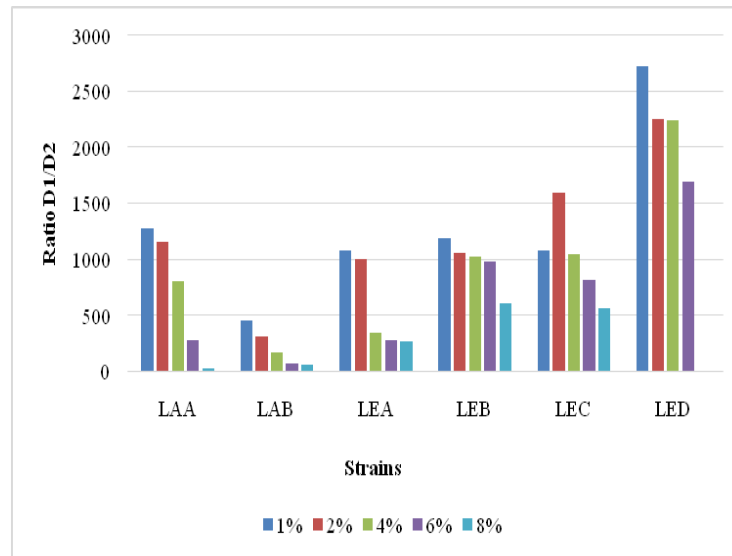
| Characteristics | Strains  |  |   |  |
|-----------------|--|--|---|--|
|                 | LEA  | LEB  | LEC   | LED  |
| Macroscopic     | Waxy, rounded and white colony                                       | Waxy, rounded and broken white colony                                | Smooth, flat and cream colony                             | Waxy, rounded and white colony                                       |
| Microscopic     | Thallus and partitioned cell with a simple fragmentation of mycelium | Thallus and partitioned cell with a simple fragmentation of mycelium | Ovoid cell of multilateral budding with a pseudo-mycelium | Thallus and partitioned cell with a simple fragmentation of mycelium |
| Glucose         | +  | +  | +   | +  |
| Galactose       | +  | -  | +   | +  |
| Maltose         | +  | -  | +   | +  |
| Saccharose      | +  | -  | +   | -  |
| Raffinose       | +  | -  | +   | -  |
| Sorbitol        | +  | -  | +   | +  |
| Celobiose       | +  | -  | -   | -  |
| Inositol        | -  | -  | -   | -  |
| Hyphes          | -  | +  | -   | +  |
| DBB test        | -  | -  | -   | -  |
| Suspected genus | <i>Candida</i>   | <i>Candida</i>   | <i>Saccharomyces</i>                                      | <i>Candida</i>   |

**Legend:**(+) Positive reaction; (-) Negative reaction; DBB= Diazonium Blue B

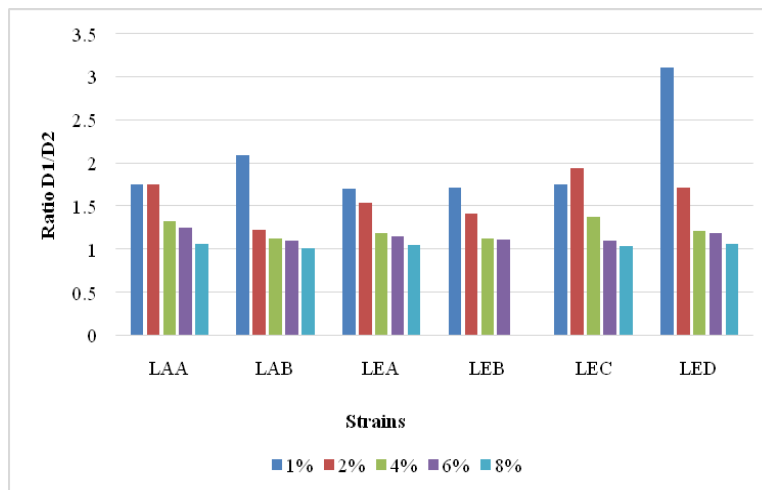
**Figure.1** Ratio D1/D2 of outstanding strains



**Figure.2** Effect of starch concentration on growth of isolates



**Figure.3** Effect on starch concentration on amyolytic activity of isolates



### Identification of isolates of sabouraud agar with chloramphenicol

Table 3 listed biochemical and morphological characteristics of LEA, LEB, LEC and LED that have high amyolytic activity. After preliminary microscopic confirmation all 4 yeasts were subjected to morphology, fermentation and assimilation tests. None of the isolates could ferment inositol whereas the majority fermented glucose (100%), maltose (75%) and raffinose (50%). All isolates were ascomycetous yeasts as revealed by

Diazonium Blue B test. Based on their assimilation of carbon compounds, the isolates LEA, LEB, LEC and LED were identified being respectively *Candida guilliermondii*, *Candida krusei*, *Saccharomyces cerevisiae* and *Candida tropicalis*.

### Isolation of fermentative microorganisms

This work aims to screen fermentatives micro-organisms with amyolytic potentials of agbelima, eblima and epoma, three fermented

foods in Togo obtained respectively from cassava, maize and millet. Microbiological analysis of different products coming from five markets of Lomé reveals the presence of lactic acid flora, yeasts and moulds. These results are the same found by Hounhouigan *et al.*, (1993) and N'tcha *et al.*, (2015) who reported that lactic acid bacteria and yeasts are predominant micro-organisms in most of native African fermented foods. According to Amoa-Awua *et al.*, (1996) and Assanvo *et al.*, (2006), lactic acid bacteria and yeasts isolated of fermented amylaceous products are only ferment germs. Also, moulds also take part in the process of fermentation. Indeed, Djoulde *et al.*, (2005) findings revealed the presence of moulds in fermented foods from cassava.

### **Selection of strains with amylolytic activity**

The isolates were tested to see their capacity to produce amylases. Globally, they were amylolytic with respectively 46.67% of lactic acid isolates, 31.04% of yeast isolates and 32% of moulds isolates. This reveals the high proportion of micro-organisms that produce amylases. Indeed, there exists a high correlation between natural starched substrates and presence of amylolytic micro-organisms during fermentation of these substrates (Haydersah, 2010). According to Yao *et al.*, (2009), the high proportion of amylolytic micro-organisms could be linked to the rate of starch in analysed products (more than 80% of dry matters). In consideration of the richness of raw materials, essentially composed of starch, it is not surprising that almost all tropical amylaceous fermented foods contain amylolytic micro-organisms with rates from 12% to 14% (Haydersah, 2010). These rates can vary during the fermentation. Thus, for pozolmexican the proportion of amylolytic micro-organisms in bacterial community varies approximately from 40% to 30% at the beginning and the end of fermentation (Diaz-

Ruiz *et al.*, 2003). The presence of amylase activity during fermentation has been previously mentioned by Tou *et al.*, 2006 and Songre-Ouattara *et al.*, 2009. Tou *et al.*, (2006) indicated during the production of ben-saalga, its micro-flora was dominated by lactic acid bacteria with ratio amylolytic bacteria/bacteria at 12%. This result is clearly lower than the ratio obtained during this work (ratio amylolytic bacteria/bacteria at 46.67%). The difference could be explained by the nature of samples, the duration of fermentation and the type of technology used for the production of ferments.

### **Effect of starch concentration on microbial growth and amylase activity**

Analysis of the effect of starch concentration on the growth and amylase activity of isolates shows globally that the utilization of starch at concentration 1% favours microbial growth and induces amylase synthesis of the studied isolates. The effect of starch concentration on bacterial development was also studied by Lagzouli *et al.*, (2007). The study of amylase activity depending on starch concentration reveals that utilization of starch at 1% concentration induces the production of amylase. This result is in concordance with Bennamoun *et al.*, (2004) who showed that starch at 0.8% is the best inductor of  $\alpha$ -amylase of *Aspergillus oryzae* and that the synthesis of enzyme decreased when the substrate concentration decreases. The decrease of starch synthesis is probably due to the increase of glucose level accumulated during the hydrolysis of starch with repression of the synthesis of amylases. Besides, starch, maltose and another saccharide are described like inductors of amylases (Moreira *et al.*, 2001). Nevertheless, sometimes when the concentrations are very large, they are easily hydrolysed in glucose and the production of enzyme is negatively affected (Ray *et al.*, 1996).



## Phenotypic characters and identification of outstanding isolates

Previous study on traditional fermented foods in Benin mentioned the presence of amylolytic lactic acid bacteria in fermented foods from maize, sorghum and millet (Tchekessi *et al.*, 2014). Another research (Songre-Ouattara *et al.*, 2009) on fermentation of millet indicates that lactic acid bacteria of *Lactobacillus* genera are known for their production of amylolytic enzymes. Djoulde *et al.*, (2005) identified strains of *Lactobacillus* of which *Lactobacillus cellobiosus*, *Lactobacillus sake*, *Lactobacillus coprophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus alimentarius* and *Lactobacillus acidophilus* like species of amylolytic lactic acid bacteria involved in the fermentation of cassava. Kermiche (2013) during the characterization of some microbial strains involved in fermented wheat revealed that germs of *Saccharomyces* genera have a high amylolytic activity. Djoulde *et al.*, (2005) added that 67% of yeasts isolated during the fermentation of cassava have  $\alpha$ -amylase activity and are in majority composed of *Saccharomyces* and *Candida* genera. Amylolytic micro-organisms used in the fermentation permits to improve the energetical and nutritional values of amylose porridges and reduce their viscosity (Nguyen *et al.*, 2007).

In conclusion, the present findings show the detailed information and further expansion on the state of knowledge about amylolytic micro-organisms in agbelima, eblima and epoma, three fermented foods in Togo. This research permitted to test the capacity of 281 isolates to produce amylases. Indeed, six isolates are revealed to have attractive enzyme potentials. That is: *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Candida guilliermondii*, *Candida krusei*,

*Saccharomyces cerevisiae* and *Candida tropicalis*. This experiment confirmed that the substrate concentration greatly influences the amylase activity of strains. Lactic acid fermentation of vegetable matters is a promising process to bring into focus some new fermented foods that have better added value with poor cost. These preliminary results could contribute to bring into focus a starter for fermentation of local amyloseous foods in order to have innovative goods having satisfactory energizing and nutritional value.

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