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Fermentative Production of Sweetened Jelly using Lactic acid-producing Amylolytic Mold Starters

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ABSTRACT: An experiment was conducted to prepare and standardise sweetened jelly like dessert food from glutinous rice and cassava by using a pure mold culture *Amylomyces rouxii* (SGK-2) under semi-controlled fermentation condition. The different substrates selected for the formulation of various treatments were boiled glutinous rice (100%) (T_1), boiled cassava (100%) (T_2), boiled glutinous rice (50%) + cassava (50%) (T_3). The different treatment combinations were mixed with two gram of rice flour based inoculum containing 10^6 cfu g^{-1} of pure mold culture *Amylomyces rouxii (SGK-2)* and fermented at room temperature for 48 hrs. After the period of incubation, a thick paste-like mass was developed with a sweet aroma. The palatability of the thick paste-like mass was increased by overlaying of the developed product with melted, sweetened China grass agar jelly and allowed to settle for 10 min. The contents were refrigerated at 20°C. The chilled, sweetened jelly was served for sensory evaluation. The results showed that the sweetened jelly had a better organoleptic acceptability score of 9.00% for the treatment (T_3) consisting of boiled glutinous rice (50%) + cassava (50%). Reducing sugar was the highest in T_1 (9.80 mg/100g) followed by T_3 (8.90 mg/100g). Total titrable acidity percentage was highest in T_1 (2.87%) followed by T_3 (2.21%). The thiamine and riboflavin content was maximum in T_3 of 0.42 mg/100g and 0.23 mg/100g respectively. Fermentation products such as amylase activity, ethanol and lactic acid was found to be highest of 0.075 mg of maltose, 2.70% and 0.20 mg/ml respectively in T_3 treatment with boiled glutinous rice (50%) + cassava (50%). Among the treatments, T_3 was highly accepted in both nutritional and sensory attributes. The study highly contributed to the applications of amylolytic mold in starch foods fermentation and opens an avenue for value addition of under utilised glutinous rice and cassava.

Keywords: *Amylomyces rouxii* (SGK2) glutinous rice, cassava, semi-controlled fermentation sweetened jelly, dessert food.

INTRODUCTION

Fermented foods are a type of functional foods that improves our immune system and prevents many diseases. Indigenous and traditional fermented foods have become a new interest and consequently provided new subjects for intellectual creation these few years. Advanced scientific knowledge on food fermentation and its microbial agent has increasingly revealed many beneficial effects which lead to new applications other than food preservation, safety, and sensory appreciation. Tapai is one of the popular traditional desserts in Malaysia and among other Asian countries which is prepared by fermenting glutinous rice (*Oryza sativa glutinous*) or using cassava tuber (*Manihot utilisima*) (Abdul Raji *et al*., 2017).

Fermented jelly is a traditional Indonesian fermented food prepared from glutinous rice which has been steamed, inoculated, and allowed to ferment for 24 to 48 h or longer at ambient temperatures (25 to 30°C). The inoculum, known as *ragi* starter consists of dry circular cakes prepared locally from rice flour and distinctive spices and contains the essential microflora. The fermentation is dependent on at least one amylolytic filamentous fungus and one or more yeasts. *Amylomyces rouxii*, a filamentous food fermenting mold resembling some species of *Rhizopus* is widely used for the preparation of this jelly. Certain foods such as Chinese yeast, Look Pang, ragi, tempeh in South East Asian countries are normally produced by these typical fungi along with other fermenting yeasts (Hesseltine, 1983). The mold is a strong amylolytic and a monotypic genus containing only the single variable species of *Amylomyces rouxii*. It is safer for human use, being food grade fungi and has been consumed long before written history (Cronk *et al*., 1979). The fermented product is a partially liquefied with cohesive paste like mass having a sweet-sour and mildly alcoholic taste which can be consumed without further processing as a dessert or snack item (Steinkraus, 1996). The most popular traditional dessert is called as

Tapai which is prepared by fermenting glutinous rice (*Oryza sativa glutinous*) or cassava (*Manihot utilisima*) tuber in Malaysia and other Indo-Asian countries (Abdul Raji *et al*., 2017).

The fungi are capable of producing glucoamylase, lactic acid, and ethanol (Knox *et al.*, 2004) during solid substrate fermentation of rice, cassava, and various other starchy substrates.

In Sikkim and Meghalaya states of India, the traditionally used amylolytic starters for the fermentative production of various ethnic alcoholic beverages are called as *Marcha.* These types of foods contained both the fungal and bacterial communities such as the Ascomycota, Zygomycota, and Lactic acid bacteria. Most filamentous fungi are used as traditional starters in India for the production of amylase and alcohol-containing fermented foods such as *marcha, hamei*, *Chowan, phut, dawdle,* and *khichri*.

The fungal starters were *Mucor circinelloides, Aspergillus sydowii, Penicillium chrysogenum, Bjerkandera adusta, Penicillium citrinum, Rhizopus oryzae, Aspergillus niger, Aspergillus flavus, Mucor indicus, Rhizopus microsporus, Rhizopus delemar, Aspergillus versicolor, Penicillium oxalicum, Penicillium polonium, Trametes hirsute* and *Cladosporium para halotolerant* (Sha *et al.,* 2017).

In India, cassava is consumed only as baked tubers, sago, fried chips, and as culinary items in Kerala, Tamil Nadu and Andra Pradesh. The development of value added sweetened cassava will prove to be a nutritious mold fermented product of commercial importance. glutinous rice, gluten-free starchy, sticky and waxy rice which is underutilized in India can be used as a substrate for the fermenting mold to develop into a novel, healthy fermented functional food. The nutritional and ethnic significance of the traditional rice variety such as glutinous rice and untapped health benefits of cassava as fermented food is the main focus for which the present study is selected to exploit underutilized glutinous rice and cassava to develop nutritious, fermented, and sweetened dessert food using pure cultures of mold *Amylomyces rouxii* (*SGK 2*).

MATERIALS AND METHODS

A. Preparation of glutinous rice and cassava

Fermented jelly was prepared under semi-controlled fermentation condition by using rice flour based *Amylomyces rouxii* (mold starter) by adopting the procedures of Siebenhandl *et al*., (2001). The different treatments such as boiled glutinous rice (T_1) (100%), boiled cassava (T_2) (100%), boiled glutinous rice (50%) + cassava (50%) (T_3) and uninoculated control were selected for the study. The glutinous rice was cleaned and soaked in water for one hour. Cassava tubers were cleaned and washed well. Then they were cooked separately and packed in food-grade sterilized polypropylene containers. Cooking is usually done to soften the substrates for microbial fermentation. The preparation of substrates for the production of fermented jelly is based on the accepted procedures developed by Siebenhandl *et al*., (2001). The packed container was cooled and brought to room temperature. The different treatments were mixed with mold inoculum and fermented at room temperature for 48 hrs. The sweetened paste like mass is usually developed with a sweety aroma after the period of incubation. To increase the palatability of the product, sweetened China grass agar jelly was poured over sweetened paste and allowed to settle for 10 min and refrigerated. Then it was served chill for organoleptic evaluation.

B. Isolation and development of pure culture of mold Amylomyces rouxii (SGK 2)

For the isolation of the mold and development of pure culture, the fermented food samples such as fermented rice and fermented finger millet porridges were collected from the houses of Madurai village of Tamil Nadu. Several mold were isolated from the samples using potato dextrose agar (PDA) medium by serial dilution technique with the dilution of 10^{-3} in three replicates by pour plate method.

The isolated mold strains were streaked on Petri plates containing sterilized potato dextrose agar medium and incubated for 4 days at 30°C. Milky white fuzzy colonies with sweet odour was formed in the Petri plates after 4 days of incubation. The pure white colonies produced greyish pink spores on 21 days after incubation. Sporangia of the mold were produced over the columella of the mycelium. The preliminary microscopical characterization and identification of the mycelial mold was carried out and was identified as *Amylomyces rouxii (*SGK-2). The pure isolated culture of *Amylomyces rouxii (*SGK-2) *was* submitted to Institute of Microbial Technology (IMTECH), Microbial Type Culture Collection Centre (MTCC), Chandigarh, India for identification and authentication. The culture was further authenticated with a strain accession number as *Amylomyces rouxii* MTCC 6586. The culture is now available at IMTECH, MTCC, Chandigarh, India as *Amylomyces rouxii* MTCC 6586 under general deposit category.

C. Preparation rice flour based starter inoculum using Amylomyces rouxii MTCC 6586

The mold mycelium cannot be added directly, hence, a pre-standardised mold inoculums of *Amylomyces rouxii* (SGK 2) developed using sterilized rice flour was used for semi-controlled fermentation of the solid substrates.

About 100g of rice flour was sterilized in the one-litre container and covered it with a double layer muslin cloth and a single layer of aluminium foil and kept at 121°C /15 psi for 20 min. Then it was cooled to room temperature at 28°C. Mold colony formed on Potato Dextrose Agar slants was scrapped off and inoculated to 100 g of sterile rice flour. To this 30 ml of sterilized deionized water was added and stirred well with heat sterilized spatula and incubated at 30°C for 4 days. Then it was stirred well and the aluminium foil was removed and covered with a muslin cloth and the inoculum was dried at 40°C for 5-7 days. Now the prepared culture was mixed well and packed in a sterilized 200 gauge polyethylene pouches (3g/packet).

The development of the inoculum was strictly carried out under sterile condition where only pure cultures of the mold were used for inoculating the sterile rice flour. And also, during viability studies using plate culture technique, rose Bengal agar medium was used which is a selective medium for mold and fungi and inhibits the growth of bacteria. Further, uniform growth of the mold *Amylomyces rouxii* were confirmed in the Petri plates. Viability studies were performed of the packed inoculum for a period of 150 days (5 months). For the viability study sample (1g) was drawn once in 30 days for analysis. The pre-standardized inoculum was used to develop fermented jelly. The development of the inoculums is strictly carried out under sterile condition where only pure cultures of the mold were used for inoculating the sterile rice flour. And also, during viability studies using plate culture technique, rose Bengal agar medium was used which is a selective medium for mold and fungi and inhibits the growth of bacteria. Further, uniformity of the mold of *Amylomyces rouxii* were formed in the Petri plates. The results of the study are given below in Table 1.

Table 1: Viability of *Amylomyces rouxii* in rice flour

Interval (Days)	Mold count (cfu g^{-1})
	Amylomyces rouxii
Initial	20.3×10^8
30	17.6×10^8
60	17.0×10^6
90	13.0×10^6
120	13.3×10^6
150	11.6×10^6

Rice flour based mold starter inoculum had the population of 20.3×10^8 cfu g^{-1} on the day of inoculation. One to two gram of inoculum is normally used for fermenting one hundred gram of the substrate and the inoculum was stored at ambient condition. Siebenhandl *et al*., (2001). The rice flour based powdered inoculum had the viability of 11.6×10^6 cfu g^{-1} on the 150^{th} day of incubation. The number of colonies declines from 20.3×10^8 to 11.6×10^6 cfu g^{-1}. However 10^6 to10^8 cfu g^{-1} is sufficient for inoculating the substrates. The mold spores will multiply in the substrate.

D. Fermentative production and standardization of sweetened jelly

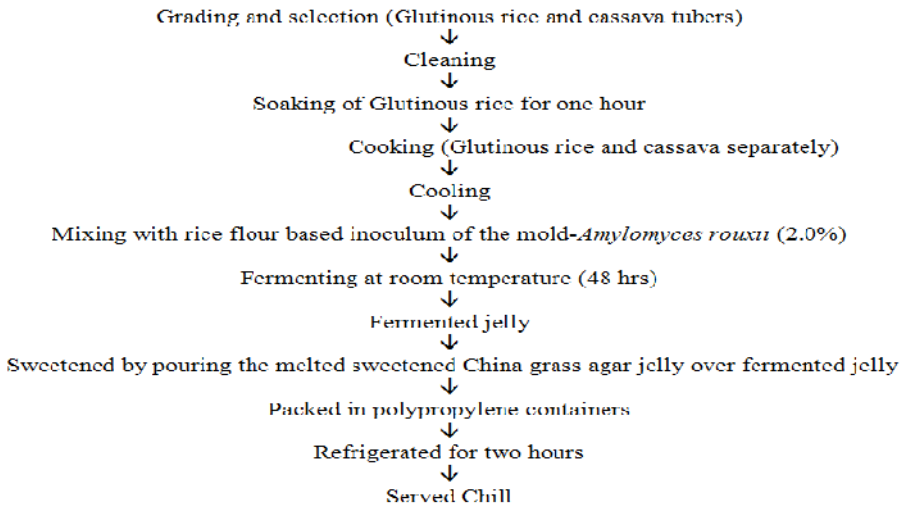
The fermentation substrates selected for the study were traditional rice cultivar of sticky/glutinous rice (*Oryza sativa glutinosa*) locally called as *puttarisi.* Tubers of tapioca or cassava *(Manihot esculenta)* were used for the study. Fermented jelly was developed by following treatments such as boiled glutinous rice (T_1) (100%), boiled cassava (T_2) (100%), boiled glutinous rice (50%) + cassava (50%) (T_3) and uninoculated control. The different treatments were mixed with pure rice flour based mold inoculum *Amylomyces rouxii* SGK 2 @ 2.0 % and fermented at room temperature for 48 hrs. The thick paste like mass was developed after incubation with the sweety aroma (Fig. 1). Sweetened China grass agar jelly was melted to boiling and poured over the thick paste like mass developed after fermentation and allowed to settle for 10 min and refrigerated. Then it was served chill for organoleptic evaluation.

Fig. 1. Development of sweetened jelly using pure culture of *Amylomyces rouxii* (SGK 2).

E. Processing of sweetened jelly

Final processing of the fermented jelly is essentially carried out to improve the taste and palatability of the final product. The taste of fermented jelly obtained from different treatments were improved by pouring sweetened China grass agar jelly over it and allowed to settle. The contents were refrigerated at 20°C. The chilled, sweetened jelly was served for sensory evaluation (Fig. 2). The various steps involved in the processing of sweetened jelly is given below.

Fig. 2. Fermented jelly overlaid with sweetened China grass agar.



F. Biochemical analysis of the sweetened jelly

The developed fermented samples were analysed for pH, reducing sugars, starch, protein, thiamine and riboflavin, lactic acid, ethanol content, fungal amylase activity by adopting the procedure of AACC, (2000). Ten g of the sample was taken and the pH of the slurry was determined by a digital pH meter calibrated with standard buffer solutions. Total Soluble Solids (TSS) were determined using hand refractometer. Total Titrable Acidity (TTA) was determined by titrating the sample with 0.1 N sodium hydroxide to the end point using phenolphthalein as an indicator and the percent lactic acid was determined Protein content was determined using the Kjeldahl distillation method on the dried sample. The ethanol content of the prepared fermented jelly was assessed using the distillation method. Mold count were determined using Rose Bengal Chloramphenicol agar at 25°C for 3 days (Weenk, 1995). The determinations were done in triplicates and the mean values were recorded.

G. Statistical analysis

The experiment was conducted in completely randomised design and the data obtained were subjected to analysis of variance (ANOVA) using MS excel program developed by Srinivasan (2017)

RESULTS AND DISCUSSION

The fermented sweetened jelly was organoleptically evaluated using 9 points hedonic scale by a panel of judges. It was observed that after fermentation, the protein content of different treatments such as T_1, T_2 and T_3 was 10.1, 3.4, and 4.5g/100g respectively. The highest protein content was recorded in T_1 treatment and it was due to the protein content of rice. Among the three treatments, maximum pH was recorded by T_3 (4.5) followed by T_1 (4.3). Reducing sugar was the highest in T_1 (9.80 mg/100g) followed by T_3 (8.90 mg/100g). Total titrable acidity was highest in T_1 (2.87%) followed by T_3 (2.21%). The thiamine and riboflavin content was maximum in T_3 (0.42 mg/100g, 0.23 mg/100g) when compared to T_1 and T_2.

Table 2: Biochemical characteristics of sweetened jelly.

Treatments	pH	Reducing Sugar (mg/100 g)	Total Titrable Acidity (%)	Starch (g/100g)	Protein (g/100g)	Thiamine (mg/100g)	Riboflavin (mg/100g)
Boiled glutinous rice (T_1) (100%) T_1	4.3	9.8	2.87	15.0	10.1	0.28	0.17
Boiled cassava (T_2) (100%), T_2	4.1	8.5	2.15	7.0	3.4	0.33	0.21
Boiled glutinous rice (50%) + cassava (50%) (T_3) T_3	4.5	8.9	2.21	21.3	4.5	0.42	0.23
SED	**0.040**	**0.034**	**0.006**	**0.294**	**0.042**	**0.004**	**0.004**
CD (0.05)	**0.084**	**0.072**	**0.013**	**0.617**	**0.089**	**0.008**	**0.009**

Table 3: Effect of fermentation on mold count, amylase activity, ethanol and lactic acid content.

Sr. No.	Mold count log (10^6cfu/g)	Fungal amylase activity (mg of maltose)	% Ethanol	Lactic acid (mg/ml)
T_1	6.74	0.042	2.20	0.18
T_2	6.71	0.063	2.30	0.16
T_3	7.73	0.075	2.70	0.20
SED	**0.0067**	**0.0003**	**0.0299**	**0.0055**
CD (0.05)	**0.0141**	**0.0008**	**0.0628**	**0.0116**

From Table 3, it was noted that the fungal amylase activity was maximum in T_3 of about 0.075 mg of maltose. The mold population after fermentation in T_3 was highest amongst the other two treatments with 7.73 x 10^6 cfu/g. Ethanol content was higher in T_3 of 2.7% (v/v). Glutinous rice in combination with mold and natural yeasts of rice and cassava produced 2.3% and 2.2% of ethanol in T_2 and T_1 treatments respectively. The result are in confirmation with the findings of Cronk *et al.,* (1979) who had reported that during fermentation of glutinous rice with *Amylomyces rouxii and yeasts such as Endomycopsis*, *Candida* and *Hansenula*, higher amounts of fusel alcohol of 192 mg/l was produced at 192 hours of fermentation; and similar observations were reported by Gayathry & Jothilakshmi, (2012) that during fermentation of millets for the production of wine with *Amylomyces rouxii (SGK 2) and yeasts*, about 10.9 % (V/V) of ethanol was formed. Ayumi *et al.,* (2004) have reported that strains of *Amylomyces rouxii* produced more organic acid namely lactic acid by fermenting the starch potato pulp. The present study also confirms the results of above study where fermentation of the starchy substrates (glutinous rice and cassava) by *Amylomyces rouxii*, resulted in the production of 0.18, 0.163 and 0.20 mg/ml of lactic acid in T_1, T_2 and T_3 treatments which indicates the probiotic nature of the culture.

Molds in *Loog-pang-khao-mak* be able to produce amylolytic enzymes such as glucoamylase and α-amylase that can break down starch into glucose and oligosaccharide, while yeasts transform glucose into alcohol. The yeast *Saccharomycopsis fibuligera* is highly efficient at producing amylolytic enzymes. Daroonpunt *et al*. (2016); Carroll *et al*. (2017).

Loog-pang-khao-mak a similar type of fermented tapai like jelly has been used for traditional fermentation of Thai fermented foods for over years. Effective glucoamylase that effectively hydrolyze starch and low alcohol producing mold and yeasts were isolated from *Loog-pang-khao-mak. Saccharomycopsis fibuligera* and the molds were identified as *Aspergillus niger*, *Aspergillus oryzae* and *Amylomyces rouxii.* (Roongrojmongkhon, *et al*., 2020). The present study also exhibited a fungal amylase activity of 0.075 mg of maltose after fermentation which are highly essential for the breakdown of starchy substrates and development of aroma and flavour of the final product.

The uninoculated control developed an off odour and was highly unacceptable in organoleptic evaluation and was rejected for further analysis. It was found that T_3 treatments had a better acceptability score of 9.00% and it has 21 per cent and 33 per cent increased in thiamine content when compared to T_2 and T_3. Regarding the riboflavin content, a 19 percent increase was recorded over the T_1 treatment.

DISCUSSION

From the results, it was observed that after fermentation, the pH was slightly acidic ranging from 4.1 to 4.5 rendering the developed product acidic and sour taste during organoleptic evaluation. Since, the process of fermentation completes in 48 hours, the fermented jelly was overlaid with melted, sweetened China grass agar for improving the palatability and hence the product was named as sweetened jelly. Yuwa *et al.,* (2019) evaluated the mixed culture of *Amylomyces rouxii* TISTR 3667 with *Zygosaccharomyces pseudorouxii* TISTR 5966 or *Zymomonas mobilis* TISTR 550 for the production of bioethanol from cassava pulp, and cellulose. It was shown that more than 15% ethanol was gained from 10% cassava pulp with 0.5% cellulase (25 g/l ethanol) compared to the system without cellulase (20 g/l). Similarly, the use of cassava and *Amylomyces rouxii* had developed an alcoholic flavoured jelly-like food with high organoleptic parameters.

Initially, the pH was found to be 6.5 and this has dropped due to fermentation by the mold. The drop in pH might have originated due to the production of lactic acid and acetic acid by the fermenting mold. Similar findings have been reported that in the development of glutinous rice paste called tapai, a Sabah's fermented beverage using traditional starters, the pH of inoculated glutinous rice was 6.6 initially and it decreased rapidly to 3.4 (Chiang *et al*., 2006). Total Titrable Acidity (TTA) was highest in T_1 (2.87 g/100g) followed by T_3 (2.21 g/100g). Fleet, (2003) has indicated that the correlation between acidity and pH is believed to be associated with both yeasts and lactic acid bacteria were well known for the production of acids especially lactic whereas some yeasts were previously reported to produce acid in alcohol fermentation to make a positive contribution to the products' flavour. At the same time, low pH and high acidity also eliminated enteropathogen, coliforms, and spoilage organisms in this product. The protein content of different treatments was 10.1, 3.4, and 4.5g/100g respectively. Reducing sugar was the highest in T_1 (9.80 mg) followed by T_3 (8.90 mg). In an experiment conducted to develop bioethanol from co-culture of yeast and *Amylomyces rouxii*, thef reducing sugar concentration, especially glucose concentration increased during fermentation (Azmi *et al*., 2008) The thiamine and riboflavin content was maximum in T_3 (0.42 mg) when compared to T_1 and T_2. From the analysis, it was found T_3 treatments had a better acceptability score (9.00) and it has a 21 percent and 33 percent increase in thiamine content when compared to T_2 and T_1. Regarding the riboflavin content, a 19 per cent increase was observed when compared to T_1.

Better glutinous rice tapai or sweetened jelly was prepared by wrapping rubber leaves. Different types of yeast such as *Crytococcus laurentii , Rhodotorula mucilaginosa , Candida famata , R. minuta* were found in rubber leaf due to which the primary fermentation process was proved to promote the growth and activity of starter cultures to enhance fermentation efficiency (Mohd Zin *et al*., 2021). In the present investigation also similar results were obtained were the finalproduct exhibited a mild alcoholic flavour due to the survival of yeast and mold during fermentation which is evidenced

by the mold count of 7.73×10^6 $cfug^{-1}$ when both glutinous rice and cassava were used as the substarte.

Halim *et al*. (2014) had reported that the incorporation of different hydrocolloids in *Tapai* ice cream, especially xanthan gum showed the best effect on overrun property and overall acceptability. *Tapai* ice cream with guar gum possessed the highest value of lightness and hardness property. In the development of fermented jelly using cassava, a similar gummy like properties were obtained which favoured for an high organoleptic acceptability score of 9.00% when used in combination with the cooked glutinous rice. Hence, use of cassava could serve as a natural source of improving the consistency of the fermented jelly.

CONCLUSION

The mold in the sweetened jelly is a strong amylolytic starter and breaks down mainly the carbohydrate of the rice or glutinous rice into simple sugars which are then further decomposed by the yeasts into alcoholic compounds. Therefore developed product has always a sweet taste, slightly sour with a soft alcoholic smell. The rice becomes soft and during the fermentation, some acids are also formed. The acids react with the alcohols resulting in a pleasant aroma of the finished product. A too-long incubation time will result in a sour product.

The result of this study indicated that starter culture development is important for the potential small-scale commercial production of sweetened jelly like products using *Amylomyces rouxii*. The overall acceptability, microbiological stability, and hygiene safety can be enhanced by pure culture fermentation. Further, nutritious food can be consumed by all age groups since it is easily digestible and contain compounds related to lactic acid fermentation by Lactic Acid Bacteria (LAB). Strains of *Amylomyces rouxii* isolated from traditional fermented foods could be screened for their properties of exo and endocellular enzymatic activities as well as potential probiotic and nutraceutical properties for application in the improvement of human health. The future research may be entrusted in the development of probiotic based mold namely *Amylomyces rouxii* and LAB formulation as starters in starchy products fermentation.

Since the experiment was semi-controlled with pure culture fermentation using *Amylomyces rouxii* (SGK 2), the sweetened jelly contained a variety of yeasts and LAB. Various flavour compounds might be present in sweetened jelly making it a favourite alcoholic beverage. Thus, controlled fermentation should be done to assess the contribution of yeasts and LAB on the flavour and aroma of this traditional alcoholic fermented beverage. There is a need for an investigation into the selection of the most suitable strain for controlled fermentation. Starter culture development is important for the potential small-scale commercial production of sweetened jelly and the improvement of its acceptability, microbiological stability, and hygiene safety. Detailed availability of nutrient values which included minerals and vitamins in sweetened jelly should be carried out. Strains isolated from sweetened jelly could be screened for their properties of exo and endocellular enzymatic activities as well as potential probiotic and nutraceutical properties for application in the improvement of human health.

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