PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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Pakistan Journal of Nutrition 9 (12): 1171-1175, 2010
ISSN 1680-5194
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Isolation and Identification of *Aspergillus oryzae* and the Production of Soy Sauce with New Aroma

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Abstract: Soy sauce is a dark brown salty liquid with a peculiar and a meaty taste. It is the chief savory-seasoning agent used in Oriental cookery, but it is becoming increasingly popular in many other regions of the world. The purpose of this study was to isolate *Aspergillus oryzae* strain from contaminated rice, soybean and wheat for using in soy sauce production with new aroma of thyme and dill. Samples of rice, soybeans and wheat assumed to be contaminated with *Aspergillus oryzae* were used in the isolation. Pure cultures obtained by culturing and subculturing on Potato Dextrose Agar (PDA) were maintained on PDA slant. All isolates were inoculated on *Aspergillus flavus* and *Parasiticus agar* (AFPA) medium to differentiate them from *Aspergillus flavus* and *Aspergillus parasiticus* based on reverse color. These isolates and the reference strain were inoculated on Czapack Yeast Extract Agar (CYA) and the macroscopic characteristics amongst these strains were compared. Slide cultures for these strains were prepared and their microscopic characteristics were compared. The preparation of the soy sauce was carried out by two stages. The first stage was Koji, which was prepared by mixing the isolates and the reference strain separately with steamed soybeans and the crushed millet was incubated for three days. The second stage involved the preparation of brine which consists of a koji and salt solution. The obtained data were analyzed using SPSS program. The results of analysis of soy sauce encouraged the use of the isolates, especially the rice isolate in soy sauce production and the addition of dill or thyme gave a specific aroma to the final product.

Key words: *Aspergillus oryzae*, soy sauce, Aroma, thyme, dill

INTRODUCTION

Aspergillus oryzae is a member of the *A. flavus* group. The *A. flavus* group also includes *A. sojae*, *A. nomius and A. parasiticus*. They are defined by the production of spore chains in radiating heads which range in color from yellow-green to olive brown. *A. flavus* and *A. parasiticus* are known to produce the potent carcinogen aflatoxin. *A. oryzae* and *A. soji* have been used for producing food grade amylase and fermentation of oriental foods for centuries (Sooriyamoorthy *et al*., 2004; Geiser *et al*., 1998).

Koji fermentation, provides enzymes such as α-amylase to liberate sugars from substrate, thereby facilitating yeast fermentation. Normally a strain of *A. oryzae* is used at 25-30°C (Sooriyamoorthy *et al*., 2004; Waites *et al*., 2001). Moromi fermentation begins by combining the fermented soy bean wheat mixture with salt brine (Sugiyama, 1984).

Soy sauce is a dark brown salty liquid with a peculiar aroma and a meaty taste. It is the chief savory-seasoning agent in Oriental cookery, but it is becoming increasingly popular in many other regions of the world (Yue, 1990).

Soy sauce has been extensively produced and studied worldwide. However, to our knowledge no soy sauce production unit or previously published research has been conducted in Gaza strip. Soy sauce is usually exported to Gaza strip. The present study shows that high quality, low priced, aromatic soy sauce can be produced in Gaza strip.

All chemical ingredients in the soy sauce were investigated. This study was undertaken to isolate and characterize the *A. oryzae* strain. This strain has been used in the production of a new aromatic soy sauce of either thyme or dill.

MATERIALS AND METHODS

Soy beans (*Glycine max*) and rice (*Oryza sativa L*) were purchased from Egypt, the wheat (*Triticum aestivum*), millet (*Panicum miliaceum*) and spices were obtained from Palestinian Ministry of Agriculture. The chemicals used in this study were purchased from Merck Chemical Company (Deisenhofen, Deutschland) and Sigma Chemical Company (N.Y., USA). The other media were purchased as the follow: *Aspergillus* differentiation agar (base) (Sigma; India), Potato dextrose agar (Himedia; India), Chloramphenicol supplement (Liofilchem; Italy).

Microorganism: The reference *Aspergillus oryzae* was obtained from Thailand (Faculty of Science, Mahidol University).

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Isolation: Samples, such as rice, wheat and soybeans assumed to be contaminated with *A. oryzae* were collected. The International Seed Testing Association Techniques (ISTA) especially (Agar plate method) was used to detect the *A. oryzae* (Khan, 1992). Isolates spores, (assumed to be *A. oryzae*) were inoculated on PDA and incubated at 30°C until sporulation (Sooriyamoorthy *et al*., 2004). Five pure cultures of isolates were obtained by sub-culturing and maintained on PDA slants for further identification.

Differentiation on AFPA selective medium: Isolates and the reference strains were inoculated in triplicates on AFPA selective medium and incubated at 30°C for 48-72 h and observed for reverse color. All the plates were incubated at 30°C and observed every two days for one week and any changes of the reverse color were recorded (Sooriyamoorthy *et al*., 2004).

Morphological characteristics: The isolates and the reference strain were plated on CYA (Czapek conc. 1 ml, K_2HPO_4 1 g, yeast extract 5 g, sucrose 30 g, agar 15 g and distilled water 1L) at 25°C for 7 days (Sooriyamoorthy *et al*., 2004). After the incubation period, all the plates were observed for macroscopic culture, such as colony diameter, colony color, conidial color, mycelial color, colony reverse, colony texture and nature of spores (Sooriyamoorthy *et al*., 2004). The microscopic characteristics were observed by preparing slide cultures, as described by Leck (1999).

Starter and Koji: The starter was prepared from spores of *A. oryzae*, 5 g of crushed millet and 2 ml of distilled water. 0.4 g of each starter was suspended in distilled water and the spores were counted using haemocytometer chamber. Different numbers of *A. oryzae* spores were determined (1.2×10^7 spores/ml for the reference *A. oryzae*, 1.33×10^7 spores/ml for the soybean isolate and 2×10^7 spores/ml for the rice isolate). In koji preparation, 0.4 g of each starter was taken and mixed with 330 g of soybeans, washed and soaked for 15 h, crushed and steamed for 30 min and drained for 3.5 h. Then, 300 g of millet was roasted for 15 min and crushed. The mixture was incubated for 72 h at 30°C and stirred twice daily (Ueki *et al*., 1994).

The brine: During the brine preparation (for the isolates and the reference), 276 g of koji, 61.4 g of NaCl and 375 ml of H_2O were mixed in a glass container. The resulting brine was incubated at 30°C for 3 months and stirred daily. Thereafter, the brine was filtered through sterile cotton cloth and the filtrate was pasteurized for 30 min, followed by the addition of thyme (*Thymus vulgaris*) or dill (*Anethum graveolens*) and held at room temperature until cool and then stored in sterilized bottles. The filtrates were analyzed for pH, ash, moisture, total solids, nitrogen, protein, salt, ethanol and calcium. The pH was measured by a pH meter (HANNA PH 211). Ash, moisture, total solids and salt percentages were determined as described by James (1996). Nitrogen and protein levels were determined using Kjeldahl method (Olvera-novoa *et al*., 1994). Ethanol percentage was determined using distillation method (Ault, 1998). Calcium levels were measured using atomic absorption spectrophotometer (A Analyst 100 Perkin-Elmer).

Data analysis: Data were analyzed using SPSS version-13. ANOVA test was used to differentiate between different numerical obtained data and any difference less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Isolation: Contaminated rice, wheat and soybeans were treated by ISTA, using agar plate method. All pure cultures on PDA varied, gradually becoming white centre, green yellow periphery and colony color. The isolates produced creamy reverse color on AFPA medium within 48 h of incubation at 30°C. The reverse color of the isolates and the reference did not change after the additional incubation period for one week. The isolates and the reference strain were compared.

Morphological characteristics: The macroscopic characteristics of the isolates and the reference were reported as shown in Table 1 and Fig. 1.

All the macroscopic characteristics of the isolates and the reference were identical, indicating that the isolates are most likely strains of *A. oryzae*.

The microscopic characteristics of the isolates were similar to that of the reference (Fig. 2), whereby the conidia, phialides, vesicles and mycelia were identical. In addition, cream reverse color was produced within 48 h when the isolates and the reference samples were inoculated on AFPA. This was in agreement with Sooriyamoorthy *et al*. (2004) and Jernejc and Cimerman (2001). From these comparable characteristics it could be concluded that the isolates are strains of *A. oryzae*.

Koji preparation: Koji was incubated for 72 h at 30°C. After 24 h of incubation, the heat of koji was raised gradually to 35°C and it started to drain and draining was continued for 60 h. *A. oryzae* started to grow during the initial 36 h and continued to grow rapidly. Firstly, the color of the fungus throughout the incubation became white and then yellowish and lastly the yellow greenish color was dominant. These changes were combined with the release of a very clear volatile aroma. Aeration was necessary for the fungi to grow and the fungi would die under anaerobic conditions as agreed with Shankar and Mulimani (2007).

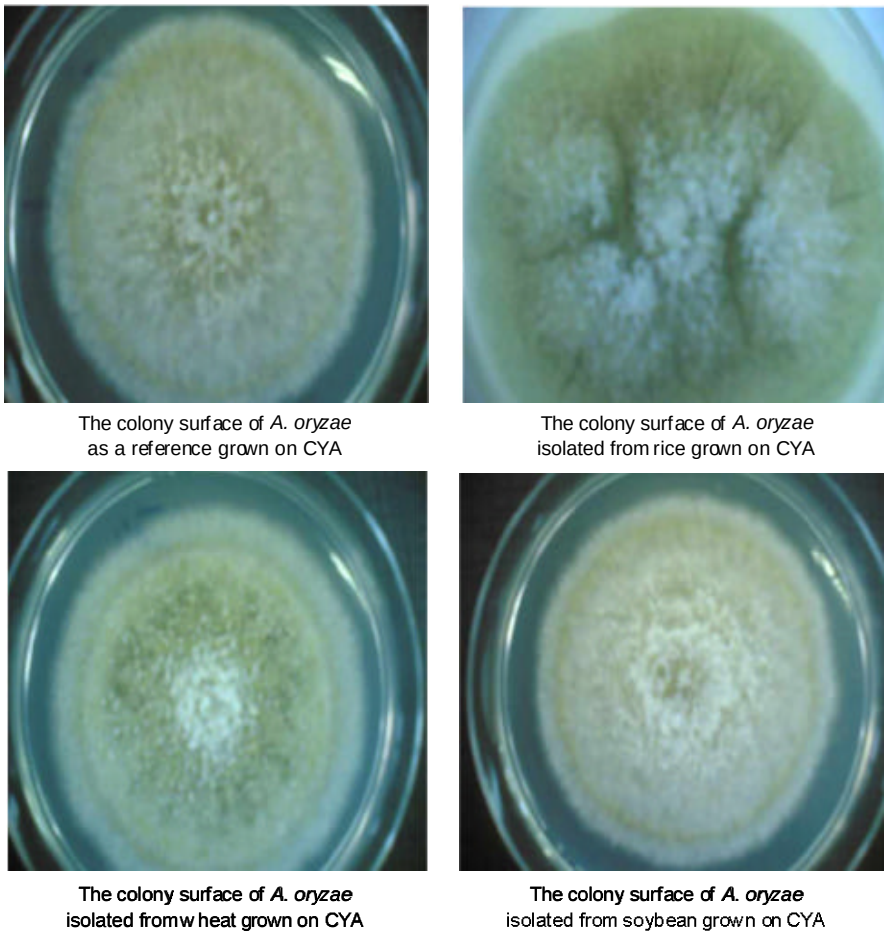

Fig. 1: The colony surface of the reference and the isolates strains

Table 1: Macroscopic characteristics of the isolates compared with the reference strain of *A. oryzae* observed after 7 days of incubation at 25°C on CYA medium

Characteristics	Soybean isolate	Rice isolate	Wheat isolate	Reference
Colony diameter	55 mm	75 mm	60 mm	56 mm
Colony color	White centre green yellow periphery	White centre green yellow periphery	White centre green yellow periphery	White centre green yellow periphery
Colony reverse	Pale yellow	Pale yellow	Pale yellow	Pale yellow
Colony texture	Wet	Wet	Wet	Wet
Conidial color	Yellow green	Yellow green	Yellow green	Yellow green
Nature of pore	Powdery	Powdery	Powdery	Powdery

Brine preparation: In the brine solution, the *Aspergillus* enzymes of koji continued to hydrolyze the soybeans and millet and as a result an excess of different kinds of sugars and amino acids were produced. These sugars and amino acids were consumed by natural microorganisms, such as salt tolerant lactic-acid bacteria (*Tetragenococcus halophila*) and yeasts (*Zygosaccharomyces rouxii* and *Candida versatilis*) during the so-called brine fermentation (Der sluis *et al*., 2001). During brine fermentation, the color was changed gradually to dark brown and a different aroma was released. Glutamic aroma was originated and an additional attractive new aroma was produced by the addition of dill or thyme. The brine was analyzed to observe the capacity of the isolates. The amount of protein in rice isolate soy sauce was higher than that in the reference strain and soy bean isolate soy sauce. Rice isolate soy sauce also contained higher levels of nitrogen, calcium, NaCl and pH (Table 2). This increase may be due to the rapid growth of this strain. Consequently, acid protease of this isolate might increase the proteolytic hydrolysis in brine fermentation,

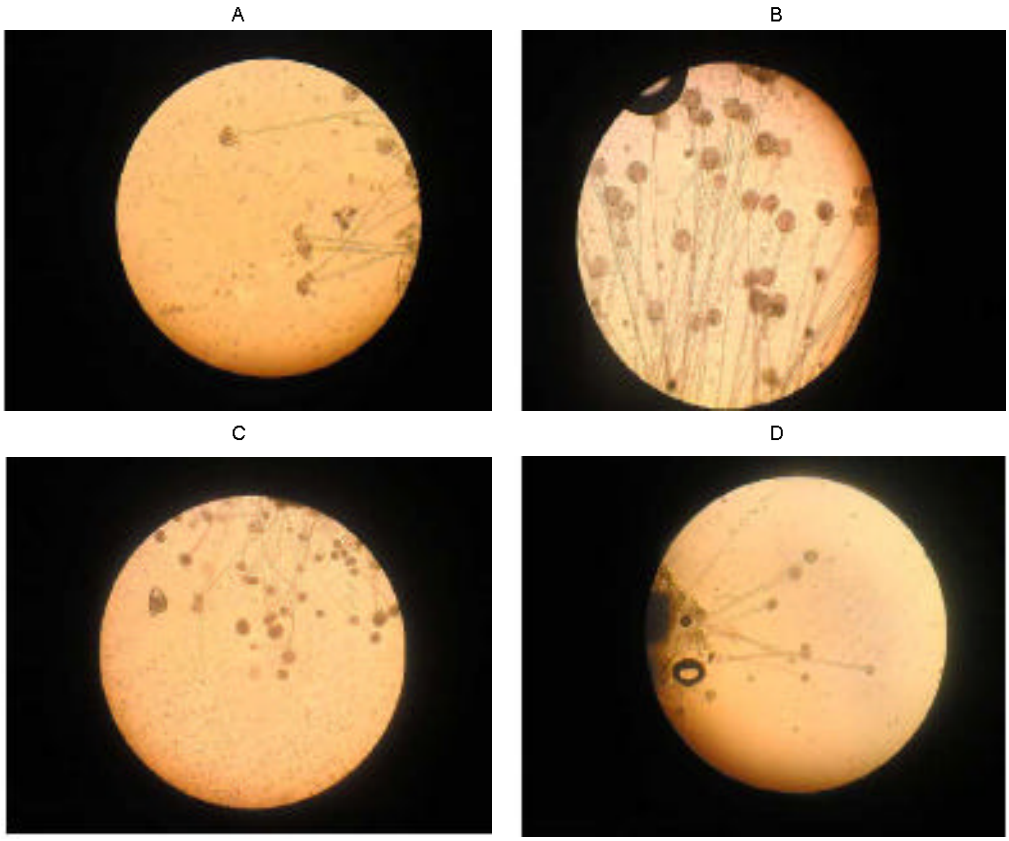

Fig. 2: Fruiting heads of A- reference strain, B- wheat isolates, C- rice isolates, D- Soybean isolates

Table 2: Chemical analyses of three kinds of soy sauce products

Types of chemical analysis	Reference soy sauce	Rice isolate soy sauce	Soy bean isolate soy sauce
pH	$4.16^{b}\pm(0.12)$	$4.65^{a}\pm(0.20)$	$4.25^{b}\pm(0.09)$
Ash %	$10.1^{b}\pm(0.10)$	$12.5^{a}\pm(0.13)$	$12.5^{a}\pm(0.14)$
Protein %	$8.1^{b}\pm(0.07)$	$9.8^{a}\pm(0.08)$	$7.2^{c}\pm(0.10)$
Nitrogen %	$1.3^{b}\pm(0.04)$	$1.5^{a}\pm(0.02)$	$1.1^{b}\pm(0.01)$
Moisture %	$76.6^{a}\pm(1.2)$	$43.3^{b}\pm(1.1)$	$78.3^{a}\pm(1.4)$
Total solids %	$23.4^{b}\pm(1.1)$	$56.7^{a}\pm(1.7)$	$21.7^{b}\pm(1.2)$
Ethanol %	$0.11^{c}\pm(0.01)$	$0.57^{b}\pm(0.08)$	$0.92^{a}\pm(0.11)$
NaCl %	$14.04^{c}\pm(1.12)$	$16.38^{a}\pm(1.52)$	$15.21^{b}\pm(1.23)$
Ca (mg/100 g)	$123.2^{a}\pm(2.63)$	$127.1^{a}\pm(3.35)$	$102.3^{b}\pm(4.12)$

Note: Wheat isolate was not used in soy sauce production and values of different superscripts in the same raw differ significantly ($p<0.05$)

resulting in good soy sauce (Iizuka and Aishimal, 1999). Nitrogen content is important parameter used for grading the quality of soy sauce product. According to the Chinese National Standard, grade A soy sauce should contain total nitrogen and amino nitrogen of more than 1.4 and 0.56%, respectively (Chou and Ling, 1998). The concentration of NaCl of the products was sufficient to stop bacterial growth. These results were also confirmed by the results of Iizuka and Aishimal (1999). They found that sodium chloride helps to destroy staphylococci in soy sauce. However, Soy sauce with salt solution containing 10-17% sodium chloride, pH 4.7 destroyed 90% of the staphylococci cells in 10% NaCl solution at 980-1440 min and of 17% at 460-530 min. For moisture and total solids, there was an opposite relation between them may be due to the strength of hydrolysis in the brine. The less moisture, the higher total solids. So, good soy sauce is produced with the available quantity of protein, nitrogen and calcium. These components were close to those of commercial soy sauce (Chou and Ling, 1998). This study indicates that rice isolate would be the best industrial starter for the production of a new aromatic soy sauce.

Conclusion: The following specific objectives were achieved:

- Isolation of the *A. oryzae* from different contaminated sources.
- Characterization of the *A. oryzae*.
- Production a new aroma of soy sauce with *Anethum graveolens* and *Thyme vulgaris*.
- Identification of the chemical gradients of the new product.

ACKNOWLEDGEMENTS

We thank the Faculty of Science at Mahidol University-Thailand for providing us with the reference strain of *A. oryzae* and the Environmental and Rural Research Center at the Islamic University-Gaza for their help in the analysis of the ingredients.

REFERENCES

Ault, A., 1998. Techniques and experiments for organic chemistry, University Science Book. USA.

Chou, C. and M. Ling, 1998. Biochemical changes in soy sauce prepared with extruded and traditional raw materials. Food Res. Int., 31: 487-492.

Der sluis, C., J. Tramper and R.H. Wijffels, 2001. Enhancing and accelerating flavour formation by salt tolerant yeasts in Japanese soy-sauce processes. Trends Food Sci. Technol., 12: 322-327.

Geiser, D., J. Pitt and J. Taylor, 1998. Cryptic Speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proc. Natl. Acad. Sci. USA., 95: 388-393.

Iizuka, K. and T. Aishimal, 1999. Differentiation of soy sauce by pattern recognition analysis of mid- and Near-IR spectra. J. Food Compost. Anal., 12: 197-209.

James, C., 1996. Analytical chemistry of foods, The Alden press, Oxford.

Jernejc, K. and A. Cimerman, 2001. Morphological Characteristics, Extracellular and Intracellular Protein and Enzyme Patterns of Five *Aspergillus* Species. Food Technol. Biotechnol., 39: 333-340.

Khan, S., 1992. Studies on fungi causing seed borne diseases of wheat and rice and their control. University of Karachi, Pakistan (unpublished).

Leck, A., 1999. Preparation of lactophenol cotton blue slide mounts. Community Eye Health, 12: 24.

Olvera-novoa, M., R. Martinez-palacios and E. Leon, 1994. Nutrition of fish and crustaceans a laboratory manual. FAO. Mexico City.

Shankar, S. and V. Mulimani, 2007. α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresour. Technol., 98: 958-961.

Sooriyamoorthy, S., K. Silva, M. Gunawardhane and C. Liieperuma, 2004. Isolation and identification of indigenous *Aspergillus oryzae* for saccharification of rice starch. Trop. Agric. Res., 16: 121-127.

Sugiyama, S., 1984. Selection of micro-organisms for use in the fermentation of soy sauce. Food Microbiol., 1: 339-347.

Ueki, T., Y. Noda, Y. Teramoto, R. Ohba and S. Ueda, 1994. Practical soy sauce production using a mixed koji-making system. J. Ferment. Bioeng., 78: 262-264.

Waites, M., N. Morgan, J. Rockey and G. Higton, 2001. Industrial microbiology/an introduction, Blackwell Science Ltd. USA.

Yue Xu (Yiao Hsu), 1990. Advances in the Soy Sauce Industry in China. J. Ferment. Bioeng., 70: 434-439.