Microbiological and Biochemical Studies of Indigenous Fermented Cereal-Based Beverages of the Sikkim Himalayas

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This is to certify that the work presented in the thesis: **Microbiological and Biochemical Studies of Indigenous Fermented Cereal-Based Beverages of the Sikkim Himalayas,** has been carried out by ***Shri Saroj Thapa*** under my guidance and supervision at Food Microbiology Laboratory of Department of Botany, Sikkim Government College, Gangtok. The results incorporated in this thesis have not been submitted for any degree elsewhere.

I also certified that Shri Thapa has followed the rules and regulation of this college in carrying out the work, and also fulfilled the conditions for submission of thesis to North Bengal University.

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CONTENTS

1. INTRODUCTION

Wine was believed to be made in the Caucasus and Mesopotamia as early as 6000 BC and the colonisation by the Romans spread wine-making all around the Mediterranean and eventually came to Northern India and China in 100 BC (Robinson, 1994; Pretorius, 2000). Alcoholic drinks have continued to be widely consumed in India since pre-Vedic times and specific reference to their consumption among the tribal people was mentioned in the *Ramayana* (300-75 BC) (Prakash, 1961).

Food fermentation is probably one of the oldest 'biotechnological processes' from which development of fermented foods and beverages, based on trial and error, has been rooted in cultural history of human being (Geisen and Holzapfel, 1996). Fermented foods and beverages are prepared by the action of microorganism(s), either spontaneously or by adding starter culture(s), which modify the substrates biochemically and organoleptically into edible products, and are thus generally palatable, safe and nutritious (Kwon, 1994; Campbell-Platt, 1994). Microorganisms bring about some biochemical changes in the substrates during fermentation such as enrichment of human diet with acceptable flavour, texture and aroma, biopreservation of food, bioenrichment of substrates with vitamins, protein and essential amino acids, and detoxification of undesirable components (Steinkraus, 1994; Stiles and Holzapfel, 1997). Filamentous fungi, yeasts and bacteria, mostly lactic acid bacteria constitute the microflora associated with the traditional fermented foods and beverages which are present in or on the ingredients, utensils, environment, and are selected through adaptation to the substrate (Hesseltine, 1983; Tamang, 1998). Making and use of fermented beverages are widespread interest enhancing the pleasure

of eating and have nutritional significance (Darby, 1979). Platt (1964) referred to traditional fermented beverage as primary example of "biological ennoblement" due to bio-enrichment with essential nutrients through fermentation.

Sikkim is a mountainous state of India with an area of 7096 sq. km and a population of 403,612 (Census of India, 1991a). The state comprises four districts→ North, East, South and West. Darjeeling, with an area of 3075 sq. km and a population of 13,35,618 (Census of India, 1991b) is a district of West Bengal in India. Excepting Siliguri, the three other sub-divisions of this district including Darjeeling, Kalimpong and Kurseong are hilly, commonly known as the Darjeeling hills, inhabiting 70 % of the district population. Topographically, culturally and ethnically, people of the Darjeeling hills and Sikkim, comprising of mostly the ethnic Nepalis, the Bhutias and the Lepcha, have similarities. The Darjeeling hills and Sikkim are located in the Eastern Himalayan regions and are ecologically grouped as the Sikkim Himalayas.

The staple crop in the Sikkim Himalayas is maize (*Zea mays* L.) and other major cereal-crops are paddy (*Oryza sativa* L.), finger millet (*Eleusine coracana* Gaertn.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Legumes including soybean [*Glycine max* (L.) Merrill] and black gram [*Vigna mungo* (L.) Hepper] are also grown.

A variety of indigenous fermented foods including dairy, cereal, vegetable, legumes, meat and alcoholic beverages are produced and consumed by the ethnic people of the Darjeeling hills and Sikkim for long centuries (Tamang *et al.*, 1988, Tamang, 2000). These foods are based on

socio-economic development status, religious and cultural practices, and have been evolved as the result of tradition and empirical experiences of generations over a period of time. Description of alcohol-drinking custom in the Sikkim Himalayas has been cited in some historical documents (Hooker, 1854, O'Malley, 1907; Risley, 1928). Indigenous fermented beverages constitute an integral part of dietary culture and have strong ritual importance among the ethnic people in the Sikkim Himalayas (Tamang *et al.*, 1996). Alcoholic beverages are exclusively prepared from locally grown cereal-grains using a starter called *marcha*. These alcoholic beverages are considered as nutritious. Traditional alcohol brewing is a home-based industry mostly done by rural women using their indigenous knowledge of alcohol fermentation.

Documentation on indigenous fermented beverages of the Sikkim Himalayas, information on microorganisms associated with these products, their role in fermentation, biochemical aspects of the various fermented beverages are not available. The proposed dissertation is aimed to study in depth the microbial diversity in indigenous fermented beverages, their role in fermentation, physico-chemical and enzymatic changes during fermentation. This will help to improve the commercial value of traditional fermented beverages, and also to preserve valuable microbial strains as genetic resources.

Objectives:

- To assess the traditional processing and utilization of available starchy substrates; equipment uses; consumption pattern; socio-economy and ethnical importance.
- To determine proximate composition of the products before and after fermentation.
- To isolate and to characterize the dominant microorganisms from the samples collected from different places in order to identify their taxonomical status, and to analyse viable cell counts.
- To study the succession during fermentation: microbial, physico-chemical and enzymatic activities.
- To select strain(s) isolated from marcha collected from different places on the basis of amylolytic activities for improving traditional starter with desirable microorganism(s).
- To evaluate the sensory properties of fermented beverages prepared by using selected strain(s).

2. REVIEW OF LITERATURE

Alcoholic foods and beverages, in which starch hydrolysis and fermentation are accomplished by amylolytic moulds and yeasts, range from very primitive Thai rice wines to highly sophisticated Japanese saké, which itself developed from a primitive beverage (Steinkraus, 1983). A list of common traditional fermented beverages of Asia is shown in Table A.

Table A. Traditional fermented beverages of Asia prepared by using starters

Type	Substrate	Starter	Nature	Country
Brem	Rice	Ragi	Liquor	Indonesia
Krachae	Rice	Loogpang	Wine	Thailand
Lao-chao	Rice	Chiu-yueh	Alcoholic beverage	China
Pachwai	Rice	Bakhar	Rice beer	India
Tapuy	Rice	Bubod	Wine	The Philippines
Tapai	Rice	Jui-piang	Wine	Malaysia
Tapé	Rice, cassava, maize, millet	Ragi	Sweet-sour alcoholic paste	Indonesia
Saké	Rice	Koji	Distilled alcoholic drink	Japan
Yakju and Takju	Rice, wheat, barley, maize	Nuruk	Alcoholic beverages	Korea

2.1. STARTERS

In Asia three types of inoculum as starter are commercially produced to convert starchy materials to sugars and subsequently to alcohol and organic acids (Hesseltine *et al.*, 1988):

1. In koji, pure cultures of *Aspergillus oryzae* and *Aspergillus sojae* are used in combination. At the same time they produce amylases that convert starch to fermentable sugars, which are then used for the second stage yeast fermentation to make miso and shoyu, while proteases are formed to break down the soybean protein.
2. In a second type, whole-wheat flour with its associated flora is moistened and made into large compact cakes, which are incubated to select certain desirable organisms. The cakes, after a period of incubation, are used to inoculate large masses of starchy material, which is then fermented to produce alcohol. Cakes contain yeasts, *Rhizopus* and *Absidia.* This inoculum is used in the so-called Kao-liang process for making alcohol.
3. The third type of starter is a mixed culture of yeast, fungi and bacteria. This starter is in the form of flattened or round balls of various sizes, compact in texture, and dry. The starter is inoculated with some previous starter. This mixed flora is allowed to develop for a short time, then dried, and used to make either alcohol or fermented foods from starchy materials. The starters have a variety of names such as ragi in Indonesia, bubod in the Philippines, Chinese yeast and chiu-chu in China and Taiwan (Hesseltine, 1983), loogpang in Thailand (Steinkraus,

1983), nuruk in Korea (Park *et al.*, 1977), which are used as starters for a number of fermentations based on rice and cassava or other cereals in Asia.

Calmette (1892) was the first to report the presence of several wild yeast species accompanied by *Amylomyces, Mucor, Aspergillus* and 30 different bacteria in starters used in Indochina to produce alcohol.

2.1.1. Ragi

Ragi is a starter used in Indonesia in the form of flat cakes where rice is used as a substrate (Saono *et al.*, 1974). During production of ragi, mainly rice or millet or cassava or other starchy bases are milled, mixed with herbs and spices, roasted together, sieved, water added and starter (ragi) from previous batch is mixed and shaped into balls. These are incubated at 25-30° C for 72 h in humid environment. Balls are dried in the sun and used as inoculum for the various fermentations. Went and Prinsen-Geerligs (1896) found *Monilia javanicus* (= *Pichia anomala*) and *Saccharomyces cerevisiae* as principal yeasts in ragi. Dwidjoseputro and Wolf (1970) reported the yeasts *Candida parapsilosis, C. melinii, C. lactosa, Hansenula subpelliculosa, H. anomala* and *H. malanga* in ragi. Addition of spices to some ragi contributes other microorganisms or may inhibit the growth of undesirable microorganisms (Soedarsono, 1972). Saono *et al.* (1974) conducted studies on mycoflora of ragi and products fermented by ragi such as tape keté la, tapé ketan hitam, oncom hitam and oncom mérah from various places in West Java and reported that *Candida* spp was dominating among yeasts, *Mucor* spp and *Rhizopus* spp were dominating among

moulds. Kato *et al.* (1976) studied the properties of glucoamylase from ragi isolates of *Saccharomycopsis fibuligera*. Saono and Basuki (1978) reported thirteen species of *Candida* from ragi of Indonesia. Hadisepoetro *et al.* (1979) reported that population of yeast in three ragi was 5.6×10^6 to 1.4×10^7, bacteria was 3×10^4 to 1.8×10^5 and mould was 3.2×10^4 to 4×10^4. Ardhana and Fleet (1989) found only one yeast *Candida pelliculosa* and one mould *Amylomyces rouxii* in four samples of ragi. Yokotsuka (1991) reported the presence of mixed cultures in ragi mainly *Rhizopus* and *Mucor* among molds; other organisms such as *Amylomyces*, *Aspergillus*, *Fusarium*, *Candida*, *Saccharomyces*, *Hansenula*, *Endomycopsis* (= *Saccharomycopsis*). Ishimaru and Nakano (1960) isolated *Streptococcus faecalis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus* in ragi in the range of 10^5 to 10^8 cfu/g. Hesseltine and Ray (1988) reported that most of the bacteria isolated from ragi belong to *Pediococcus pentosaceus* and *Streptococcus faecalis*, which may produce secondary products from the glucose formed by the amylolytic yeasts and moulds always found in the starters. Ardhana and Fleet (1989) reported the presence of bacteria in all four samples studied, which included *Bacillus coagulans*, *B. brevis*, *B. stearothermophilus* and an unidentified species of *Acetobacter* at the level of 10^3 to 10^4 cfu/g.

Saono *et al.* (1984) prepared ragi by using pure cultures of the selected molds and yeasts, *Amylomyces rouxii* strains AU-3 and CB-3, and *Saccharomyces cerevisae* strains RM-1and K-3 and pure culture of *Rhizopus formosaensis* and also prepared brem from this improved ragi. Elegado and Fujio (1993) isolated two polygalacturoniase producing strains

of *Rhizopus* spp from ragi and studied the enzyme stability in wide range of pH from 2-11 and tolerance at 50° C for 20 min. Uchimura *et al.* (1991) revealed that there is a higher variability rate of *Pediococcus pentosaceus* in older ragi than younger ones and the result suggested that rod-shaped bacteria cannot survive for a long time under dry conditions in ragi.

2.1.2. Bubod

Bubod is used as a starter in the Philippines (Tanimura *et al.*, 1977). Rice and ginger are powdered, and mixed thoroughly with enough water to have a consistency that permits rolling the material into a ball and flattening it. The discs are coated with 1-3 month old bubod and incubated in rice straw for 36 h at room temperature and sun-dried. Tanimura *et al.* (1977) found *Mucor, Rhizopus* and filamentous yeasts in bubod. Kozaki and Uchimura (1990) reported the presence of *Mucor circinelloides*, *M. grisecyanus*, *Rhizopus cohnii*, *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera* in bubod. Sanchez (1986) reported that the molds present in bubod ranged from 10^3 to 10^5 cfu/g, yeasts from 10^7 to 10^8 cfu/g, and lactic acid bacteria from 10^5 to 10^7 cfu/g. Hesseltine and Kurtzman (1990) reported that *Saccharomycopsis fibuligera* was dominant in bubod.

2.1.3. Koji

Koji is mould-culture and is prepared from steamed-cooked cereal. The substrate is usually rice, or sometimes steamed legume beans. The steamed substrate is spread on trays usually made of bamboo strips to depth of 5-7 cm which are stacked with gaps of about 10 cm in between to allow air circulation. It is followed by inoculation with 0.1 % mould spores, *tane-koji*

and incubated at 23-25° C. The rise in temperature due to the growth of mould is kept within the range 35-45° C by stirring and turning koji top to bottom on trays at about 20 h and 40 h, normally fermented for 3 days, when mould mycelium spread throughout mass, and before sporulation (Lotong, 1985). The mould used is *Aspergillus oryzae*, which is used for starch saccharification in saké manufacture (Inoue *et al.*, 1992). Since koji is not cultivated in a closed system, koji is a mixture of several microorganisms. At an early stage of cultivation, yeast grows on steamed rice grain and after that, about 20 h after inoculation of seed koji, koji mold begins to grow. Koji usually contains 10^2/g saké yeast, 10^2 to 10^5 g film-foaming yeasts, 10^2 /g lactic acid bacteria, 10^4 to 10^6 /g micrococci, 10^7 /g bacilli, etc. Kodama and Yoshizawa (1977) studied the biochemical changes occurring in koji and found the increase of reducing sugar from 0.2 % to 21.4 %. Tanaka (1982) studied enzyme activity of steamed or unsteamed glutinous rice-koji inoculated with *Aspergillus oryzae* and *Rhizopus jawanicus* and found that α-amylase was 1527 U/g in *Aspergillus* and 100 U/g in *Rhizopus* in steamed rice koji, whereas 1255 U/g and 100 U/g in unsteamed rice koji, respectively.

2.1.4. Nuruk

Nuruk is the starter for preparing Korean alcoholic drink yakju and takju. Historically the substrate for nuruk was rice but presently it is wheat (Park *et al.*, 1977). Generally, nuruk is prepared by natural inoculation of molds, bacteria, and yeasts; however, it can be prepared by inoculation with *Aspergillus usamii*. Traditionally nuruk is prepared by moistening wheat

flour, kneaded and molded into a ball [0.8-1.6 kg (dry weight)] and fermented for 17 d at 30° C to 45° C, dried for 2 weeks and cured for 1-2 months at room temperature (Park *et al.*, 1977). Kim (1968) isolated *Aspergillus oryzae* (10^7 cfu/g), *A. niger* (10^7 cfu/g), *Rhizopus* sp (10^6 cfu/g), anaerobic bacteria (10^7 cfu/g), aerobic bacteria (10^6 to 10^7 cfu/g) and yeasts (10^5 cfu/g) from nuruk.

2.1.5. Chiu-yueh

Chiu-yueh or peh-yueh is the Chinese starter for lao-chao, fermented rice product of China. It is a gray-white ball containing yeasts and fungi grown on rice flour which is closely related to Indonesian ragi. Wei and Jong (1983) isolated yeasts and moulds from chiu-yüeh and tested the ability of these microorganisms to convert steamed glutinous rice into a good quality lao-chao.

2.1.6. Chou or Chu

It is moulded cooked cereal which acts like, malt, as rich source of enzymes with substrate in various fermentations (Campbell-Platt, 1987). Chu is ball, cake or brick (20 × 22 × 4.5 cm) shaped and made from moistened raw rice, wheat, sorghum or barley flour. The principal amylolytic enzyme producers of chu are *Rhizopus* and *Mucor* (Yokotsuka, 1991). The microorganisms of wheat chu were found to be *Rhizopus japonicus, R. hangchon, R. chinensis, Absidia, Mucor, Monilia, Aspergillus, Lactobacillus* and *Acetobacter* (Otani, 1973; Iizuka, 1979).

2.1.7. Loogpang

Loogpang is the starter commonly used in Thailand to prepare alcoholic drink and vinegar (Vachanavinich *et al.*, 1994). In loogpang, organisms are grown on bran (Steinkraus, 1983). The main ingredient of this starter is rice flour with the addition of different type of spices and microorganisms. The microorganisms are originated from the inoculum or surrounding place of preparation of previous batch (Vachanavinich *et al.*, 1994). Pichyangkura and Kulprecha (1977) found that the molds *Amylomyces*, *Rhizopus*, *Aspergillus*, *Mucor*, and *Absidia* in loogpang. Dhamcharee (1982) showed that the molds present in loogpang from different places in Thailand were *Rhizopus*, *Mucor*, *Amylomyces*, *Penicillium*, and *Aspergillus*, and the main yeast genera were *Endomycopsis* (= *Saccharomycopsis*), *Hansenula*, and *Saccharomyces*. Sukhumavasi *et al.* (1975) isolated a strain of *Endomycopsis* (= *Saccharomycopsis*) *fibuligera* from loogpang with high glucoamylase activity. Uchimura *et al.* (1991) reported the presence of *Saccharomycopsis fibuligera* and *Pediococcus* sp. in loogpang.

2.1.8. Bakhar

Bakhar is a starter culture used to make pachwai, rice wine in eastern part of India and contains *Rhizopus* sp., *Mucor* sp., and at least one species of yeast (Hutchinson and Ram-Ayyar, 1925). Ginger and other plant materials are dried, ground and added to rice flour. Water is added to make a thick paste and a small round cake of 1.0-1.5 cm in diameter are formed and inoculated with powdered cakes from previous batches. The cakes are then wrapped in leaves, allowed to ferment for 3 d and then sun-dried

(Hutchinson and Ram Ayyar, 1925). Ray (1906) reported the presence of *Saccharomyces* sp. in bakhar.

2.1.9. Marcha

Murcha (correctly spelled as marcha) is a ball-like starter, used to ferment starchy materials into fermented beverage in Nepal, Bhutan and the Darjeeling hills and Sikkim in India (Tamang and Sarkar, 1995). Kobayashi *et al.* (1961) reported *Rhizopus oryzae, Mucor praini* and *Absidia lichtheimi* in marcha samples collected from Sikkim. Hesseltine *et al.* (1988) isolated *Mucor* and *Rhizopus* spp. in marcha. Tamang and Sarkar (1995) identified the microorganism of marcha of the Darjeeling Hills and Sikkim as *Pediococcus pentosaceus, Saccharomycopsis fibuligera, Pichia anomala, Mucor circinelloides,* and *Rhizopus chinensis.* Batra and Miller (1974) reported *Hansenula anomala* var. *schneggii* (= *Pichia anomala)* in marcha. In Bhutan, marcha is called chang-poo, in which the dominating microorganism belonged to the genus *Saccharomycopsis,* moulds were *Penicillium* sp. and *Aspergillus* sp. (Uchimura *et al.*, 1990).

2.1.10. Mana

Mana is a granular type of starter culture prepared from steamed rice or wheat particularly in Nepal (Karki, 1994). Mana contains *Aspergillus oryzae,* mucoraceae, yeast and lactic acid bacteria (Nikkuni *et al.*, 1996).

2.2. ALCOHOLIC BEVERAGES PRODUCED BY STARTER

2.2.1. Tapé

Tapé is a sweet-sour paste with an alcoholic flavour, prepared from glutinous rice or cassava or other cereals by using starter ragi in Indonesia (Ko, 1972). During preparation of tapé, glutinous rice is washed, soaked, steamed until it is well cooked and sticky, cooled to room temperature on a woven bamboo tray, sprinkled with powdered ragi, packed in small banana leaves and fermented for 2 to 3 d at room temperature and soft juicy mass of tapé is produced. With continued incubation more liquid is produced in the product (Saono *et al.*, 1977). There are various substrates used to prepare tapé, they are cassava (tapé ketala), glutinous rice (tapé ketan), corn (tapé jagung), and millet (tapé cantel). Ko (1972) noted the fall of pH in tapé ketan from ~ 6.0 to 3.5, generally remaining at pH 4.0. Ethanol content ranged from 3 % v/v (Tanuwidjaja, 1972) to as high as 8.5 % v/v (Cronk *et al.*, 1977). A combination of *Aspergillus rouxii* and *Endomycopsis* (= *Saccharomycopsis*) *burtonii* reduced total solids by 50 % in 192 h at 30° C, which raised the crude protein in tapé ketan by 16.5 % on a dry basis (Cronk *et al.*, 1977, 1979). Cronk *et al.* (1977) also found that rice lipids were hydrolysed during tapé ketan fermentation. Ardhana and Fleet (1989) studied the tapé ketan fermentation using ragi as starter showing the importance of bacteria in the overall fermentation along with mould and yeast. Suprianto *et al.* (1989) while studying on tapé production by inoculating ragi found that the inoculation of *Streptococcus* in a mixed culture of *Rhizopus* and *Saccharomycopsis* produced a higher level of

aroma. He also found that liquefaction was not caused by amylases of *Saccharomycopsis* even though it produced high activity of ∝-amylase.

2.2.2. Brem

Indonesian brem are of three types, (i) *brem madiun*, which is yellowish-white in colour, sweet-sour in flavour and is prepared in blocks of 0.5 × 5 to 7 cm; (ii) *brem wonogiri*, which is sweet flavoured, very soluble, white, and thin circular blocks of 5 cm diameter; and (iii) *brem bali*, which is a famous alcoholic liquor produced in Bali. All the three brems are made from the liquid portions of tapé ketan. The filtrate of tapé ketan is boiled down, poured onto table covered with banana leaves or polythylene sheeting, and left to cool to ambient temperature over 8-12 h (*brem madiun*) or sun dried for 1 day to produce *brem wonogiri* (Campbell-Platt, 1987). Keeping the liquid portion of tapé ketan for 7 months, during which time solids precipitate leaving a clarified brem, known as *brem bali*. It is decanted and bottled (Basuki, 1977). Saono *et al.* (1984) produced brem wine by using improved ragi and observed that this wine had a grape aroma and more desirable than commercial one. Winarno (1986) reported that the alcohol content of brem wine to be 6.13 %.

2.2.3. Tapai

Tapai is the Malaysian fermented food-beverage. It is consumed as a desert but in East Malaysia it is the rice wine with lighter colour and less sweetness (Merican and Yeoh, 1989). Tapai is produced by adding pulverized ragi or jui-piang to washed, cooked and cooled glutinous rice on

a woven bamboo tray, covered with cloth and incubated at room temperature (25-26° C) for 3 days, stirring the fermenting mash at least once a day to keep the surface moist. After 1 week, wine or brandy is added to the mash as a preservative and allowed to ferment for an additional 25 days and wine is collected by immersing a strainer-like collection vessel into the mash (Wong and Jackson, 1977). There are two types of tapai, namely, tapai pulut (made by fermented glutinous rice) and tapai ubi (made from tapioca or cassava) (Merican and Yeoh, 1989). *Candida* spp, *Saccharomycopsis fibuligera*, *Amylomyces rouxii*, *Mucor circinelloides*, *M. javanicus*, *Hansenula* spp, *Rhizopus oryzae*, and *R. chinensis* have been found to be present in tapai (Wang and Hesseltine, 1970; Ko, 1972). According to Cronk *et al.* (1977) the protein content of rice is doubled to about 16% after fermentation as a result of losses of total solids and synthesis of proteins by the microorganisms. Merican and Norrijah (1985) showed that the organisms necessary to produce a good tapai pulut consist of a mixture of *Amylomyces rouxii, Endomycopsis* (= *Saccharomycopsis*) *fibuligera* and *Hansenula anomala*, and for a good quality tapai ubi the essential microorganisms are *Amylomyces rouxii,* and *Endomycopsis* (= *Saccharomycopsis*) *fibuligera.*

2.2.4. Tapuy

Tapuy is a popular rice wine of the Ifugao race of the Philippines, which is sweet though acidic and aromatic (Tanimura *et al.*, 1977). Tapuy is prepared by washing, cooking and cooling glutinous rice, placed in a clay pot and pulverized bubod is sprinkled over it. The pot is covered with

cheesecloth and incubated in a cool place for 2-3 d (Tanimura *et al.*, 1977). Uyenco and Gacutan (1977) isolated *Endomycopsis* (= *Saccharomycopsis*) *fibuligera, Rhodotorula glutinis, Debaromyces hansenii, Candida parapsilosis* and *Trichosporon fennicum,* homofermentative and heterofermentative lactic acid bacteria, including some species of *Leuconostoc.* Tanimura *et al.* (1977) studied biochemical changes in tapuy and found that reducing sugar ranged from 4.1 to 5.2 %; pH from 3.3 to 4.9; total acidity from 6.55 to 22.49 ml of 0.1(N) NaOH/100 ml; and ethanol from 13.5 to 19.1 %.

2.2.5. Lao-chao

Lao-chao is a popular Chinese fermented food with a sweet taste and mild alcoholic flavour with a fruity aroma, made from rice by using chiu-yueh or peh-yueh as starters (Wang and Hesseltine, 1970). It is served as a dessert and is also a traditional diet for new mothers who believe that it helps them regain their strength (Wei and Jong, 1983). The process of manufacture is identical to Indonesian tapé keatan. Wei and Jong (1983) studied the flora of lao-chao and found *Rhizopus, Amylomyces, Torulopsis,* and *Hansenula.* Pure culture fermentation method of lao-chao was developed by Wang and Hesseltine (1970) and showed that a good fermented rice was made when a mold, *Rhizopus chinensis* NRRL 3671, and a yeast, *Endomycopsis* (= *Saccharomycopsis*) sp. NRRL Y7067, used as inocula instead of a commercial starter.

2.2.6. Yakju and Takju

Yakju and Takju are Korean alcoholic beverages, made from rice by using nuruk (Park *et al.*, 1977). The lower or diluted concentration of yakju is known as takju. During yakju preparation, steamed and cooled rice is mixed with nuruk and fermented and liquid pressed from the fermenting mass is filtered under pressure, aged and bottled (Park *et al.*, 1977). Microbial studies of yakju revealed the presence of many yeasts, *Bacillus* spp. and *Lactobacillus* sp. and *Leuconostoc* spp. (Shin and Cho, 1970; Kim, 1970; Kim and Lee, 1970; Lee and Rhee, 1970). Kim and Lee (1970) reported that *Saccharomyces cerevisiae* is the most important organism in alcohol production while *Hansenula* spp. play an important role in flavour development. Kim (1963) and Chung (1967) found various organic acids formation during yakju (takju) fermentation and recorded a total acidity reaching 0.44 to 0.62 % as lactic acid. Kim and Choi (1970 a & b) while studying nutritional changes observed the increase of thiamine during first two days and riboflavin decrease for first 3 d and increase markedly during the following 2 d.

2.2.7. Ewhaju

Ewhaju is a traditional wine prepared from rice by using nuruk in Korea (Kim and Kim, 1993). They suggested that brewed ewhaju could be remained with high quality for long period without any addition of preservatives/heat treatment, also found out that stored ewhaju contained high amylases activity which might contribute to digestion.

2.2.8. Krachae or Nam-khaao

Krachae or nam-khaao or sato is the Thai rice wine, prepared from Thai rice using loogpang as starter (Vachanavinich *et al.*, 1994). Lotong (1985) noted that moulds play an important role at the initial stage of fermentation for cleavage of sugar polymers present in rice to substantial sugars which can be used for substrates for simultaneous fermentation by yeast and lactic acid bacteria. Ko (1982) also noted that moulds produce sugars from starch, and subsequently yeast converts sugars to alcohol. Lim (1991) reported that lactic acid bacteria play a role in formation of flavour and taste in krachae.

2.2.9. Khao-maak

Khao-maak is a fermented glutinous rice beverage prepared by using loogpang, common in Thailand (Phittankpol *et al.*, 1995). It is semi-solid, juicy, white coloured, with sweet taste and slightly alcoholic. It is eaten as a dessert. If the fermentation is prolonged to 10 d, rice wine (krachae or nam-khaao) is made, if prolonged to 17 d, vinegar is made (Phittankpol *et al.*, 1995).

2.2.10. Basi

Basi is a traditional alcoholic beverage of the Philippines made by fermenting boiled, freshly extracted sugarcane juice with a mixture of yeast, bacteria and moulds or with organisms found in 'samac' (*Macharanga tanarius* or *M. gradifolia* Linn.) leaves, bark, or fruit (Tanimura *et al.*, 1978). Kozaki (1976) reported that the dominant organisms in basi are *Saccharomyces*, *Saccharomycopsis* and lactic acid bacteria. Sanchez and Kozaki (1984) developed an improved method for

preparation basi (sugarcane wine) which was more acceptable in all the sensory attributes than the traditionally prepared basi.

2.2.11. Saké

Saké is a Japanese rice wine, which is clear, pale yellow, containing 15 to 20 % alcohol. Polished rice is washed, steeped in water and steamed for 30-60 min, then cooled. Mixed with moulded rice koji and yeast moto, and water to form main mass moromi. Main fermentation in open tanks in cool conditions, starting at about 10° C, increasing to about 15° C. After 3 weeks' fermentation, filtered to give fresh saké, which settled, re-filtered, pasteurized and blended and diluted with water before bottling (Yoshizawa and Ishikawa, 1989). Murakami (1972) has reported the first organisms developed in the mash under traditional fermentation conditions to be the nitrate-reducing microorganism such as *Pseudomonas, Achromobacter, Flavobacterium, or Micrococcus* spp. These are followed, or possibly accompanied by *Leuconostoc mesenteroides* var *saké* and *Lactobacillus saké* followed by yeasts fermentation (Kodama and Yoshizawa, 1977). The highly refined saké brewed by the most skillful brewers using very highly polished rice at low temperatures of 9 to 11° C for 25 to 30 days is known as gonjoshu (Kodama and Yoshizawa, 1977). Changes in the various substances (starch, proteins, minerals, ether extractable lipids, and moisture) in rice grains with changes in milling ratio was studied by Yoshizawa and Ishikawa (1974) for saké making. Yoshizawa (1966) and Koizumi (1968) found the presence of n-propanol, i-butanol, i-amylalcohol, and their acetic acid esters, together with the ethyl esters of butyric acid,

and myristic acids in saké in lagre amount. Yoshizawa and Ishikawa (1989) noted that the temperature of the mash has a great influence on the quality of the saké production. Most lactic acid bacteria that spoil saké are homofermentative rods and are more tolerant to ethanol and acid than non-spoilers. *Lactobacillus casei* and *Lactobacillus leichmanii* are more tolerant (Inoue *et al.*, 1992).

2.2.12. Ruhi

Ruhi is an Indian drink primarily produced by the tribal people in Nagaland and in the eastern hill regions (Dahiya and Prabhu, 1977). It is a strong alcoholic beverage made by boiled rice fermentation. Boiled rice is spread on a mat, cooled and mixed with starter and nosan leaves, put into a cone shaped bamboo basket and fermented for 24 h. An earthenware pot is placed under the cone to collect the liquefied rice as it ferments. The juice is collected and transferred to new boiled rice about 3-4 time in succession. The total liquid collected becomes the first quality ruhi (Dahiya and Prabhu, 1977). They reported the ethanol content ranging from 12 to 14 % v/v, pH about 4.0, reducing sugars 2.5 % with total sugar of 3.0 % w/v from ruhi.

2.2.13. Madhu

Madhu is a alcoholic drink of Nagaland and eastern hill regions of India. It is a low-alcohol containing rice wine where soaked rice is the substrate. Raw rice is soaked for 2 h in cold water, drained and ground to a paste, slurry prepared with hot water to which cold water (15 L for 10 kg rice) and starter is added and fermented for 2-3 d (winter) and 1 d (summer). This

drink is used as an early morning meal in which salt is added to taste. *Mucor* and *Rhizopus* along with yeast and lactic acid bacteria were found in madhu fermentation (Dahiya and Prabhu, 1977).

2.2.14. Pachwai

Pachwai is a rice beer, prepared by adding powdered bakhar to steamed rice and fermented for 24 h after which the whole mass is transferred to an earthenware jar, water is added and fermentation continued. The beer is ready to drink in 1 or 2 d when it develops a characteristic alcoholic flavour (Hutchinson and Ram-Ayyar, 1925; Batra and Millner, 1976).

2.2.15. Millet beverage

In old literatures of Darjeeling and Sikkim, there are mentions of fermented millet beverages of the Darjeeling hills and Sikkim (Hooker, 1854; Gorer, 1938). The fermented beverage is also known as chang by the Sikkimese (Risley, 1928) and chi by the Lepcha (Gorer, 1938). Similar product called chhang has been reported from Ladakh region by Bhatia *et al.*, (1977). Thumba (correctly spelled toongbaa), the fermented beverage common in Darjeeling, Sikkim and Nepal has been reported by Hesseltine (1965, 1979) and Batra and Millner (1976).

3. MATERIALS AND METHODS

3.1. CULTURE MEDIA

Anaerobic agar (HiMedia M228)

Arginine hydrolysis test medium (Thornley, 1960)

Peptone	1.0 g
Arginine	10.0 g
NaCl	5.0 g
$K_2HPO_4.3H_2O$	0.3 g
Phenol Red	0.01 g
Agar	4.0 g
Distilled water	1 L
pH	7.2-7.4

Ascospore agar (HiMedia M804)

Basal medium (Gordon *et al.*, 1973)

Diammonium hydrogen phosphate	1.00 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.2 g
Bromocresol purple	0.4 g
Distilled water	1 L
pH	7.0

Davis and Mingioli's broth (Davis and Mingioli, 1950)

K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
Sodium citrate.$3H_2O$	0.5 g
$MgSO_4.7H_2O$	0.1 g
$(NH4)_2 SO_4$	1.0 g
Glucose (sterilized separately)	10.0 g
Distilled water	1 L
pH	7.0

Fermentation basal medium (Wickerham, 1951)

Yeast extract powder	4.5 g
Peptone	7.5 g
Demineralised water	1 L
Bromothymol blue	
(Till sufficiently dense green colour appears)	

Gelatin agar (Sneath and Collins, 1974)

Beef Extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Gelatin	10.0 g
Distilled water	1 L
pH	7.2

Malt extract agar (HiMedia M137)

MRS agar (HiMedia M 641)

MRS broth (HiMedia M 369)

MRS broth (de Man *et al.*, 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
K_2HPO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	20.0 g
Tween 80	1.0 g
Sodium acetate	5.0 g
$MgSO_4.7H_2O$	0.58 g
$MnSO_4.4H_2O$	0.28 g
Distilled water	1 L
pH	6.2-6.4

Milk agar (Gordon *et al.*, 1973)

Skim milk powder (HiMedia RM1254)	5 g in 50 ml distilled water
Agar	1 g in 50 ml distilled water

Autoclaved separately at 121° C for 20 min, cooled to 45° C, mixed together and poured into petri-dishes. The plates were allowed to dry the surface of the agar.

Nitrate broth (Gordon *et al.*, 1973)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1L
pH	7.0

Plate Count agar (HiMedia M091A)

Potato Dextrose agar (HiMedia M096)

Starch agar (Gordon *et al.*, 1973)

(a) Starch (HiMedia RM089)	10% (w/v)
(b) Tryptone	5.0 g
Yeast extract	15.0 g
Potassium dihydrogen phosphate	3.0 g
Agar	20.0 g

[(a) and (b) were mixed and autoclaved after making the volume up to 1L]

Yeast extract-malt extract (YM) agar (HiMedia M424)

Yeast Morphology agar (HiMedia M138)

Yeast Nitrogen Base (HiMedia M139)

3.2. REAGENTS

Burke's iodine solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Crystal violet stain (HiMedia S012)

Ehrlich-Böhme reagent (Iswaran, 1980)

p-Dimethylaminobenzaldehyde	1.0 g
95 % ethanol	95 ml
Concentrated HCl	20 ml

Malachite green (1 % w/v) solution (HiMedia S020)

Nitrate reduction test reagent

Solution A

Sulphanilic acid	0.8 g
5 N acetic acid	100 ml
(Glacial acetic acid: water, 1: 2.5)	

Solution B

∝-Napthylamine	0.5 g
5 N acetic acid	100 ml

The solutions A and B were mixed in equal quantities just before use.

Reagents for alcohol determination

N/5 $K_2Cr_2O_7$

$K_2Cr_2O_7$	9.807 g
Distilled water	1 L

8% KI

KI	8.0 g
Distilled water	92 ml

(Store in a brown bottle)

N/10 $Na_2S_2O_3$

$Na_2S_2O_3$	25.0 g
Distilled water	1 L

Reagents for reducing sugar and total sugar estimation

Reagent A

Anhydrous sodium carbonate	25.0 g
Sodium potassium tartarate	25.0 g
Sodium hydrogen carbonate	20.0 g
Anhydrous sodium sulphate	200.0 g

These are dissolved in 800 ml distilled water and diluted to 1 L.

Reagent B

$CuSO_4.5H_2O$	30.0 g
Distilled water with 4drops of conc.H_2SO_4	200 ml

Reagent C

(a) Ammonium molybdate	25.0 g
Distilled water with 21ml of conc. H_2SO_4	450 ml
(b) Disodium hydrogen arsenate heptahydrate	3.0 g
Distilled water	25 ml

Solution (b) was added to solution (a) slowly with stirring, then diluted to 500 ml, kept at 37° C to 40° C overnight and stored in a brown bottle.

Reagent D (Somogy copper solution)

25 ml of Reagent A was mixed with 1 ml of Reagent B. Freshly prepared Reagent D was used.

Reagents for phosphorus

Molybdovanadate reagent

Forty g of NH_4 molybdate.$4H_4O$ was dissolved in 400 ml of hot distilled water and allowed to cool. Two g of NH_4 metavanadate was dissolved in 250 ml of hot distilled water and cooled; 200 ml 70 % $HClO_4$ was added. Molybdate solution was gradually added to vanadate solution with stirring and diluted to 2 L.

Phosphate standard solution

A pure 1.9174 g of dried (105° C for 2 h) KH_2PO_4 was dissolved in 1 L distilled water. Freshly prepared solution was used.

Reagents for α-amylase assay

100 mM Tris - HCl buffer, pH 7.0

Dissolve 1.5% soluble starch in 100mM Tris (hydroxymethyl) aminomethane – HCl buffer, pH was adjusted to 7.0.

Stop solution

0.5 N Acetic acid – 0.5 N HCl (5:1)

Iodine solution

I_2 = 0.01%
KI = 0.1%

Reagent for glucoamylase assay

100 mM Acetate buffer, pH 5.0

0.1 M solution of acetic acid
0.1 M solution of sodium acetate
pH was adjusted to 5.0

3.3. EXPERIMENTAL

3.3.1. Collection of samples

Marcha samples were collected directly from their place of preparation in different traditional marcha-making villages located in the Darjeeling hills and Sikkim. Fermented beverages samples were collected from different places and markets of the Darjeeling hills and Sikkim aseptically in pre-sterile bottles, which were kept in an icebox carrier and transported, immediately to the laboratory for analyses.

3.3.2. Survey

Survey was conducted in randomly selected 100 houses in three sub-divisions of the Darjeeling hills namely Darjeeling, Kalimpong and Kurseong and 100 houses in four districts of Sikkim - North, West, South and East, representing the major ethnic communities of the Nepalis, Bhutias and Lepchas. Information was collected on indigenous knowledge of traditional beverage fermentation technology, ingredient uses, equipment uses, mode of consumption and socio-cultural importance of the product, using an questionnaire lasting for 1 h. Amount of fermented beverages consumed in every meal by each person was weighed directly by a portable weighing balance (Ishida, Germany) and daily per capita consumption was estimated as g/capita/day. Feeding frequency of fermented beverage by each family was also recorded in percentage.

Table B. Questionnaire on consumption of fermented beverages in the Darjeeling hills and Sikkim

- Name of the Informant:
- Ethnic Group:
- Name of (i) Village/Revenue Block:; (ii) Sub-division; (iii)District:
- Approximate number of population of the Village
- Nearest Market
- Kindly provide information on fermented beverages:

Fermented beverages	Local name	Raw material	Consume (daily/times per week/occasionally)	Whether prepare at home or market purchase/both
Kodo ko jaanr				
Bhaati jaanr				
Makai ko jaanr				
Gahoon ko jaanr				
Other beverages				
Raksi				

- Method of preparation
- Mode of consumption
- Equipment uses
- Socio-economy of the product, if any
- Do you perform any ritual or worship any particular god(s) or goddess(es) with fermented products you consume/drink?

3.3.3. Microbial analysis

3.3.3.1. Isolation of microorganisms

Ten g of sample was blended with 90 ml of 0.85 % (w/v) sterile physiological saline contained on a rotary shaker (120 rpm) for 30 min. Decimal dilution series were prepared in sterile diluent. One ml of appropriately diluted suspension of sample was mixed with the molten media and poured into plates. Total viable counts were determined using plate count agar (HiMedia M091A) and incubated at 30° C for 2 days. Moulds and yeasts were isolated on potato dextrose agar (HiMedia M096) and yeast extract-malt extract agar (HiMedia M424), respectively supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate and incubated aerobically at 28°C for 3 days. The lactic acid bacteria (LAB) were selectively isolated on MRS agar (HiMedia M641) plates supplemented with 1 % $CaCO_3$ and incubated anaerobically in an Anaerobic Gas-Pack system (HiMedia LE002) at 30° C for 3 days. Colonies were either selected randomly or all sampled if the plate contained less than 10 colonies. The purity of the isolates was checked by streaking again on fresh agar plates of the isolation medium, followed by microscope examination. Colonies appeared were counted as colony forming units (cfu) per g sample. The isolated strains were picked up on slants of their respective media and kept at 4° C. Cultures were sub-cultured in every two months. Identified strains were deposited and preserved in cryotubes at -20° C at the Food Microbiology Laboratory of the Department of Botany, Sikkim Government College, Gangtok, Sikkim, India.

3.3.3.2. Characterisation of moulds

The general morphology of moulds was examined after growing on potato dextrose agar (HiMedia M096) for 2 days at 28° C. Presence or absence of rhizoid and stolon were observed. Size of sporangium and sporangiospore were measured with a standardised ocular micrometer.

3.3.3.3. Characterisation of yeasts

3.3.3.3.1. *Cell morphology*

Sterile yeast morphology agar (Hi Media M138) slants were inoculated with an actively growing (24 h-old) yeast culture and incubated at 28° C for 3 days and observed for cell morphology and mode of vegetative reproduction (Kreger-van Rij, 1984). Dimension of cells were measured with a standardized ocular micrometer.

3.3.3.3.2. *Pseudo- andTrue-mycelium*

For observation of pseudo-mycelium and true-mycelium of yeast isolates, slide culture method described by Kreger-van Rij (1984) was followed. A petri-dish, containing U-shaped glass rod supporting two glass slides, was autoclaved at 121° C for 20 min. The molten potato dextrose agar (HiMedia M096) was poured onto the slides. The solidified agar on the slides was inoculated very lightly with yeast isolates in two lines along each slide. Four sterile coverslips were placed over part of the lines. Some sterile water was poured into the petri-dish to prevent the agar from drying out. The culture was then incubated at 28° C for 4 days. The slides were taken out of the petri-dish and the agar was wiped off from the back of the slide. The

edges of the streak under and around the coverslips were examined microscopically for the formation of pseudo-mycelium or true- mycelium.

3.3.3.3.3. *Characteristics of asci and ascospore*

Sterile ascospore agar (HiMedia M804) slants were streaked with a 24 h-old yeast isolates, incubated at 28° C for 3 days and examined at weekly intervals up to 4 weeks for observation of asci and ascospores. A heat fixed smear was flooded with 5 % w/v aqueous malachite green (HiMedia S020) for 30 to 60 sec, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (HiMedia S027) for 30 sec and observed under the microscope (Kreger-van Rij, 1984).

3.3.3.3.4. *Reduction of nitrate*

Cultures were grown in 5 ml nitrate broth incubated at 28° C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red or yellow colour, indicating the presence of nitrate. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate, i.e. absence of reduction (Norris *et al.*, 1981).

3.3.3.3.5. *Growth at 37° C*

Slants of malt-extract agar (HiMedia M137) were inoculated with cells of young yeast isolates and incubated at 37° C for 4 days and then observed the growth (Yarrow, 1998).

3.3.3.3.6. *Sugar fermentation*

The method was based on Kreger-van Rij (1984). Cells were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes of 10 ml of fermentation basal medium (Wickerham, 1951) supplemented with 2 % w/v sterile sugars containing Durham tubes, were inoculated with the above yeast culture and incubated at 28° C and were shaken regularly to observe gas accumulation in the inverts.

3.3.3.3.7. *Sugar assimilation*

The method was based on Kreger-van Rij (1984). Yeast isolates were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (HiMedia M139) and carbon source were inoculated with cultures and incubated at 28° C for 3 to 7 days. Control test tube was made by adding 0.5 ml of yeast nitrogen base (HiMedia M139) in 4.5 ml of sterilized distilled water (devoid of any carbon source). Assimilation of carbon sources was observed by comparing with the control.

3.3.3.4. Characterization of bacteria

3.3.3.4.1. *Gram staining*

The method of Bartholomew (1962) was followed. A 48 h-old drop of cell suspension of bacterial culture was taken on grease-free slide and a smear was made. It was then heated-fixed, flooded by crystal violet stain for 1 min, and washed for 5 sec with water. The smear was flooded with Burke's

iodine solution, allowed to react for 1 min, and washed again for 5 sec with water. Holding the slide against a white surface, 95 % ethanol was poured drop-wise from the top edge of the slide until no more colour came out from the lower edge of the slide. After washing with water, the smear was stained with safranine for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

3.3.3.4.2. *Cell morphology*

An air-dried (not heated-fixed) smear of a 24-h old bacterial culture was stained for 30 sec with safranin, washed in water, air-dried (Norris *et al.*, 1981) and observed under oil-immersion objective. Cell dimension was measured with a standardized ocular micrometer.

3.3.3.4.3. *Motility*

A drop of a 24 h-old culture in MRS (HiMedia M369) broth was used to prepare a hanging drop in a cavity slide. The drop of culture was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) for motility test following the method of Harrigan (1998).

3.3.3.4.4. *Production of catalase*

A 24 h-old slant culture was flooded with 0.5 ml of 10 % hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.*, 1981).

3.3.3.4.5. *Gas ($C0_2$) production from glucose*

It was observed in MRS broth without citrate and containing inverted vials (Schillinger and Lücke, 1987). Accumulation of gas in the inverts indicates positive result.

3.3.3.4.6. *Anaerobic growth*

Anaerobic agar (HiMedia M228) was put into culture tubes in sufficient amount so that to give 7.5 cm depth of the medium and sterilized by autoclaving at 121° C for 20 min. Tubes were inoculated with a small (outside diameter 1.5 mm) loopful of 24 h-old MRS broth culture by stabbing up to the bottom of the column. They were incubated at 30° C for 3 and 7 days and observed for growth along the length of the stab (anaerobic) (Claus and Berkeley, 1986).

3.3.3.4.7. *Hydrolysis (decomposition) of casein*

Milk agar plates were streaked with 24 h-old cultures at the centre and examined after incubation at 30° C for 7 days for any clearing of casein around and underneath the growth (Gordon *et al.*, 1973).

3.3.3.4.8. *Hydrolysis of gelatin*

MRS agar (HiMedia M641) plates containing 1 % w/v gelatin were streaked with bacterial culture at the centre and incubated at 30° C for 3 and 5 days. Plates were then flooded with 10 ml of 1 N sulphuric acid saturated with ammonium sulphate. Hydrolysis was indicated by a clear zone, under

and around the growth, in contrast to opaque precipitate of unchanged gelatin (Sneath and Collins, 1974).

3.3.3.4.9. *Hydrolysis of arginine*

Culture tubes containing arginine hydrolysis test medium were inoculated by stabbing and immediately after this, a layer (~ 1 cm) of sterile mineral oil was added over the stab. The tubes were incubated at 30° C for 3 days and observed for the change in colour from yellow to red indicating the formation of ammonia from arginine (Lelliott *et al.*, 1966).

3.3.3.4.10. *Hydrolysis of starch*

Starch agar plates were streaked with 24 h-old cultures and incubated at 30° C for 3 days. Flooded the plates with iodine solution for 15-30 min and observed and measured the clear zone underneath (after the growth was scrapped off).

3.3.3.4.11. *Production of indole*

Cells were grown at 30° C in 10 ml Davis and Mingioli's broth, prepared by replacing ammonium sulphate with L-tryptophan (0.1 % w/v) and supplementing with yeast extract (0.02 % w/v). Ehrlich-Böhme reagent (1-2 ml) was layered on 3, 5 and 7 days old broth culture. Formation of a red ring at the culture-reagent interface was considered as an indication of positive result (Iswaran, 1980).

3.3.3.4.12. *Reduction of nitrate*

The method followed was the same as described in 3.3.3.3.4.

3.3.3.4.13. *Salt tolerance*

Salt tolerance was tested by inoculating a loopful of culture in MRS broth (HiMedia M369) supplemented with 4 %, 6.5 %, 10 % and 18 % sodium chloride, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987).

3.3.3.4.14. *Growth at different pH*

The pH of MRS broth (HiMedia M369) was adjusted to different levels using 1 N HCl or 10 % w/v NaOH. The medium was then distributed into tubes containing 10 ml in each tube. They were autoclaved, cooled and inoculated with 0.2 ml of 48 h-old cultures. The tubes were incubated at 30° C for 24 h-old and observed for growth (Hesseltine and Ray, 1988). The different pH used was 4.2, 7.5 and 8.5.

3.3.3.4.15. *Growth at different temperatures*

MRS broth (HiMedia M369) was inoculated with cultures and incubated at 15° C and 45° C for 3 days, respectively and the growth was observed (Schillinger and Lücke, 1987).

3.3.3.4.16. *Sugar Fermentation*

Sugar fermentation test of bacterial cultures for 49 sugars were carried out by using API 50 CHL system (bioMérieux, France). Cultures were grown at

30° C for 48 h on MRS agar (HiMedia M641). The growth was harvested in 2 ml sterile normal saline which was used to prepare suspensions, corresponding to 10^7 cells/ml. The incubation box was prepared by distributing about 10 ml of sterile water into the honeycombed base of the 50 CHL trays. The strips were unpacked, placed them in the trays and the tubes were filled with the bacterial suspension. The inoculated strips were kept slightly tilted and incubated at 30° C for 48 h. The strips were read by referring to the manufacturer's interpretation table. All spontaneous reactions were recorded.

3.3.3.5. Identification

Moulds were identified according to Schipper (1976, 1984) and Hesseltine (1991). Identified representative strains of moulds were sent to International Institute of Mycology, Surrey, U.K. for confirmation. Yeast strains were identified according to the criteria laid down by Kreger-van Rij (1984) and Kurtzman and Fell (1998). For identification of bacterial species, taxonomic keys laid down in Bergey's Manual of Systematic Bacteriology, volume 2 (Sneath *et al.*, 1986) and keys described by Wood and Holzapfel (1995) were followed.

3.3.3.6. Preliminary screening of amylolytic activity

All isolates were streaked on surface-dried plates of starch agar and incubated at 28° C for mould and yeasts and at 30° C for lactic acid bacteria. After 3 days plates were flooded with iodine solution and observed for clear zone in and around the colony. If positive, diameter of

the clearing zones was measured by a scale, which was used as an assessment of amylolytic activity.

3.3.4. Physico-chemical analysis

3.3.4.1. *pH*

Ten g of sample was blended with 20 ml of carbon-dioxide free-distilled water in a homogeniser for 1 min and the pH of the slurry was determined directly using pH-meter (Type 361, Systronics) calibrated with standard buffer solutions (Merck).

3.3.4.2. *Temperature*

The temperature (° C) change of the fermenting substrates during beverage fermentation was recorded directly by a thermometer.

3.3.4.3. *Moisture*

Moisture content of sample was calculated by drying 2.5–3.0 g of well-mixed sample at 135 ± 1° C for 2 h to constant weight (AOAC, 1990).

3.3.4.4. *Titratable acidity*

Titratable acidity of sample was calculated by titrating the filtrates of a well blended 10 g sample in 90 ml carbon-dioxide free distilled water with 0.1 N sodium hydroxide to end point of phenolphthalein (0.1 % w/v in 95 % ethanol) (AOAC, 1990).

3.3.4.5. *Ash*

A sample (~ 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace preheated to 550° C for 3 h. The crucible was transferred directly to a desiccator, allowed to cool to room temperature and weighed immediately (AOAC, 1990). The process of heating for 30 min, cooling and weighing was repeated until the difference between two successive weighing was ≤ 1 mg.

3.3.4.6. *Alcohol*

Alcohol content of sample was determined by dichromate oxidation method (AOAC, 1990). The 10 ml of extract was pipetted in a 500 ml round-bottomed flask where 1 g of $CaCO_3$ and 100 ml of distilled water was added and distilled. The distillate was collected for 15 min and diluted to 100 ml with distilled water (after coming to room temperature). Diluted distillate was pipetted out into a conical flask with stopper to which 10 ml of N/5 $K_2Cr_2O_7$ and 10 ml of concentrated H_2SO_4 were added and allowed to stand for 1 h. After this, stopper was removed and 100 ml of distilled water was added, followed by addition of 8 % KI and immediately titrated with N/10 $Na_2S_2O_3$ using freshly prepared 1 % starch (HiMedia RM089) solution as the indicator. Alcohol content was calculated in percentage.

Alcohol (%) =

$(V_1-V_2) \times f_2 \times 0.00115 \times 100/V_3$ 100/S (multiply by 250/E of diluted extract used).

V_1 = titration volume of N/10 $Na_2S_2O_3$ against 10 ml of N/5 $K_2Cr_2O_7$ (blank test without sample)

V_2 = titration volume of N/10 $Na_2S_2O_3$ against the distillate

f_2 = factor of N/10 $Na_2S_2O_3$

100 = total volume of the distillate

V_3 = pipetting volume of the distillate for the reaction

100 = %

S = sample size

250 = total volume of the diluted extract

E = ml of extract taken for alcohol distillation.

3.3.4.7. *Reducing sugar*

Reducing sugar content of sample was determined by modified colorimetric method of Somogyi (1945) using glucose as standard solution. To 1 ml of extract in a 20 ml capped glass tube, 1 ml of Reagent D was added and heated in a vigorously boiling water-bath for 20 min. Allowed to cool for 5 min in running tap water, and 1 ml of Reagent C was added and shaken the test tube until no bubbles were evolved. After standing for 20 min diluted to 25 ml with distilled water and absorbance was measured at 520 nm in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Reducing sugar was calculated in percentage.

$$\text{Glucose (\%)} = (As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 250/V_2 \times 100/10$$

As = absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G] = concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

250 = total volume of extract (ml)

100 = %

10 = sample size for preparation of extract

3.3.4.8. *Total Sugar*

Total sugar was determined by determining reducing sugar in hydrolysed sample with HCl (AOAC, 1990). In a 300 ml conical flask fitted with condenser, 2 g of sample was blended in 20 ml of distilled water to which 160 ml of distilled water and 20 ml of HCl (25 %) were added. It was heated in vigorously boiling water bath for 3 h, cooled in a running tap water, neutralized with 10 % NaOH using pH meter (Type 361, Systronics) and diluted to 500 ml with distilled water. It was filtered and the filtrate was taken for determining reducing sugar as described above. Total sugar was calculated in percentage.

Total sugar (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 500/V_2 \times 100/S$

As= absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G]= concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

500 = total volume of extract (ml)

100 = %

S = sample size for preparation of extract

3.3.4.9. *Nitrogen*

The method described in AOAC (1990) was followed. Approximately 1 g of sample was taken in a digestion flask, 0.7 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25 ml of concentrated H_2SO_4 were added to it. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round-bottomed flask, and mixed with approximately 100 ml of distilled water and 25 ml 4 % w/v aqueous Na_2S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40 % w/v NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard 0.1 N H_2SO_4 containing about 5 drops of methyl red indicator (HiMedia I007). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard 0.1 N NaOH solution. The blank determination on reagents was considered for correction. Nitrogen was calculated in percentage.

N (%) =

(ml of standard acid × N of standard acid) – (ml of standard NaOH × N of standard NaOH) ×1.4007/weight of sample (g).

3.3.4.10. *Protein*

Protein content was determined by multiplying total nitrogen, estimated by micro-Kjeldahl method, by 6.25 (AOAC, 1990).

3.3.4.11. *Fat*

Fat content was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in the desiccator for cooling. The weight (W_1) of the round-bottomed flask was taken. A cellulose thimble (dry and fat free) was taken and in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether having boiling range 40-60° C, on heating mantle at 60° C for 5 h. The flat bottomed flask was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated in percentage.

Fat (%) = $W_2 - W_1$/Sample weight x 100

3.3.4.12. *Crude fibre*

Crude fibre content was determined using fibertec extraction (Tecator, model 1010 M6, Sweden) following the method of AOAC (1990). One g of the pre-dried sample was weighed and weight of the sample was noted (W_1). The 150-ml of hot 0.128 N H_2SO_4 was added into the column. Tap

water was opened for cooling of the condensers. Drops (2-4) of octanol was added to prevent foaming and heated to boiling for 30 min. Cold water tap was opened for the water suction pump and filtration was started. Sediment was removed from the filter surface by applying reversed pressure, washed three times with hot deionized water in each column. The 150 ml of 0.223 M KOH solution was added to sample and a few drops of octanol was added and boiled for 30 min. As in the previous case here also the heater was put off and filtered and if necessary sediments were removed by applying reversed pressure, washed 3 times with hot deionized water in each column. It was made sure that no residue remained on the inside wall of the column. The crucibles were removed from the Hot Extractor Unit to the Cold Extractor Unit by using the crucible holder. The valves of Cold Extractor Unit were closed and filled each crucible containing the sample with 25 ml of acetone, filtered it out by placing the valve in vacuum position. It was repeated for three times for each sample, removed the crucibles and transferred them to a crucible stand, left them at room temperature, until nearly all acetone was is gone in order to avoid the burning of the fibres during the drying process. The crucibles were dried at 100° C overnight, cooled to room temperature in a desiccator and weighed them afterwards (W_2). The sample was ashed in the crucible at 500° C for at least 3 h and final weight was taken (W_3). Crude fibre was calculated in percentage.

Crude fibre (%) = $W_2 - W_3/W_1 \times 100$

W_1 = sample weight

W_2 = crucible + residue weight

W_3 = crucible +ash weight

3.3.4.13. *Carbohydrate*

Carbohydrate content was calculated by difference (Standal, 1963):

100 – (% protein + % fat + % ash)

3.3.4.14. *Minerals*

The method was based on AOAC (1990). The ash after heating the sample (2 g) at 550° C for 3 h was dissolved in 5 ml of 20 % HCl. The solution was evaporated to dryness on a hot plate at a temperature of 100-110° C and in an oven at 110° C for 1 h. The minerals in the dried residue were dissolved in about 10 ml of 100 % HCl and the solution was heated on a hot plate at a temperature of 100-110° C for 3-4 times. The solution was made up to 100 ml with 1 % HCl. Calcium, magnesium, manganese, copper, iron, zinc, sodium and potassium were estimated in an atomic absorption spectrophotometer (Model 2380, Perkin-Elmer).

Phosphorus was determined by colorimetric method (AOAC, 1990). A series of standard solutions (0.0, 0.1, 0.2, 0.3 and 0.4 mg P_2O_5/ml) were prepared. Five ml aliquots was pipetted into 100 ml volumetric flasks, and 50 ml distilled water and 4 ml 70 % $HClO_4$ were added. To each flask 20 ml molybdovanadate reagent was added, diluted to volume with distilled water, thoroughly shaken and allowed to stand for 15 min. Determination of blank as 'A' and standards in set of matched cells against distilled water as reference was done. Standards for 'A' were corrected and was plotted against concentration in mg P_2O_5/ml solution. The ash after heating the

sample (5 g) at 550° C for 3 h was dissolved in 4 ml of 70% $HClO_4$ and 20 ml of distilled water. The solution was transferred to 100 ml volumetric flask and 20 ml molybdovanadate reagent was added, diluted to 100 ml, mixed thoroughly and absorbance at 400 nm was measured in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Blank and standard were carried through entire determination. 'A' of blank was subtracted from that of sample.

3.3.4.15. *Energy*

The energy value of a sample was determined by multiplying its percent protein, fat and carbohydrate contents by the factors 4, 9 and 4, respectively and adding all the multiplication values to get Kcal per 100 g (Gopalan *et al.*, 1995).

3.3.5. Enzymatic activities

3.3.5.1. *Enzymatic profiles*

The enzymatic profiles of selected strains of moulds, yeasts and bacteria isolates were assayed following the method of Arora *et al.* (1990) in API zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: phosphatase alkaline, esterase (C4), esterase lipase (C8), lipase (C14), leucine, valine and cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Cultures were grown on MRS agar (HiMedia M641) and growth was

harvested in 2 ml sterile normal saline which was used to prepare suspension of 10^7 cells/ml. The strip was unpacked and 2 drops of cell suspensions was inoculated in each cupule of the strip containing ready-made enzyme substrates and incubated at 30° C for 6 h. After incubation, 1 drop of ready-made zym A and zym B reagents was added and observed for colour development based on the manufacturer's colour chart.

3.3.5.2. *α-amylase activity assay*

The blue value method of Fuwa (1954) as modified by Kawaguchi *et al.* (1992) was followed for α-amylase activity. Cultures were grown on broth medium (1.0 % soluble starch, 1.0 % yeast extract, 1.0 % peptone, and 0.3 % NaCl, pH 7.2) in shaking incubator (180 rpm) at 28° C for 48 h. The cultures were immediately centrifuged in refrigerated automatic centrifuge (C-24, Remi) at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and 1.5 % soluble starch dissolved in 100 mM Tris-HCl buffer, pH 7.0 were pre-incubated separately at 37° C for 5 min in water-bath shaker (RSB-12, Remi). Then, the reaction mixture was started by adding 1.0 ml of 1.5 % soluble starch (HiMedia RM089) to 0.5 ml enzyme solution and incubated at 37° C for 10 min. The reaction was stopped by the addition of 2.5 ml of stop solution. The 100 ml of the reaction mixture was added to potassium iodide solution, left at room temperature for 20 min and absorbance at 660 nm of the resulting solution was measured in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). One unit of α-amylase activity (liquefying

activity) was defined as the amount of α-amylase which produced 10 % fall in the intensity of blue colour at the above condition.

3.3.5.3. *Glucoamylase activity assay*

Glucoamylase activity was determined according to modified method of Ueda and Saha (1983). Cultures were grown on broth medium (1.0 % soluble starch, 1.0 % yeast extract, 1.0 % peptone, and 0.3 % NaCl, pH 7.0) in shaking incubator (180 rpm) at 28° C for 48 h and were immediately centrifuged in refrigerated automatic centrifuge (C-24, Remi) at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The reaction mixture containing 2 ml of 1 % soluble starch (HiMedia RM 089) in 2 ml of 100 mM acetate buffer (pH 5.0) and 0.5 ml of enzyme solution was pre-incubated separately at 40° C for 5 min in a water-bath shaker (RSB-12, Remi). The 2 ml of 1 % soluble starch dissolved in 100 mM acetate buffer was added to enzyme solution and incubated at 40° C for 10 min. After a 10 min reaction, 1 ml of the reaction mixture was taken and glucose was determined by calorimetric method (Somogyi, 1945). One unit of glucoamylase activity (saccharifying activity) was defined as the amount of enzyme, which liberated 1 mg glucose in 1 min under the above condition. Unit of activity was expressed as mg glucose released per ml per 10 min.

3.3.6. Microbial and physico-chemical changes during fermentation

Jaanr was prepared in the laboratory following traditional method. Dry seeds of finger millet {*Eleusine coracana* (L.) Gaertn.), local variety

'mudke kodo}, rice (*Oryza sativa* L.) and dry seeds of maize (*Zea mays* L.) were purchased from Gangtok market. Seeds were cleaned, washed, cooked and powdered marcha (2 %), collected from Aho village, was added when the temperature of cooked substrates was around 30° C. Inoculated seeds were put into pre-sterile bottles with loosely covered lids and fermented at 28° C for 2 days for saccharification. After 2 days, lids were tightly capped to make anaerobic condition and kept for 8 days at 28° C. Samplings were made at every one day interval till 10^{th} day for microbial, physico-chemical and enzymatic analysis.

3.3.7. Testing of isolates for producing kodo ko jaanr

3.3.7.1. *Preparation of inocula*

Kodo ko jaanr was prepared in the laboratory using selected strains of moulds, yeasts and lactic acid bacteria, previously isolated from marcha samples. The suspension of mould cultures was made by adding 5 ml of sterile distilled water to each 4-day-old slant culture on potato dextrose agar (HiMedia M096), and mycelia and sporangia were scraped off the agar with an sterile inoculating wire. Yeasts and lactic acid bacterial inocula were prepared by introducing 5 ml each of sterile distilled water onto 48 h-old slant culture on yeast extract-malt extract agar (HiMedia M424) and 72 h-old slant culture on MRS (HiMedia M641), respectively. Tubes were agitated for 30 sec in a cyclomixer (Remi). Number of cells in the suspension was determined using a Neubauer's counting chamber and a phase contrast microscope (Olympus CH3-BH-PC, Japan). Cell suspensions of selected strains were prepared (10^5 to 10^6 cells/ml) and 2 ml

of each mixture was inoculated to 100 g sterilised (121° C for 15 min) and cooled to ~ 40° C seeds of finger millet. Inoculated seeds were saccharified in loosely capped pre-sterile bottles at 28° C for 2 days, and after saccharification, lids of the bottles were tightly capped and fermented at 28° C for 6 days. The rationale behind selecting the inoculum size was based on the observation on conventional method of adding ~ 2 % of marcha in boiled seeds of finger millets during kodo ko jaanr preparation.

The products were evaluated physico-chemically and organoleptically using the method as described in this chapter.

3.3.7.2. *Sensory evaluation*

Sensory properties of product was evaluated in terms of aroma, taste, texture, colour and general acceptability as method described by Meilgaard *et al.* (1990). Kodo ko jaanr samples produced by selected strains of moulds, yeasts and lactic acid bacteria, previously isolated from marcha were organoleptically evaluated by a panel of 7 judges with score rate of 1, bad and 5, good considering market jaanr as control with scoring rate of 3, moderate (Table C).

Table C. Format for sensory evaluation of kodo ko jaanr produced by selected strains, isolated from marcha

Please use market kodo ko jaanr as a control with scoring rate of 3 (moderate)

Sample code:..... Name:.....

Attribute	Score					Comment
Aroma:						
Mild alcoholic	Weak				Strong	
	1	2	3	4	5	
Taste:						
Mild sweet	Weak				Strong	
	1	2	3	4	5	
Slightly acidic (sour)	Weak				Strong	
	1	2	3	4	5	
Texture:	Hard				Soft	
	1	2	3	4	5	
Colour:	Bad				Good	
	1	2	3	4	5	
General Acceptability:	Bad				Good	
	1	2	3	4	5	

3.3.8. Consumers' Preference Trial

Market samples of kodo ko jaanr as well as kodo ko jaanr prepared in the laboratory by using a mixture of selected isolates were served to 50 consumers representing different ethnic groups of people of the Sikkim Himalayas who were familiar with jaanr. The 9-point scale used in this study ranged from 'dislike extremely' (score, 1) to 'like extremely' (score, 9) (IS, 1971).

3.3.9. Statistical analysis

The data were analysed by determining standard deviation (SD), standard error of measurement (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran, 1989).

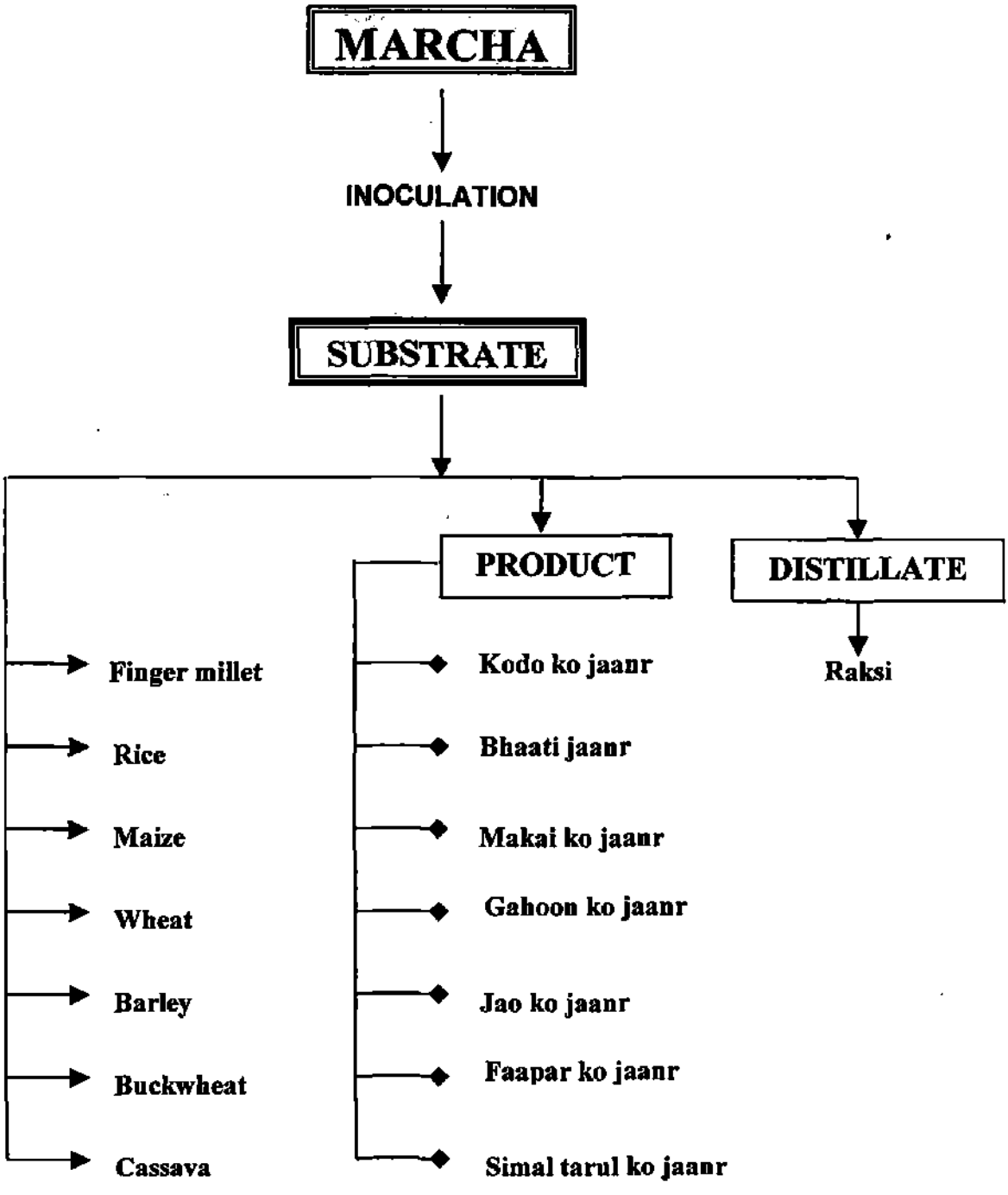
4. RESULTS

4.1. SURVEY ON FERMENTED BEVERAGES

Survey was conducted using questionnaire in the Darjeeling hills and Sikkim. The traditional methods of preparation, equipment used, mode of consumption, feeding frequency, socio-economy and ethnical importance of indigenous fermented beverages (Table 1) were documented. Survey data showed that in rural areas majority of people prepared indigenous fermented beverages (57.6 % the Darjeeling hills and 76.7% in Sikkim) for home consumption.

Table 1: Traditional fermented beverages of the Darjeeling hills and Sikkim

Product	**Substrate**	**Consumption rate (%)**	
		The Darjeeling hills	**Sikkim**
Marcha (starter)	Rice, wild herbs, spices		
Common Alcoholic Beverages:			
Kodo ko jaanr	Finger millet	95.0	85.0
Bhaati jaanr	Rice	55.0	40.0
Makai ko jaanr	Maize	45.0	35.0
Gahoon ko jaanr	Wheat	15.0	25.0
Lesser-known Alcoholic Beverages:			
Simal tarul ko jaanr	Cassava tuber	< 10.0	< 10.0
Jao ko jaanr	Barley	< 10.0	< 10.0
Faapar ko jaanr	Buck wheat	< 10.0	< 10.0
Distilled liquor:			
Raksi	Starchy substrates	85.0	60.0


MARCHA
INOCULATION
SUBSTRATE
PRODUCT
DISTILLATE
Raksi
Finger millet
Rice
Maize
Wheat
Barley
Buckwheat
Cassava
Kodo ko jaanr
Bhaati jaanr
Makai ko jaanr
Gahoon ko jaanr
Jao ko jaanr
Faapar ko jaanr
Simal tarul ko jaanr

4.2. MARCHA

Marcha is the traditionally prepared mixed dough inocula used as a starter culture for production of various indigenous alcoholic beverages. Marcha is a dry, round to flattened, creamy white to dusty white, solid ball like starter ranging from 1.9 cm to 11.8 cm in diameter with the weight ranging from 2.3 g to 21.2 g (Plate 1).

4.2.1. Synonym of marcha

Marcha is a Nepali word. Different ethnic communities of this region call it by their own dialect such as *khesung* by Limboo, *bharama* by Tamang, *bopkha* or *khabed* by Rai, *phab* by Bhutia, and also by the Tibetan, and *buth/thanbum* by the Lepcha.

4.2.2. Traditional method of preparation

A traditional method of marcha preparation practiced by the Limboo women in Aho village in East Sikkim was cited. During marcha preparation, glutinous rice (*Oryza sativa* L.) is soaked in water for 6-8 h at ambient temperature. Unheated soaked rice is crushed in a foot-driven heavy wooden mortar by a pestle. In 1 kg of grinned rice, ingredients added include roots of 'guliyo jara' or 'chitu' (*Plumbago zeylanica* L.), 2.5 g; leaves of 'bheemsen paate' (*Buddleja asiatica* Lour), 1.2 g; flowers of 'sengreknna' {*Vernonia cinerea* (L.) Less}, 1.2 g; ginger, 5.0 g; red dry chilli, 1.2 g; and previously prepared marcha as a mother culture, 10.0 g. The mixture is then made into paste by adding water and kneaded into flat cakes of varying sizes and shapes, and placed individually on the ceiling floor made up of bamboo stripes above the kitchen, bedded with fresh fronds of ferns, locally called 'pire uneu'

{*Glaphylopteriolopsis erubescens* (Wall ex Hook.) Ching}, and covered with dry ferns (Plate 2 & 3) and jute bags. These are left to ferment for 1-3 days, the longer period being used under the colder condition. Completion of fermentation is indicated by distinct alcoholic and ester-aroma and puffy/swollen appearance of marcha. Finally, cakes of marcha are sun dried for 2-3 days (Fig 1). Marcha is stored at room temperature and in dry place for more than a year.

In Jhosing and Tibuk villages in North Sikkim, root-barks and flowers of wild herbs locally called 'marcha jar' (*Polygala arillata* Buch. Hum.) are mixed and grinded with soaked water during marcha preparation.

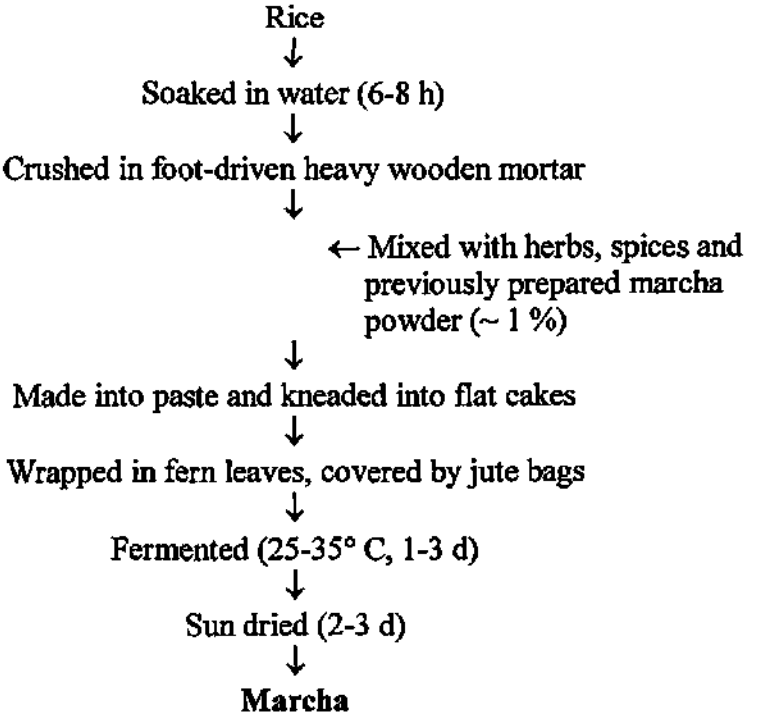


Fig 1. Flow sheet of marcha preparation in East Sikkim

Plate 1. Marcha of varying sizes

Plate 2. Marcha preparation by the Limboo woman in Aho village.

Plate 3. Kneaded dough of marcha are incubated in fern leaves.

Plate 4. Marcha is sold in Gangtok market.

4.2.3. Socio-economy

Marcha is produced at household level in few villages in the Sikkim Himalayas (Table 2 and Fig 2) exclusively by the rural women belonging to the Limboo, Rai and the Lepcha. Men can help women in collection of wild herbs and pounding the herbs during marcha preparation. This art of technology is protected as hereditary trade and passes from mother to daughters.

Table 2. Important marcha-making villages in the Sikkim Himalayas

Village	Location	Altitude (Feet)	Linked Market	Sample code	Dominant marcha-maker
The Darjeeling Hills:					
Nor Busty	Darjeeling	2300	Bijanbari, Darjeeling	MN	Rai and Limboo
Kashyong	Kalimpong	4500	Algarah, Kalimpong, Rhenock	MK	Limboo and Rai
Mangzing	Kalimpong	1800	Kalimpong, Lava, Gorubathan	MM	Limboo, Rai and Lepcha
Sikkim:					
Jhosing	North	3200	Magan, Gangtok	MJ	Limboo
Tibuk	North	3120	Magan, Gangtok	MT	Limboo
Chhejo	West	6800	Geyzing, Legsep	MC	Limboo
Lingchom	West	5000	Geyzing, Yoksum	ML	Limboo
Salghari	South	4150	Jorethang	MS	Limboo
Barnyak	South	4000	Namchi, Ravangla	MB	Limboo and Rai
Aho	East	3630	Gangtok, Pakyong	MA	Limboo
Kopchey	East	3510	Rhenok, Rongli	MKo	Limboo

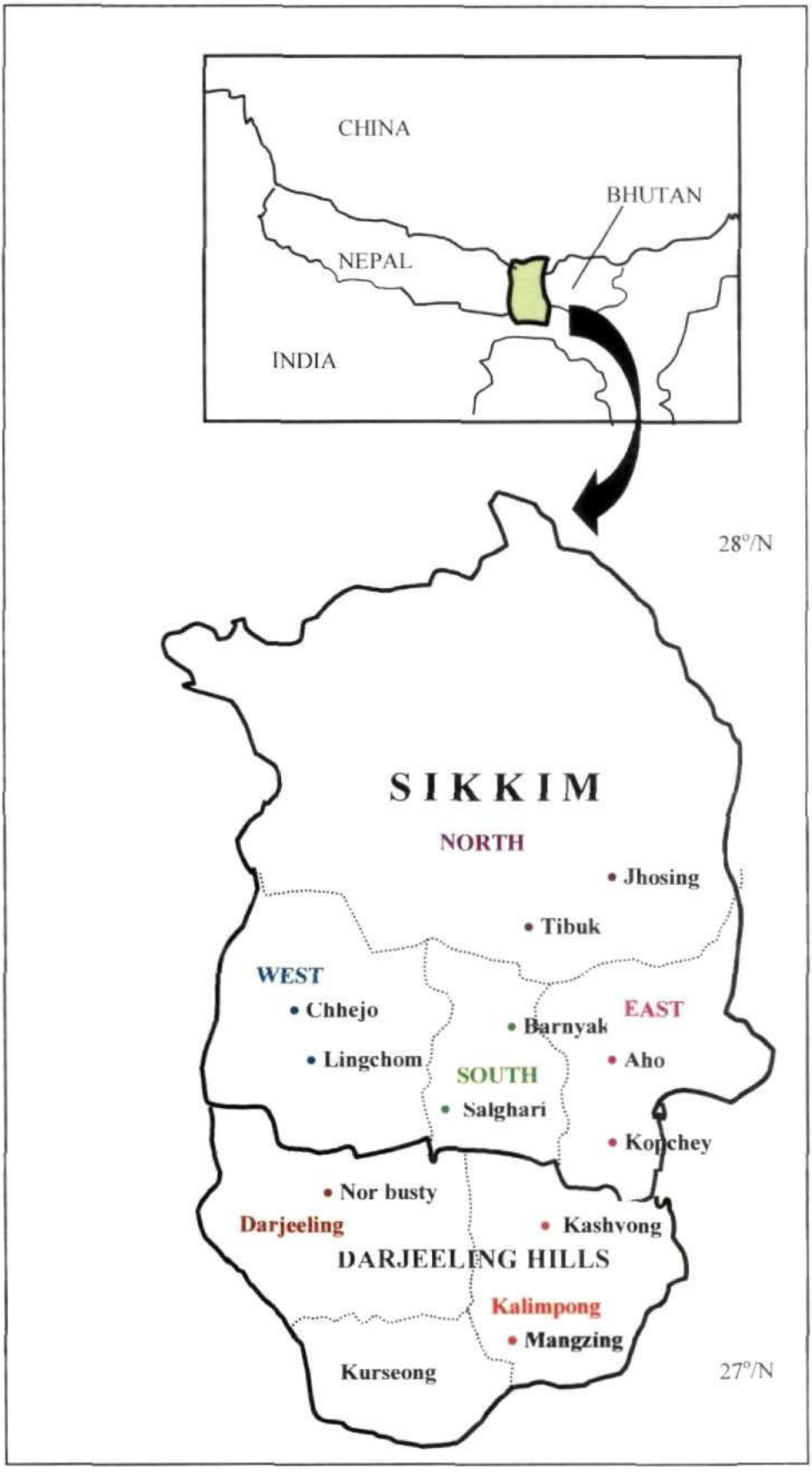


Fig 2. Location map of marcha-making villages in the Sikkim Himalayas.

Some people are economically dependent upon this product. Marcha is sold in local markets, called "haats" (Plate 4). Cost of marcha depends on its size. Small-sized marcha (~2.0 cm) costs about 0.50 paise each, while the large-sized (~12 cm) marcha is sold at Rs.5.00 per piece.

4.2.4. Similar Product

Marcha is similar to ragi of Indonesia, nuruk of Korea, bubod of the Philippines, loogpang of Thailand and chiuyueh of China. These starter cultures are used to prepare alcoholic beverages from starchy substrates.

4.2.5. Microorganisms

Sixty-six samples of marcha were collected from main source of marcha-making villages of the Darjeeling hills and Sikkim as shown in Table 2. Samples were analysed for microbial load (Table 3). Mould population in marcha was detected at the level of 10^6 cfu/g, whereas the loads of yeasts and lactic acid bacteria were 10^8 cfu/g and 10^7 cfu/g, respectively. Total viable count was at the level of 10^8 cfu/g. Out of 733 strains of microorganisms isolated from marcha samples, 152 isolates were filamentous moulds, 321 were yeasts and 260 were lactic acid bacteria.

Table 3. Microbial load of marcha collected from marcha-making villages

Source	$\times 10^7$ cfu/g dry weight			
	Mould	Yeast	LAB	Total Viable Count
Nor Busty	0.2 (0.1-0.3)	3.3 (2.8-3.8)	3.5 (2.8-4.1)	6.9 (5.3-9.8)
Kashyong	0.2 (0.1-0.3)	5.6 (4.6-6.7)	3.7 (1.6-6.5)	9.1 (5.9-11.8)
Mangzing	0.2 (0.1-0.3)	0.7 (0.5-0.9)	0.3 (0.2-0.4)	1.4 (0.6-2.3)
Jhosing	0.3 (0.1-0.2)	8.1 (5.8-10.4)	4.7 (1.4-7.7)	15.0 (14.0-18.0)
Tibuk	0.1 (0.08-0.14)	23.0 (15.0-29.0)	13.0 (6.0-18.0)	37.0 (34.0-40.0)
Chhejo	0.2 (0.1-0.2)	9.7 (6.3-11.0)	0.5 (0.3-0.7)	12.0 (8.0-16.0)
Lingchom	0.3 (0.1-0.4)	6.7 (6.3-13.0)	1.0 (0.3-1.2)	10.0 (7.0-15.0)
Salghari	0.2 (0.1-0.3)	21.0 (15.0-26.0)	16.0 (4.0- 30.0)	28.0 (24.0-33.0)
Barnyak	0.08 (0.05-0.1)	18.0 (15.0-21.0)	7.8 (6.8-8.5)	29.0 (24.0-33.0)
Aho	0.3 (0.2-0.3)	15.0 (13.0-18.0)	18.0 (16.0-20.0)	34.0 (31.0-36.0)
Kopchey	0.15 (0.1-0.2)	1.0 (0.4-1.8)	2.0 (0.9-3.6)	3.5 (1.8-5.3)

LAB, lactic acid bacteria
Data represent the means of 6 samples from each source. Ranges are given in parentheses.

4.2.5.1. Characterisation and identification of moulds

On the basis of morphology and presence or absence of stolon and rhizoids, all 152 strains of filamentous moulds isolates from each source were classified into two genera: *Mucor* (94 strains) and *Rhizopus* (58 strains) belonging to family Mucoraceae (Table 4).

Table 4. Selection of representative strains of moulds isolated from marcha[a]

Source	Number of strains[b] isolated	Stolon and rhizoids	Grouped strains	Representative strains
Nor Busty	12	Absent	6	MN:Mu1
		Present	6	MN:Rh1
Kashyong	14	Absent	8	MK:Mu2
		Present	6	MK:Rh2
Mangzing	7	Absent	7	MM:Mu1
Jhosing	13	Absent	8	MJ:Mu1
		Present	5	MJ:Rh1
Tibuk	20	Absent	10	MT:Mu1
		Present	10	MT:Rh1
Chhejo	16	Absent	8	MC:Mu1
		Present	8	MC:Rh2
Lingchom	10	Absent	10	ML:Mu2
Salghari	16	Absent	8	MS:Mu3
		Present	8	MS:Rh2
Barnyak	10	Absent	10	MB:Mu1
Aho	18	Absent	10	MA:Mu1
		Present	8	MA:Rh3
Kopchey	16	Absent	9	MKo:Mu4
		Present	7	MKo:Rh1

[a] Number of samples was six from each source.

[b] All strains had aseptate mycelia.

Table 5. Characteristics of representative strains of moulds isolated from marcha

Strain	Sporangium		Sporangiospore		Identification
	Shape	Diameter (μ m)	Shape	Diameter (μ m)	
MM:Mu1	Globose, borne circinately	30-68	Ellipsoidal to oval	5.6-7.2	*Mucor*
MJ:Mu1	Globose	18-52	Ellipsoidal to oval	4.8-7.2	
MA:Mu1	Globose, borne circinately	34-92	Ellipsoidal to oval	4.2-5.6	
MC:Mu1	Globose	28-80	Spherical, ellipsoidal to oval	2.4-5.6	
MS:Mu3	Globose, borne circinately	32-80	Ellipsoidal to oval	4.5-5.6	
MJ:Rh3	Globose to sub-globose	72-144	Round to ellipsoidal	4.0-8.0	*Rhizopus*
MA:Rh3	Globose	52-116	Round	2.0-5.0	
MC:Rh2	Globose to sub-globose	44-120	Round to ellipsoidal	5.0-8.0	
MS:Rh1	Globose to sub-globose	72-148	Round to ellipsoidal	4.0-8.5	
MKo:Rh2	Globose	76-160	Round	3.0-6.0	

Following the taxonomical keys of Schipper (1976, 1984) and Hesseltine (1991), representative strains MM:Mu1 (IMI 375454), MA:Mu1 and MS:Mu3 which had repeatedly branched sporangiophores with many sporangia borne circinately (Plate a) were identified as *Mucor circinelloides* forma *circinelloides* van Tieghem. Representative strains MJ:Mu1 (IMI 375452) and MC:Mu1 were identified *Mucor* sp. close to *M. hiemalis* sensu lato. Sporangial walls of these isolates were

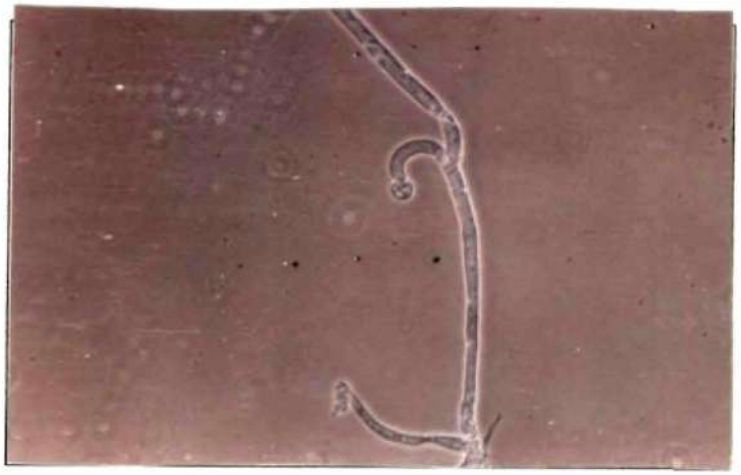


Plate (a). *Mucor circinelloides* forma *circinelloides* MJ:Mu1 (PDA, 3 d, 28° C), isolated from marcha, showing circinately borne sporangium in phase contrast micrograph (× 480).

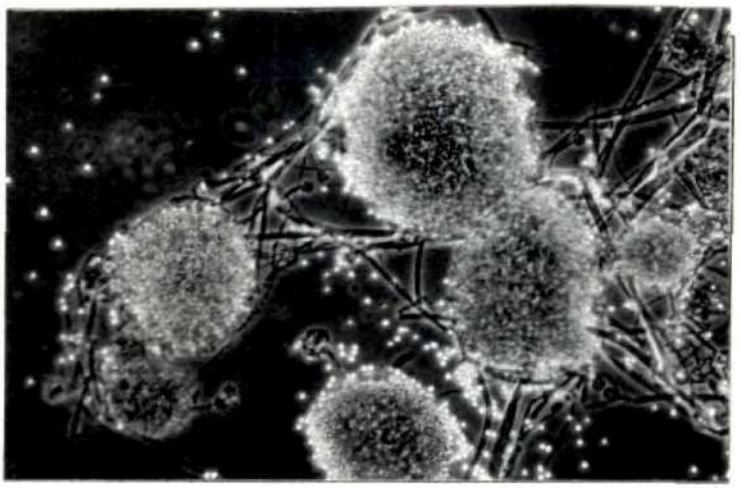


Plate (b). *Mucor circinelloides* forma *circinelloides* MJ:Mu1 (PDA, 7 d, 28° C), isolated from marcha, showing deliquesced sporangia with sporangiospores in phase contrast micrograph (× 250).

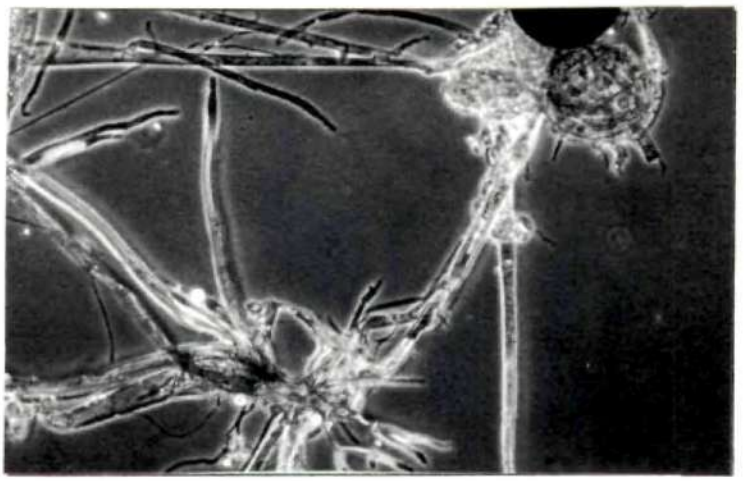


Plate (c). *Rhizopus chinensis* MJ:Rh3 (PDA, 7 d, 28° C), isolated from marcha, showing rhizoids, sporangiophores and sporangia in phase contrast micrograph (× 250).

mostly deliquesced (Plate b). Representative strains MJ:Rh3, MC:Rh2 and MS:Rh1 were identified as *Rhizopus chinensis* Saito (Plate c). Representative strains MA:Rh3 (IMI 375453) and MKo:Rh1 were identified as *Rhizopus stolonifer* variety *lyococcus* (Ehrenb.) Stalp. & Schipper. Confirmation of identity of these strains was done at International Mycological Institute, Surrey, U.K. Prevalence of *Mucor* and *Rhizopus* was 100 % and 73 %, respectively in sixty-six samples collected from eleven different marcha-making villages in the Sikkim Himalayas.

4.2.5.2. Characterisation and identification of yeasts

Four genera of yeasts were selected on the basis of colony, cell morphology, vegetative reproduction and type of ascospore among 321 yeasts isolates (Table 6a & b).

Table 6a. Selection of representative strains of yeasts isolated from marcha of the Darjeeling hills

Source	Number[a] of strains isolated	Colony	Cell Shape	Mycelium	Ascospore	Grouped strains	Representative strains
Nor Busty	30	Ds	O-Cy	True	Hat-shaped	10	MN:YD3
		Ss	O-E	Pseudo	Hat-shaped	8	MN:YP1
		Ss	O-E	Pseudo	Globose	6	MN:YS1
		Fs	O-E	–	–	6	MN:YC2
Kashyong	32	Ds	O-Cy	True	Hat-shaped	10	MK:YD1
		Ss	O-E	Pseudo	Hat-shaped	10	MK:YP1
		Ss	O-E	Pseudo	Globose	6	MK:YS5
		Fs	O-E	–	–	6	MK:YC1
Mangzing	33	Ds	O-Cy	True	Hat-shaped	15	MM:YD3
		Ss	O-E	Pseudo	Hat-shaped	10	MM:YP2
		Ss	O-E	Pseudo	Globose	8	MM:YS1

[a]Number of samples was six from each source. All isolates reproduced by multilateral budding.

Ds, dusty surface; Ss, smooth surface; Fs, fringed surface; O-Cy, Oval to cylindrical; O-E, Oval to ellipsoidal.

Table 6b. Selection of representative strains of yeasts isolated from marcha of Sikkim

Source	Number[a] of strains isolated	Colony	Cell Shape	Mycelium	Ascospore	Grouped strains	Representative strains
Jhosing	26	Ds	O-Cy	True	Hat-shaped	12	MJ:YD1
		Ss	O-E	Pseudo	Hat-shaped	8	MJ:YP1
		Ss	O-E	Pseudo	Globose	6	MJ:YS2
Tibuk	35	Ds	O-Cy	True	Hat-shaped	15	MJ:YD2
		Ss	O-E	Pseudo	Hat-shaped	12	MT:YP2
		Ss	O-E	Pseudo	Globose	8	MT:YS1
Chhejo	29	Ds	O-Cy	True	Hat-shaped	10	MC:YD2
		Ss	O-E	Pseudo	Hat-shaped	8	MC:YP1
		Ss	O-E	Pseudo	Globose	6	MC:YS1
		Fs	O-E	–	–	5	MC:YC1
Lingchom	28	Ds	O-Cy	True	Hat-shaped	10	ML:YD2
		Ss	O-E	Pseudo	Hat-shaped	7	ML:YP1
		Ss	O-E	Pseudo	Globose	6	ML:YS7
		Fs	O-E	–	–	5	ML:YC1
Salghari	26	Ds	O-Cy	True	Hat-shaped	12	MS:YD2
		Ss	O-E	Pseudo	Hat-shaped	8	MS:YP1
		Ss	O-E	Pseudo	Globose	6	MS:YS1
Barnyak	24	Ds	O-Cy	True	Hat-shaped	10	MB:YD1
		Ss	O-E	Pseudo	Hat-shaped	8	MB:YP2
		Ss	O-E	Pseudo	Globose	6	MB:YS2
Aho	32	Ds	O-Cy	True	Hat-shaped	12	MA:YD1
		Ss	O-E	Pseudo	Hat-shaped	8	MA:YP2
		Ss	O-E	Pseudo	Globose	6	MA:YS2
		Fs	O-E	–	–	6	MA:YC3
Kopchey	26	Ds	O-Cy	True	Hat-shaped	10	MKo:YD2
		Ss	O-E	Pseudo	Hat-shaped	8	MKo:YP1
		Ss	O-E	Pseudo	Globose	8	MKo:YS3

[a]Number of samples was six from each source. All isolates showed by multilateral budding. Ds, dusty surface; Ss, smooth surface; Fs, fringed surface; O-Cy, Oval to cylindrical; O-E, Oval to ellipsoidal.

Sugar fermentation and assimilation tests of randomly selected representative strains of yeasts were carried out (Table 7). Following the taxonomical keys described by Kreger-van Rij (1984) and Kurtzman and Fell (1998), representative strains MC:YD2 and MKo:YD2 had dusty and dry surfaced colonies with horn-like projections made up of many strands of mycelia when grown on agar plates. There were 2 to 4 hat-shaped ascospores per ascus. All of them fermented starch and assimilated sucrose. These strains were identified as *Saccharomycopsis fibuligera* (Lindner) Klöcker (Plate d). Representative strains MN:YP1 and MJ:YP1 had glistening surfaced colonies on agar plates, showed 1 to 4 hat-shaped ascospores per ascus and could not ferment starch. Asci were deliquescent. They were identified as *Pichia anomala* (E.C. Hansen) Kurtzman (Plate e). Representative strains MK:YS5 and MS:YS1 had smooth surfaced colonies, showing globose ascospores and fermented starch. They were identified as *Saccharomyces cerevisiae* Meyen ex Hansen (Plate f). Representative strains MN:YC2 and ML:YC1 showed fringe-surfaced colonies without ascus and ascospore and fermented only glucose and trehalose. They were identified as *Candida glabrata* (Anderson) Meyer et Yarrow (Plate g).

Prevalence of all strains of yeasts in marcha samples was 100 % except *Candida glabrata* showing only 45 % in sixty-six samples analysed.

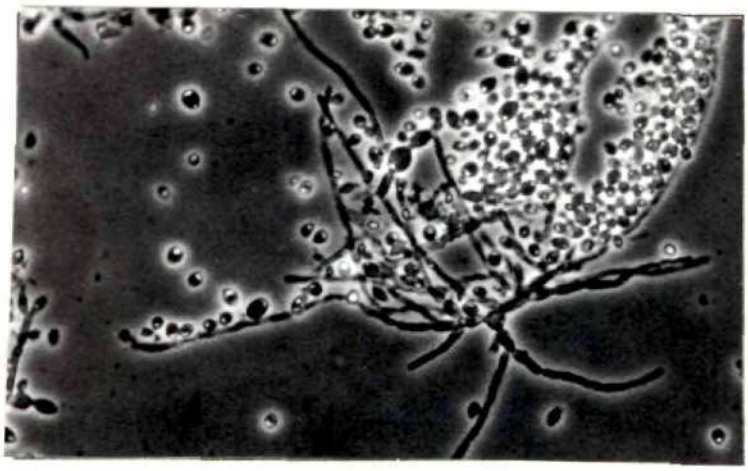


Plate (d). *Saccharomycopsis fibuligera* MC:YD2 (YM agar, 5 d, 28° C), isolated from marcha, showing oval to cylindrical cells with true mycelia in phase contrast micrograph (× 480).

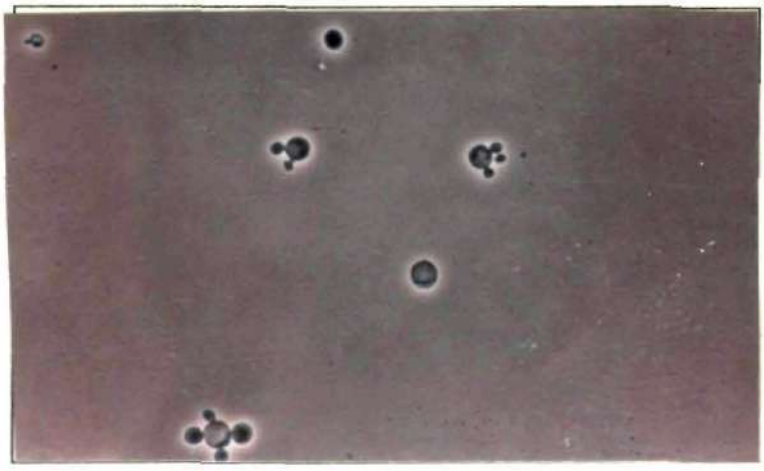


Plate (e). *Pichia anomala* MN:YP1 (YM agar, 3 d, 28° C), isolated from marcha, showing oval cells with multilateral budding in phase contrast micrograph (× 480).

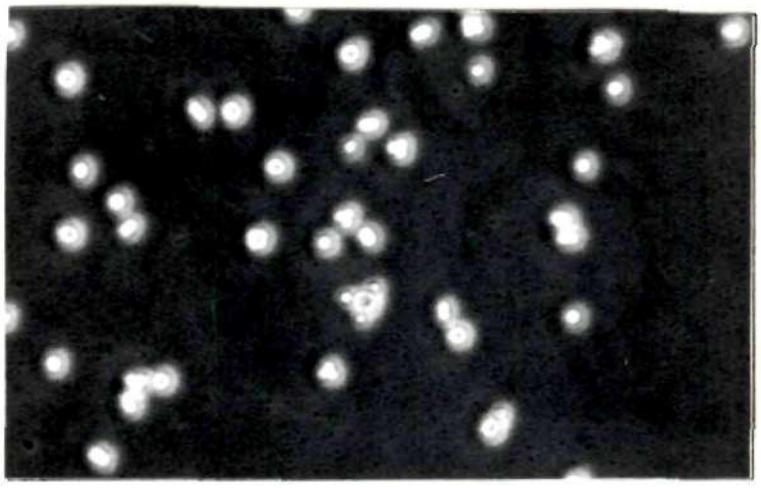


Plate (f). *Saccharomyces cerevisiae* MK:YS5 (Ascospore agar, 7 d, 28° C), isolated from marcha, showing globose ascospores in phase contrast micrograph (× 480).

Table 7. Characteristics of representative strains of yeasts isolated from marcha

Parameter	MC:YD2	MKo:YD2	MN:YP1	MJ:YP1	MK:YS5	MS:YS1	MN:YC2	ML:YC1
Cell width (μm)	2.0-5.0	2.0-5.0	1.6-4.5	1.6-3.2	1.6-4.5	1.6-4.0	0.8-2.4	0.6-2.7
Cell length (μm)	3.0-7.0	3.0-7.0	1.9-5.4	1.9-5.4	2.0-5.0	2.0-5.0	1.6-4.0	1.3-3.7
Nitrate reduction	–	–	+	+	–	–	–	–
Growth at 37^0 C	+	+	+	+	+	+	+	+
Sugar fermentation:								
Glucose	+	+	+	+	+	+	+	+
Galactose	–	–	–	–	+	+	–	–
Lactose	–	–	–	–	–	–	–	–
Maltose	+	+	+	+	+	+	–	–
Raffinose	–	+	+	+	+	+	–	–
Sucrose	+	+	+	+	+	+	–	–
Starch	+	+	–	–	+	+	–	–
Trehalose	–	–	+	–	–	–	+	+
Sugar assimilation:								
Arabinose	–	–	+	$+_w$	–	–	–	–
Cellobiose	+	+	+	+	–	–	–	–
Galactose	–	–	+	+	+	+	–	–
Glycerol	–	–	–	–	–	–	–	$+_w$
Inositol	+	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–
Maltose	+	+	+	+	+	+	–	–
Melibiose	+	+	–	–	+	+	–	–
Mannitol	–	+	+	+	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–
Raffinose	+	+	+	+	+	+	–	–
Sucrose	+	+	+	+	+	+	–	–
Starch	+	+	+	+	+	+	–	–
Trehalose	–	–	+	+	+	+	+	+
Xylose	–	–	–	+	–	–	–	–
Identification	*Saccharomycopsis*		*Pichia*		*Saccharomyces*		*Candida*	

+, positive; –, negative; $+_w$, weak positive

4.2.5.3. Characterisation and identification of bacteria

Out of 260 lactic acid bacteria strains isolated from sixty-six samples of marcha, 195 strains were coccii-tetrads and 65 strains were non-sporeforming rods (Table 8).

Table 8. Selection of representative strains of LAB isolated from marcha

Source	Number[a] of strains isolated	Cell Shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Nor Busty	35	Coccus	–	+	20	MN:C1
		Rod	+	–	15	MN:R5
Kashyong	20	Coccus	–	+	20	MK:C7
Mangzing	30	Coccus	–	+	20	MM:C3
		Rod	+	–	10	MM:R1
Jhosing	30	Coccus	–	+	20	MJ:C2
		Rod	+	–	10	MJ:R2
Tibuk	15	Coccus	–	+	15	MT:C1
Chhejo	15	Coccus	–	+	15	MC:C7
Lingchom	30	Coccus	–	+	20	ML:C3
		Rod	+	–	10	ML:R8
Salghari	20	Coccus	–	+	20	MS:C7
Barnyak	25	Coccus	–	+	15	MB:C9
		Rod	+	–	10	MB:R4
Aho	25	Coccus	–	+	15	MA:C1
		Rod	+	–	10	MA:R5
Kopchey	15	Coccus	–	+	15	Mko:C1

[a]Number of samples was 6 from each source

[b]All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile

All isolates of lactic acid bacteria were Gram-positive, non-sporeforming, non-motile, catalase negative and facultative anaerobes; they did not hydrolyse casein, gelatin and starch (Table 9a). Representative strains MN:C1, MM:C3, MA:C1 and MKo:C1 were coccii in tetrads, grew well in 4 % and 6.5 % NaCl but not in 18 % NaCl, produced no gas from glucose. Phenotypic and sugar fermentation characterisation of coccii representative strains MK:C7, MJ:C2, MT;C1, and MS:C7 were identical to strains MN:C1 whereas strains MC:C7, MC:C3, and MB:C9 were identical to MA:C1. All rod-shaped lactic representative strains MN:R5, MJ:R2, MB:R4 and MA:R5 produced gas from glucose. Rod-shaped lactic strain MM:R1 was identical to MN:R5 whereas strain ML:R8 was identical to MA:R5. Following sugar fermentation pattern of isolates using API 50 CHL system (Table 9b) and the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), coccii-tetrads were identified as *Pediococcus pentosaceus* Mees (Plate h). *P. pentosaceus* is closely related to *P. acidilactici* due to hydrolysis of arginine, but is distinguished from *P. acidilactici* due to fermentation of maltose and trehalose, and no growth at 50° C. Rod strains were identified as heterofermentative *Lactobacillus bifermentans* Kandler, Schillinger and Weiss (Plate i) due to similar characters such as gas from glucose, no hydrolysis of arginine, growth at 15° C but no growth at 45° C, fermented ribose, D-xylose and L-arabinose. However, *Lb. bifermentans* has been placed in Group B (facultative heterofermentative lactobacilli) by Hammes and Vogel (1995).

Prevalence of *P. pentosaceus* was 100 %, whereas that of *Lb. bifermentans* was only 54 % in sixty-six samples analysed.

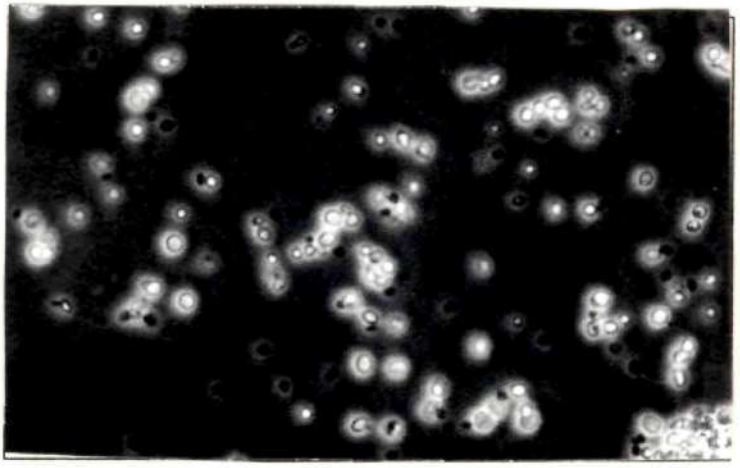


Plate (g). *Candida glabrata* MN:YC2 (YM agar, 3 d, 28° C), isolated from marcha, showing oval to ellipsoidal cells in phase contrast micrograph (× 480).

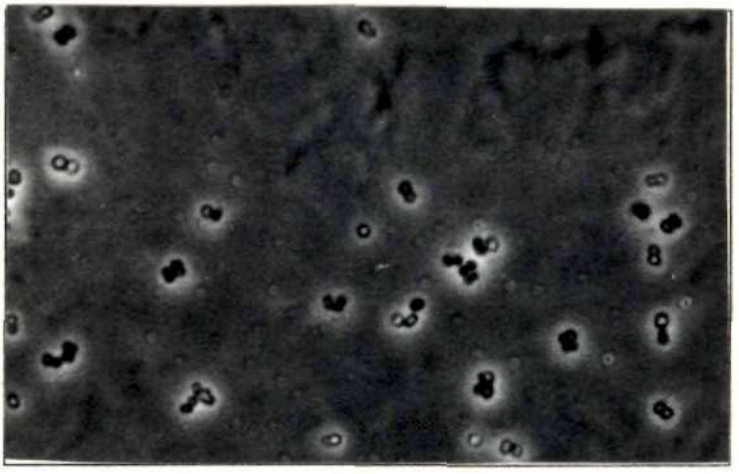


Plate (h). *Pediococcus pentosaceus* MA:C1 (MRS agar, 3 d, 30° C), isolated from marcha, showing coccoid cells in tetrads in phase contrast micrograph (× 1200).

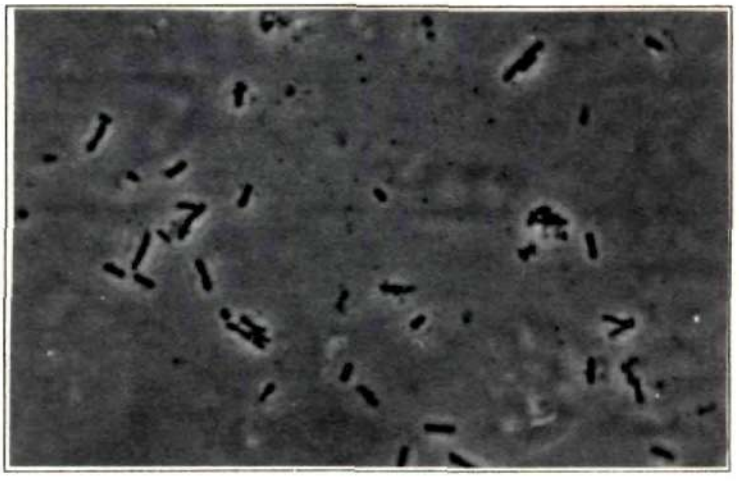


Plate (i). *Lactobacillus bifermentans* MA:R5 (MRS agar, 3 d, 30° C), isolated from marcha, showing non-sporeforming rod cells in phase contrast micrograph (× 1200).

Table 9a. Phenotypic characterisations of representative strains of LAB isolated from marcha

Parameter	MN:C1	MM:C3	MA:C1	MKo:C1	MN:R5	MJ:R2	MB:R4	MA:R5
Cell shape	Ct	Ct	Ct	Ct	R	R	R	R
Cell diameter ((µm)	0.2-0.7	0.2-0.6	0.2-0.6	0.4-0.7				
Cell width (µm)					0.2-0.3	0.2-0.3	0.2-0.3	0.2-0.3
Cell length (µm)					0.8-2.3	0.8-2.2	1.0-2.0	1.0-2.4
Anaerobic growth	+	+	+	+	+	+	+	+
Hydrolysis of:								
Casein	–	–	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–	–	–
Arginine	+	+	+	+	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–	–	–
Growth in NaCl:								
4.0 %	+	+	+	+	+	+	+	+
6.5 %	+	+	+	+	+	+	+	+
10.0 %	+	+	+	+	+	+	+	+
18.0 %	–	–	–	–	–	–	–	–
Growth in pH:								
4.2	+	+	+	+	+	+	+	+
7.5	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+
Growth at:								
15° C	+	+	+	+	+	+	+	+
45° C	–	–	–	–	–	–	–	–

Ct, coccii-tetrad; R, rod in chain with 2-3 cells

Table 9b. Sugar fermentation of LAB strains using API 50 CHL system

Parameter	MN:C1	MM:C3	MA:C1	MKo:C1	MN:R5	MJ:R2	MB:R4	MA:R5
Glycerol	+	+	–	–	–	–	+	–
Erythritol	+	+	–	–	–	–	–	–
D-Arabinose	+	+	–	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+
L-Xylose	–	–	–	–	–	–	–	$+_w$
Adonitol	+	+	–	–	–	–	+	–
β-Methyl-D-Xyloside	–	–	–	–	–	–	$+_w$	–
Galactose	+	+	+	+	+	$+_w$	–	$+_w$
D-Glucose	–	–	+	+	+	–	+	+
D-Fructose	–	–	+	+	+	+	+	+
D-Mannose	–	$+_w$	+	+	–	–	+	–
L-Sorbose	$+_w$	–	–	–	–	–	–	–
Rhamnose	+	+	–	–	–	–	+	–
Dulcitol	+	+	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	$+_w$
Sorbitol	+	+	–	–	–	–	–	–
α-Methyl-D-Mannoside	+	+	–	–	–	$+_w$	+	–
α-Methyl-D-Glucoside	–	–	–	–	$+_w$	$+_w$	+	+
N-Acetyl-Glucosamine	+	+	+	+	$+_w$	–	+	+
Amygdalin	+	+	+	+	–	–	–	–
Arbutin	–	–	–	–	–	–	–	–
Esculin	–	–	–	–	–	–	–	–
Salicin	$+_w$	–	$+_w$	–	–	–	–	–
Cellobiose	+	+	+	+	–	–	–	–
Maltose	+	+	+	+	+	+	+	$+_w$
Lactose	–	–	–	+	–	–	–	–

Parameter	MN:C1	MM:C3	MA:C1	MKo:C1	MN:R5	MJ:R2	MB:R4	MA:R5
Melibiose	–	–	–	–	$+_w$	$+_w$	+	$+_w$
Sucrose	–	–	–	–	–	–	–	–
Trehalose	+	+	+	+	–	–	–	–
Inulin	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–
Gentiobiose	+	+	+	+	–	–	–	–
D-Turanose	–	–	–	–	–	–	–	–
D-Lyxose	–	–	–	–	–	–	–	–
D-Tagatose	+	+	+	+	–	–	–	–
D-Fucose	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	–	–	–	–
L-Arabitol	–	–	–	–	–	–	–	–
Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
2-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
5-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	$+_w$
Identification	*Pedicoccus*				*Lactobacillus*			

4.2.6. Composition of marcha

Marcha is slightly acidic in nature containing pH 5.58 with 0.1 % acidity. Marcha contained 14 % moisture and 1.4 % ash (Table 10).

Table 10. Proximate composition of marcha

Parameter	Kashyong	Salghari	Jhosing	Aho	Chhejo
pH	5.77 (5.76-5.78)	6.20 (6.16-6.24)	5.28 (5.27-5.29)	5.43 (5.43-5.43)	5.20 (5.18-5.22)
Acidity (%)	0.10 (0.10-0.10)	0.10 (0.10-0.10)	0.10 (0.10-0.11)	0.10 (0.10-0.10)	0.12 (0.11-0.13)
Moisture (%)	15.3 (14.6-15.8)	13.9 (12.6-14.2)	16.2 (13.3-16.8)	13.3 (12.2-13.6)	14.0 (12.3-15.0)
Ash (%)	1.8 (1.2-2.0)	2.6 (2.3-2.7)	1.0 (0.8-1.3)	0.9 (0.7-1.2)	0.9 (0.7-1.3)

Data represent the means of 5 samples from each source. Ranges are given in parentheses.

4.2.7. Enzymatic activities of isolates

Enzymatic profiles of selected strains of moulds, yeasts and lactic acid bacteria isolates of marcha were assayed using API zym (bioMérieux, France) galleries (Table 11). Marcha isolates showed relatively weak esterase and lipase activities as compared with phosphatase activities. Preliminary screenings of amylolytic activities of all isolates of marcha were tested in starch agar plates. On the basis of amylolytic activity (showing >1.0 mm hydrolysis zone in starch agar plate), 5 strains of *Rhizopus* spp., 6 strains of *Mucor* spp., 7 strains of *Saccharomycopsis fibuligera*, 5 strains of *Pichia anomala*, 5 strains of *Saccharomyces cerevisiae* and 4 strains of *Candida glabrata* were selected for α-amylase and glucoamylase assays (Table 12). None of the lactic acid bacteria showed amylolytic activity, hence they were not selected for amylolytic enzyme assay. *Saccharomycopsis fibuligera* MS:YD4 showed highest liquefying activity (α-amylase) and *Rhizopus chinensis* MJ:Rh3 showed highest saccharifying activity (glucoamylase).

Table 11. Enzymatic profiles using API zym system of representative strains of microorganisms isolated from marcha

Enzyme	Activity (nanomoles)								
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Control (without enzyme)	0	0	0	0	0	0	0	0	0
Phosphatase alcaline	≥40	≥40	0	5	5	20	≥40	0	0
Esterase (C4)	0	0	5	5	5	5	10	5	0
Esterase Lipase (C8)	0	≥40	5	5	0	10	20	5	0
Lipase (C14)	0	0	0	0	0	0	0	0	0
Leucine arylamidase	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Valine arylamidase	0	5	5	5	5	0	0	≥40	≥40
Cystine arylamidase	0	5	0	0	0	0	0	5	5
Trypsin	0	0	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0
Phoshatase acid	≥40	5	≥40	5	5	10	10	5	5
Napthol-AS-BI-phosphohydrolase	≥40	≥40	5	10	10	10	10	5	5
α-galactosidase	0	0	0	0	0	0	20	10	0
β-galactosidase	0	0	0	0	0	0	0	≥40	0
β-glucuronidase	0	0	0	0	0	0	0	0	0
α-glucosidase	5	5	0	0	0	0	0	≥40	0
β-glucosidase	≥40	≥40	0	0	0	0	0	≥40	5
N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	10
α-mannosidase	10	0	0	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0	0	0	0

A = *Saccharomycopsis fibuligera* MS:YD4; **B** = *Pichia anomala* MN:YP1; **C** = *Saccharomyces cerevisiae* MJ:YS2:Y2; **D** = *Mucor* sp. (close to *M. hiemalis*) MJ:Mu1; **E** = *Mucor circinelloides* MM:Mu1; **F** = *Rhizopus chinensis* MJ:Rh3; **G** = *Rhizopus stolonifer* var *lycoccus* MKo:Rh1; **H**= *Lactobacillus bifermentans* MA:R5; **I** = *Pediococcus pentosaceus* MA:C1

Data represent the means of 2 replicate sets.

Table 12. Amylolytic activities of selected strains isolated from marcha

Strain	∝-amylase (U/ml)	Glucoamylase (U/ml)
Rhizopus stolonifer var *lycococcus* MKo:Rh1	5.3	71.3
Rhizopus stolonifer var *lycococcus* MA:Rh3	2.8	59.3
Rhizopus chinensis MT:Rh1	0.7	67.9
Rhizopus chinensis MJ:Rh3	1.5	96.3
Rhizopus chinensis MK:Rh1	0.4	87.4
Mucor sp (close to *M. hiemalis*) MJ:Mu1	0.7	37.0
Mucor circinelloides ML:Mu1	0.4	16.3
Mucor circinelloides MS:Mu1	1.8	15.4
Mucor sp (close to *M. hiemalis*) MC:Mu1	1.6	8.9
Mucor circinelloides MM:Mu2	1.2	11.7
Mucor circinelloides MA:Mu7	1.6	24.0
Saccharomycopsis fibuligera MK:YD1	4.5	40.2
Saccharomycopsis fibuligera MKo:YD2	4.2	47.0
Saccharomycopsis fibuligera MA:YD4	4.0	81.5
Saccharomycopsis fibuligera MS:YD4	6.6	43.1
Saccharomycopsis fibuligera MC:YD3	5.6	63.3
Saccharomycopsis fibuligera MA:YD5	5.3	46.0
Saccharomycopsis fibuligera MN:YD1	4.8	62.8
Pichia anomala MC:YP3	1.4	45.7
Pichia anomala MN:YP1	2.4	30.8
Pichia anomala MA:YP2	4.3	23.1
Pichia anomala MA:YP3	2.0	37.0
Pichia anomala MA:YP8	2.2	24.5
Saccharomyces cerevisiae MJ:YS1	4.1	18.8
Saccharomyces cerevisiae MJ:YS2	5.6	24.5
Saccharomyces cerevisiae MS:YS2	4.6	24.5
Saccharomyces cerevisiae MA:YS3	1.2	9.1
Saccharomyces cerevisiae MA:YS7	0.8	11.7
Candida glabrata MS:YC2	1.2	19.4
Candida glabrata MS:YC4	1.6	27.1
Candida glabrata MS:YC5	1.7	35.8
Candida glabrata MS:YC6	1.3	3.1

4.3. KODO KO JAANR

Kodo ko jaanr is the most common fermented beverage prepared from dry seeds of finger millet [*Eleusine coracana* (L) Gaertn.], locally called 'kodo' in the Darjeeling hills and Sikkim. Finger millet is sown in June and is harvested in December (Plate 5). Some indigenous local varieties of finger millet of these regions are 'mudke', 'nangrey', 'fyakre', 'nangkatwa', etc. Hybrid varieties of finger millet such as PR-202, HR-374 and VL- 101 have also been introduced to these regions. Finger millets, grown in these regions, are mostly utilized for preparation of alcoholic beverages. Non-fermented dough of grounded millet is also consumed as baked bread commonly called kodo ko roti, which is a traditional food.

4.3.1. Synonym of kodo ko jaanr

Jaanr is common name for all alcoholic beverages in Nepali. Different ethnic groups call it by their own dialect such as *mandokpenaa thee* by Limboo, *sampicha ummaak* by Rai, *naarr paa* by Gurung, *saangla chi* by Tamang, *chirs shyaabu* by Sunwar, *paadaare haan* by Magar, *gyaar chyyaang* by Sherpa, *minchaa chhyaang* by Bhutia, and *mong chee* by Lepcha.

4.3.2. Traditional method of preparation

During traditional method of kodo ko jaanr preparation, seeds of finger millet are cleaned, washed and cooked for about 30 min in an open cooker. Excess water is drained off and spread on a mat made up of bamboo locally called mandro for cooling. Powdered marcha (1 to 2 %) is sprinkled over

cooked seeds (Plate 6), mixed thoroughly and packed in a bamboo basket lined with fresh fern, locally called 'thadre unioon' *(Thelypteris erubescens* Well ex Hook.) or banana leaves, then covered with sack clothes, and kept for 2-4 days at room temperature for saccharification. During saccharification sweet aroma is emitted out and the saccharified mass is transferred into an earthen pot or into specially made bamboo basked called 'septu' and made it air-tight and fermented for 3-4 days during summer and 5-7 days in winter at room temperature (Fig 3).

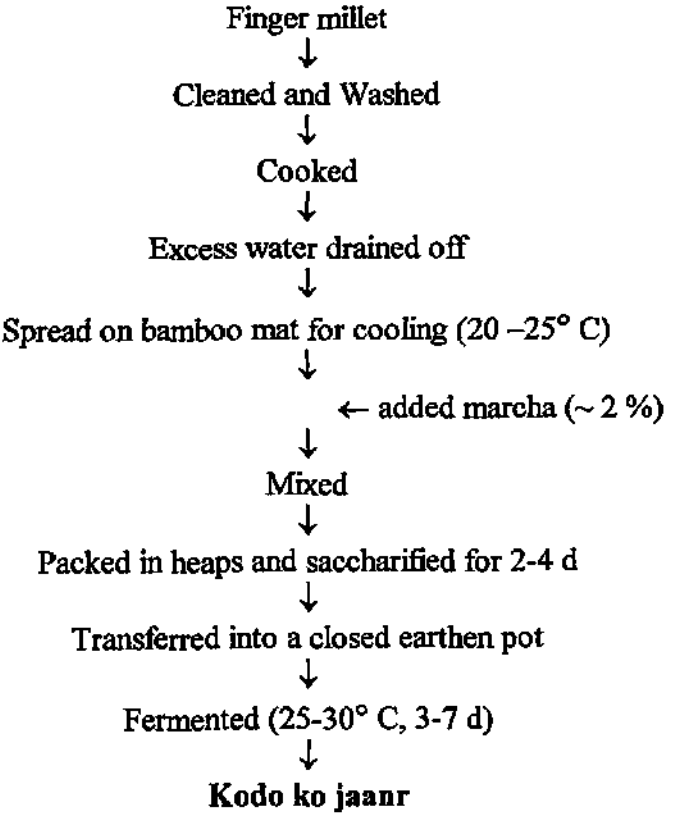


Fig 3. Flow sheet of kodo ko jaanr preparation in East Sikkim

Good quality of jaanr has sweet taste with mild alcoholic flavour (Plate 7). Prolonged fermentation makes the product bitter in taste and more alcoholic. Sour taste and unpleasant flavour of jaanr is unacceptable to consumers.

4.3.3. Mode of consumption

Kodo ko jaanr is consumed in an unique way in the Himalayan regions. About 200-500 g of kodo ko jaanr is put into a vessel called toongbaa (Plate 8 & 9) and lukewarm water is added up to the edge of the toongbaa. After 10-15 min, milky white extract of kodo ko jaanr is sipped through a narrow bamboo straw called pipsing having a hole in a side near the bottom to avoid passing of grits (Plate 10). Water can be added 2-3 times after sipping up the extract. Guests are served with toongbaa along with fried meat or pickles. Alternately, thick milky white liquid pressed from the kodo ko jaanr is filtered using a filter called chhapani under pressure. Such liquor is believed to be good tonic for ailing persons and post-natal women. After consumption, grits of kodo ko jaanr are used as fodder for pigs and cattle. This is a good example of total utilization of substrate as food and fodder.

Feeding frequency of kodo ko jaanr has been summarised in Table 13. About 70 % of people consume kodo ko jaanr daily in rural areas of the Sikkim Himalayas. Per capita daily consumption of kodo ko jaanr extract in the Darjeeling hills and Sikkim is 5.1 ml and 6.5 ml, respectively.

Plate 5. Finger millets "kodo" field in West Sikkim

Plate 6. Kodo ko jaanr preparation by the Bhutia woman. Cooked millets are placed on *mandro* (bamboo-made mat) and powdered marcha is added.

Plate 7. Finger millets seeds and kodo ko jaanr

Plate 8. Fermented grits of finger millets are filled in *toongbaa.*

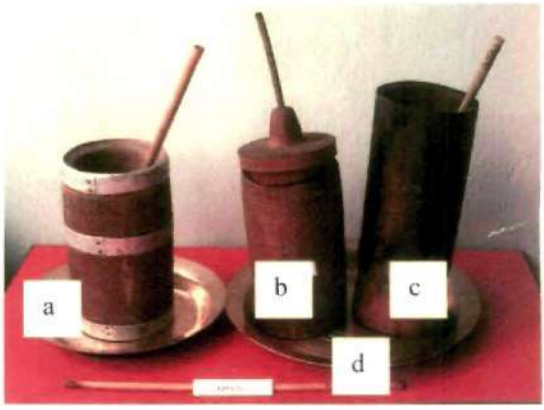


Plate 9. *Toongbaa*: (a) silver-lined wooden *toongbaa*; (b) with lid; (c) bamboo-made *toongbaa*; (d) straw called *pipsing*

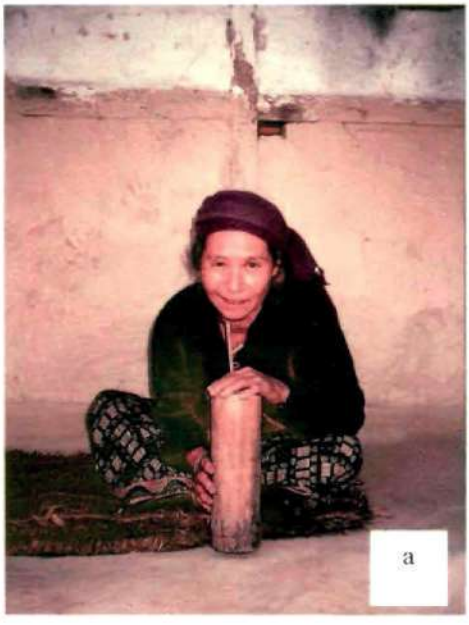


Plate 10. (a) Kodo ko jaanr extract is sipped through the *pipsing* (a) by Rai woman in bamboo-made *toongbaa* and (b) by Lepcha man in wood-made *toongbaa* in Sikkim.

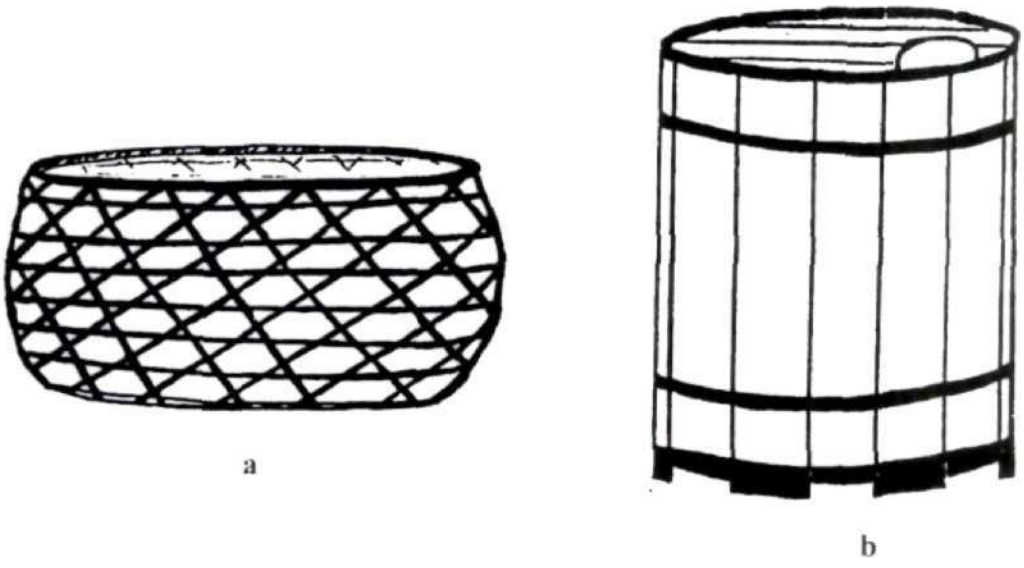


Plate 11. (a) Bamboo-made *septu* and (b) wood-made *septu*

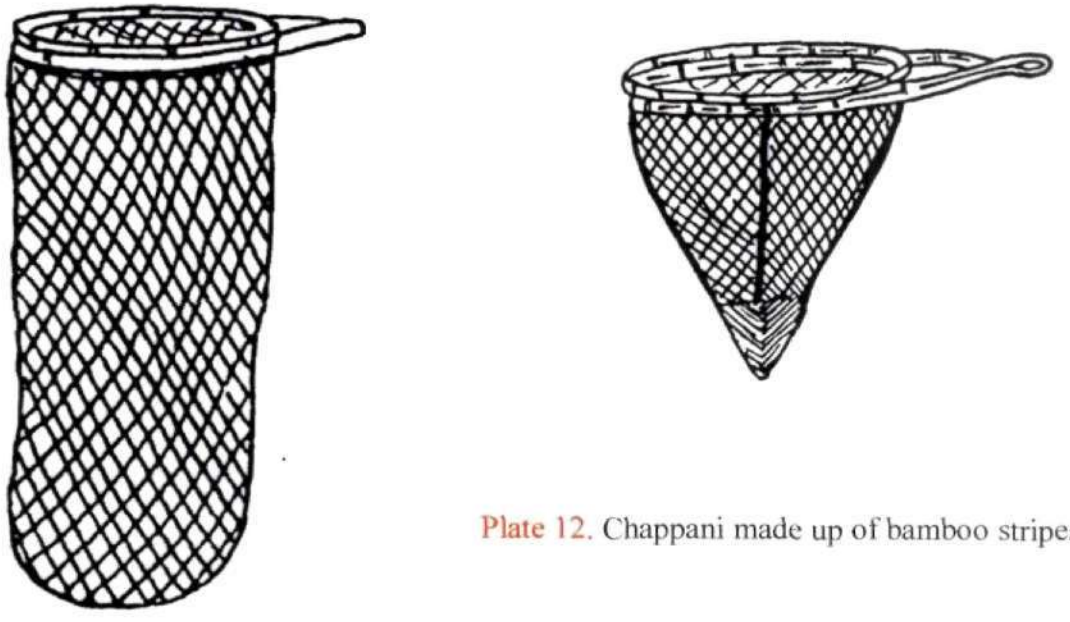


Plate 12. Chappani made up of bamboo stripes

bamboo straw called *pipsing*. *Toongbaa* is made up of wood or bamboo or sometime earthenware (Plate 9 a-c). Usually wooden toongbaa is decorated with silver lining and is provided with a lid.

Pipsing: It is a narrow straw, made up of bamboo having a hole in opposite sites near the bottom to avoid passing of grits during sipping of jaanr from *toongbaa* (Plate 9 d).

4.3.5. Ethnical Importance

In the Sikkim Himalayan regions social activities require provision and consumption of appreciable quantities of alcoholic beverages by the 'matwali' castes meaning alcohol drinkers of the non-Brahmin Nepali ethnic community mostly Limboo, Rai, Gurung, Magar, Tamang, Sunwar, Newar and Sherpa; the Bhutia and the Lepcha communities. Ethnical importance of fermented beverages was documented during survey and noted in discussion chapter.

4.3.6. Microorganisms

Forty samples of kodo ko jaanr, collected from different places of the Darjeeling hills and Sikkim, were analysed for microbial load (Table 14). Average load of yeasts and total viable counts was detected at the level of 10^7 cfu/g whereas the population of lactic acid bacteria was comparatively less (~10^5 cfu/g). Filamentous mould was not recovered in finish product. Out of 161 strains of microorganisms isolated, 81 isolates were yeasts and 80 isolates were lactic acid bacteria.

Table 14. Microbial load of kodo ko jaanr samples

Source	$\times 10^7$cfu/g fresh weight		
	Yeast	LAB	Total Viable Count
Rongli	1.1 (0.2-2.1)	0.03 (0.001-0.1)	1.1 (0.7-1.5)
Namchi	1.8 (1.7-1.9)	0.03 (0.02-0.04)	3.7 (1.5-5.8)
Aho	1.7 (1.0-2.5)	0.1 (0.05-0.2)	3.0 (1.5-4.5)
Kalimpong	1.0 (0.8-1.2)	0.2 (0.1-0.3)	1.4 (1.0-2.2)

Data represent the means of 10 samples from each source. Ranges are given in parentheses.

4.3.6.1. Characterisation and identification of yeasts

Representative strains of yeasts were selected on the basis of colony, cell morphology, vegetative reproduction and type of ascospore and were grouped into three genera (Table 15). These representative strains of yeasts were identified as *Pichia anomala* (E.C. Hansen) Kurtzman, *Saccharomyces cerevisiae* Meyen ex Hansen and *Candida glabrata* (Anderson) Meyer et Yarrow (Table 16). *Saccharomycopsis fibuligera* was not recovered in kodo ko jaanr samples. *Pichia anomala* and *Saccharomyces cerevisiae* were present in all samples showing 100 % prevalence whereas *Candida glabrata* showed only 40 % prevalence in forty samples of kodo ko jaanr analysed.

Table 6. Selection of representative strains of yeasts isolated from kodo ko jaanr samples[a]

Source	Number of strains isolated	Colony	Cell Shape	Mycelium	Ascospore	Grouped strains	Representative strains
Rongli	16	Ss	O-E	Pseudo	Hat-shaped	8	KR:YP2
		Ss	O-E	Pseudo	Globose	8	KR:YS1
Namchi	20	Ss	O-E	Pseudo	Hat-shaped	10	KN:YP3
		Ss	O-E	Pseudo	Globose	10	KN:YP4
Aho	25	Ss	O-E	Pseudo	Hat-shaped	8	KA:YP1
		Ss	O-E	Pseudo	Globose	10	KA:YS3
		Fs	O-E	–	–	7	KA:YC4
Kalimpong	20	Ss	O-E	Pseudo	Hat-shaped	10	KK:YP1
		Ss	O-E	Pseudo	Globose	6	KK:YS1
		Fs	O-E	–	–	4	KK:YC1

[a]Number of samples was 10 from each source. All isolates reproduced by multilateral budding.

Ss, smooth surface; Fs, fringed surface; O-E, Oval to ellipsoidal.

Table 16. Characteristics of representative strains of yeasts isolated from kodo ko jaanr

Parameter	KR:YP2	KN:YP3	KA:YS3	KA:YS1	KA:YC4
Cell width (µm)	1.1-3.2	1.3-3.2	1.6-3.2	1.6-4.0	0.8-2.5
Cell length (µm)	1.6-3.8	1.9-5.0	1.6-5.3	1.9-4.8	1.6-3.8
Nitrate reduction	+	+	–	–	–
Growth at 37°C	+	+	+	+	+
Sugar fermentation:					
Glucose	+	+	+	+	+
Galactose	–	–	+	+	–
Lactose	–	–	–	–	–
Maltose	+	+	+	+	–
Raffinose	+	+	+	+	–
Sucrose	+	+	+	+	–
Starch	–	–	+	+	–
Trehalose	+	+	–	–	+
Sugar assimilation:					
Arabinose	+	$+_w$	–	–	–
Cellobiose	+	+	–	–	–
Galactose	+	+	+	+	–
Glycerol	–	–	–	–	$+_w$
Inositol	–	–	–	–	–
Lactose	–	–	–	–	–
Maltose	+	+	+	+	–
Melibiose	–	–	+	+	–
Mannitol	+	+	–	–	–
Rhamnose	–	–	–	–	–
Raffinose	+	+	+	+	–
Sucrose	+	+	+	+	–
Starch	+	+	+	+	–
Trehalose	+	+	+	+	+
Xylose	–	+	–	–	–

Identification	*Pichia*	*Saccharomyces*	*Candida*

4.3.6.2. Characterisation and identification of bacteria

Out of 80 strains of lactic acid bacteria, isolated from forty samples of kodo ko jaanr, 44 strains were coccii-shaped cells in tetrads and 36 strains were non-sporeforming rods (Table 17). Species of lactic acid bacteria were identified as *Pediococcus pentosaceus* Mees and *Lactobacillus bifermentans* Kandler, Schillinger and Weiss (Table 18). Prevalence of both of them was 100 % in finish products.

Table 17. Selection of representative strains of LAB isolated from kodo ko jaanr[a]

Source	Number of strains isolated	Cell Shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Rongli	18	Coccus	–	+	10	KR:C1
		Rod	+	–	8	KR:R2
Namchi	20	Coccus	–	+	12	KN:C2
		Rod	+	–	8	KN:R3
Aho	20	Coccus	–	+	10	KA:C1
		Rod	+	–	10	KA:R1
Kalimpong	22	Coccus	–	+	12	KK:C1
		Rod	+	–	10	KK:R1

[a]Number of samples was 10 from each source

[b]All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile

Table 18a. Phenotypic characters of representative strains of LAB isolated from kodo ko jaanr

Parameter	KR:C1	KN:C2	KA:C1	KK:C1	KR:R2	KN:R3	KA:R1	KK:R1
Cell shape	Ct	Ct	Ct	Ct	R	R	R	R
Cell diameter (μm)	0.2-0.5	0.2-0.6	0.2-0.5	0.4-0.7				
Cell width (μm)					0.2-0.3	0.2-0.4	0.2-0.3	0.2-0.3
Cell length (μm)					0.8-2.3	0.8-2.2	1.0-2.2	1.0-2.3
Anaerobic growth	+	+	+	+	+	+	+	+
Hydrolysis of:								
Casein	–	–	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–	–	–
Arginine	+	+	+	+	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–	–	–
Growth in NaCl:								
4.0 %	+	+	+	+	+	+	+	+
6.5 %	+	+	+	+	+	+	+	+
10.0 %	+	+	+	+	+	+	+	+
18.0 %	–	–	–	–	–	–	–	–
Growth in pH:								
4.2	+	+	+	+	+	+	+	+
7.5	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+
Growth at:								
15° C	+	+	+	+	+	+	+	+
45° C	–	–	–	–	–	–	–	–

Ct, coccii-tetrad; R, rod in chain with 2-3 cells

Table 18b. Sugar fermentation of LAB strains using API 50 CHL system

Parameter	KR:C1	KN:C2	KA:C1	KK:C1	KR:R2	KN:R3	KA:R1	KK:R1
Glycerol	+	+	–	–	–	–	+	–
Erythritol	+	+	–	–	–	–	–	–
D-Arabinose	+	+	–	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+
L-Xylose	–	–	–	–	–	–	–	$+_w$
Adonitol	+	+	–	–	–	–	+	–
β-Methyl-D-Xyloside	–	–	–	–	–	–	$+_w$	–
Galactose	+	+	+	+	+	$+_w$	–	$+_w$
D-Glucose	–	–	+	+	+	–	+	+
D-Fructose	–	–	+	+	+	+	+	+
D-Mannose	–	$+_w$	+	+	–	–	+	–
L-Sorbose	$+_w$	–	–	–	–	–	–	–
Rhamnose	+	+	–	–	–	–	+	–
Dulcitol	+	+	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	$+_w$
Sorbitol	+	+	–	–	–	–	–	–
α-Methyl-D-Mannoside	+	+	–	–	–	$+_w$	+	–
α-Methyl-D-Glucoside	–	–	–	–	$+_w$	$+_w$	+	+
N-Acetyl-Glucosamine	+	+	+	+	$+_w$	–	+	+
Amygdalin	+	+	+	+	–	–	–	–
Arbutin	–	–	–	–	–	–	–	–
Esculin	–	–	–	–	–	–	–	–
Salicin	$+_w$	–	$+_w$	–	–	–	–	–
Cellobiose	+	+	+	+	–	–	–	–
Maltose	+	+	+	+	+	+	+	$+_w$

Parameter	KR:C1	KN:C2	KA:C1	KK:C1	KR:R2	KN:R3	KA:R1	KK:R1
Lactose	–	–	–	+	–	–	–	–
Melibiose	–	–	–	–	$+_w$	$+_w$	+	$+_w$
Sucrose	–	–	–	–	–	–	–	–
Trehalose	+	+	+	+	–	–	–	–
Inulin	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–
Gentiobiose	+	+	+	+	–	–	–	–
D-Turanose	–	–	–	–	–	–	–	–
D-Lyxose	–	–	–	–	–	–	–	–
D-Tagatose	+	+	+	+	–	–	–	–
D-Fucose	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	–	–	–	–
L-Arabitol	–	–	–	–	–	–	–	–
Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
2-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
5-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	$+_w$
Identification	*Pediococcus*				*Lactobacillus*			

4.3.7. Proximate composition

Proximate composition of finger millet and kodo ko jaanr is presented in (Table 19). Average pH, acidity and alcohol content of the product was 4.1, 0.27 % and 4.8 %, respectively.

Table 19. Proximate composition of unfermented and fermented finger-millet

Parameter	Unfermented	Fermented (Kodo ko jaanr)			
	Cooked finger-millet	Rongli	Namchi	Aho	Kalimpong
pH	6.4 (6.3-6.5)	3.9 (3.7-4.1)	4.3 (3.8-4.4)	3.9 (3.5-4.3)	4.2 (3.8-4.5)
Moisture (%)	66.0 (64.0-72.0)	68.8 (64.5-72.8)	65.7 (62.8-78.2)	71.7 (62.4-78.2)	72.5 (67.2-79.9)
Acidity (%)	0.01 (0.01-0.01)	0.26 (0.23-0.31)	0.25 (0.21-0.29)	0.33 (0.25-0.50)	0.25 (0.20-0.34)
Alcohol (%)	0.1 (0.05-0.1)	2.4 (1.8-3.7)	7.1 (3.2-8.7)	5.2 (3.5-7.0)	4.5 (3.0-6.6)
Ash (% DM)	4.9 (4.5-5.5)	5.8 (4.8-7.1)	4.1 (3.5-5.3)	4.6 (4.2-6.0)	5.8 (4.8-6.0)
Fat (% DM)	2.4 (1.7-2.9)	2.0 (1.8-2.5)	2.0 (1.6-2.6)	1.8 (1.6-2.2)	2.1 (1.7-2.9)
Protein (% DM)	10.0 (9.5-11.0)	9.2 (8.2-10.5)	9.5 (8.5-10.8)	8.5 (8.2-10.5)	9.8 (8.3-11.0)
Crude fibre (% DM)	6.7 (5.8-7.0)	7.7 (6.8-8.7)	ND	ND	10.9 (10.6-11.3)
Carbohydrate (% DM)	82.7 (80.6-84.3)	83.0 (79.9-85.2)	84.4 (81.3-86.4)	85.1 (81.3-86.0)	82.3 (80.1-85.2)
Energy (Kcal/100g DM)	392.4 (375.7-407.3)	386.8 (368.6-405.3)	393.6 (373.6-412.2)	390.6 (372.4-405.8)	387.3 (368.9-410.9)

Data represent the means of 5 samples from each source. % DM, percentage on dry matter basis. Ranges are given in parentheses. ND, not determined.

Moisture content of the product was slightly higher in fermented product than unfermented cooked millet. No remarkable change was observed in ash, fat and protein contents of kodo ko jaanr over the substrate. Crude fibre content increased during fermentation in kodo ko jaanr. Calorie content of the unfermented millet and fermented product was almost same. Remarkable increase in mineral contents such as calcium, magnesium, manganese, iron, potassium and phosphorous was observed in jaanr (Table 20).

Table 20. Mineral contents of raw and fermented finger millets

Mineral	**mg/100 g dry matter**	
	Finger millet	**Kodo ko jaanr**
Calcium	206	281
Magnesium	76	118
Manganese	3.6	9.0
Copper	0.8	2.2
Iron	8.7	24
Zinc	1.0	1.2
Sodium	28	39
Potassium	252	398
Phosphorus	228	326

Data represent the means of 2 samples.

4.3.8. Successional studies during kodo ko jaanr fermentation

Kodo ko jaanr was prepared in the laboratory following the traditional method by using marcha, collected from Aho village, as mentioned in 3.3.6. Successional studies were carried at every 1 day interval within a range of 0-10 days.

4.3.8.1. Microbial changes

Table 21 shows the changes in microbial population in fermenting finger millet seeds during kodo ko jaanr fermentation. Mould population, which was originated from marcha, declined significantly ($P<0.05$) every day during fermentation and finally disappeared after 5 d (Fig 4). Population of yeasts increased significantly ($P<0.05$) from 10^5 cfu/g to 10^7 cfu/g within 2 d, and remained relatively constant at the same level till the end of fermentation. Subsequently, load of lactic acid bacteria increased significantly ($P<0.05$) from 10^6 cfu/g to 10^8 cfu/g in first day and decreased significantly ($P<0.05$) to a level of 10^3 cfu/g at the end. Total viable counts increased significantly ($P<0.05$) within first day and decreased in every interval of 1 d.

Table 21. Microbial changes during kodo ko jaanr fermentation

Fermentation time (days)	Log cfu/g			
	Mould	Yeast	LAB	Total Count
0	4.2 ± 0.29^{a}	5.2 ± 0.21^{e}	6.0 ± 0.16^{ef}	6.2 ± 0.05^{g}
1	3.1 ± 0.46^{b}	7.5 ± 0.08^{b}	8.2 ± 0.13^{a}	8.3 ± 0.16^{a}
2	2.4 ± 0.49^{c}	7.8 ± 0.13^{a}	7.9 ± 0.13^{b}	8.1 ± 0.08^{b}
3	1.8 ± 0.21^{d}	7.8 ± 0.13^{a}	7.9 ± 0.08^{b}	8.3 ± 0.08^{a}
4	< DL	7.8 ± 0.08^{a}	6.5 ± 0.21^{c}	7.9 ± 0.08^{c}
5	0	7.6 ± 0.08^{b}	6.3 ± 0.25^{d}	7.7 ± 0.08^{d}
6	0	7.6 ± 0.16^{b}	6.0 ± 0.16^{ef}	7.7 ± 0.08^{d}
7	0	7.3 ± 0.08^{bcd}	5.9 ± 0.17^{f}	7.3 ± 0.08^{e}
8	0	7.4 ± 0.13^{bc}	5.2 ± 0.08^{g}	7.4 ± 0.08^{e}
9	0	7.2 ± 0.08^{d}	4.7 ± 0.25^{h}	7.2 ± 0.08^{f}
10	0	7.3 ± 0.08^{bcd}	3.8 ± 0.33^{i}	7.3 ± 0.09^{e}

Data represent the means ± SD of three batches of fermentation. Data were transformed into logarithmic values. DL, detection limit (10 cfu/g).

Values bearing different superscripts in each column differ significantly ($P<0.05$).

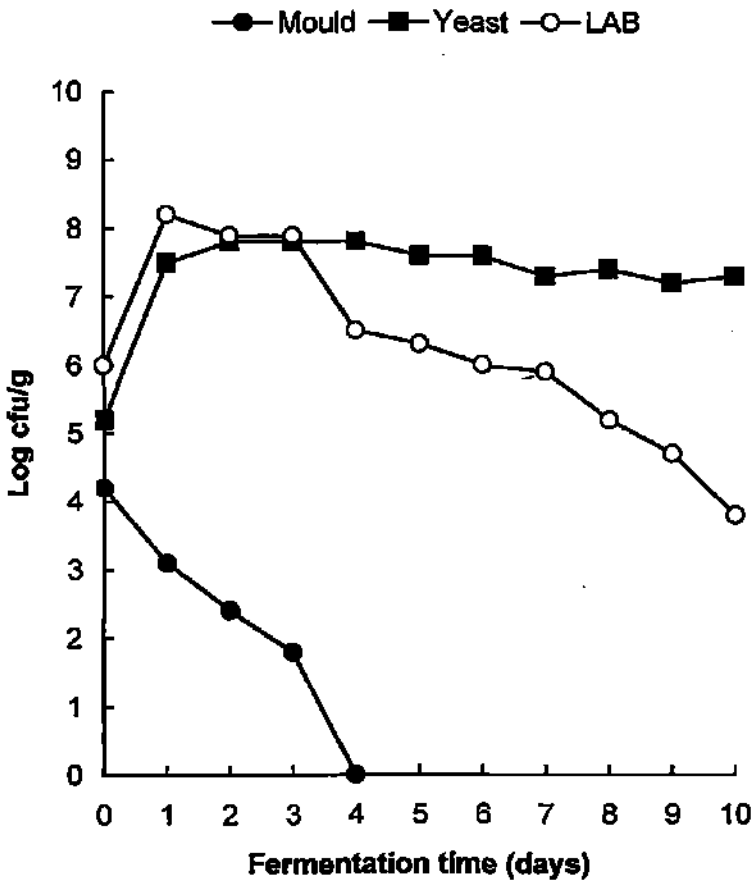


Fig 4. Changes in microbial load during kodo ko jaanr fermentation. Values are the means of three batches of fermentation. LAB, lactic acid bacteria.

4.3.8.2. Physico-chemical and enzymatic changes

Temperature of fermenting finger millet increased significantly ($P<0.05$) from 26° C to 30° C within 2 d and decreased to 28° C till the end during kodo ko jaanr fermentation (Table 22). The mean pH value decreased significantly ($P<0.05$) from 6.37 to 4.10 within 2 d of fermentation and after 2 d, decline in pH was non-significant. Titratable acidity increased significantly ($P<0.05$) from 0 d to 4 d, and remained the same till the end (Fig 5). Alcohol content increased significantly ($P<0.05$) from 0.1 % to 6.9 % within 6 d and slightly decreased to 6.5 % on 10 d.

Table 22. Physico-chemical changes during kodo ko jaanr fermentation

Fermentation time (days)	Temperature (°C)	pH	Acidity (%)	Alcohol (%)
0	26.0 ± 0.00^{f}	6.37 ± 0.01^{a}	0.01 ± 0.00^{e}	0.1 ± 0.11^{h}
1	28.8 ± 0.05^{c}	4.44 ± 0.01^{b}	0.08 ± 0.01^{d}	0.5 ± 0.08^{g}
2	30.0 ± 0.13^{a}	4.10 ± 0.01^{e}	0.14 ± 0.01^{c}	2.7 ± 0.08^{f}
3	29.5 ± 0.00^{b}	4.07 ± 0.02^{e}	0.18 ± 0.01^{b}	3.1 ± 0.08^{e}
4	29.0 ± 0.00^{c}	4.07 ± 0.01^{e}	0.24 ± 0.01^{a}	4.1 ± 0.08^{d}
5	29.0 ± 0.05^{c}	4.08 ± 0.01^{e}	0.23 ± 0.00^{a}	5.5 ± 0.08^{c}
6	28.8 ± 0.21^{c}	4.08 ± 0.01^{e}	0.23 ± 0.01^{a}	6.9 ± 0.21^{a}
7	28.3 ± 0.08^{d}	4.07 ± 0.01^{e}	0.22 ± 0.01^{a}	6.8 ± 0.13^{a}
8	28.0 ± 0.00^{e}	4.07 ± 0.02^{e}	0.23 ± 0.01^{a}	6.8 ± 0.08^{a}
9	28.0 ± 0.00^{e}	4.07 ± 0.01^{e}	0.22 ± 0.01^{a}	6.6 ± 0.08^{b}
10	28.0 ± 0.00^{e}	4.07 ± 0.01^{e}	0.23 ± 0.01^{a}	6.5 ± 0.08^{b}

Data represent the means ± SD of three batches of fermentation. Values bearing different superscripts in each column differ significantly ($P<0.05$).

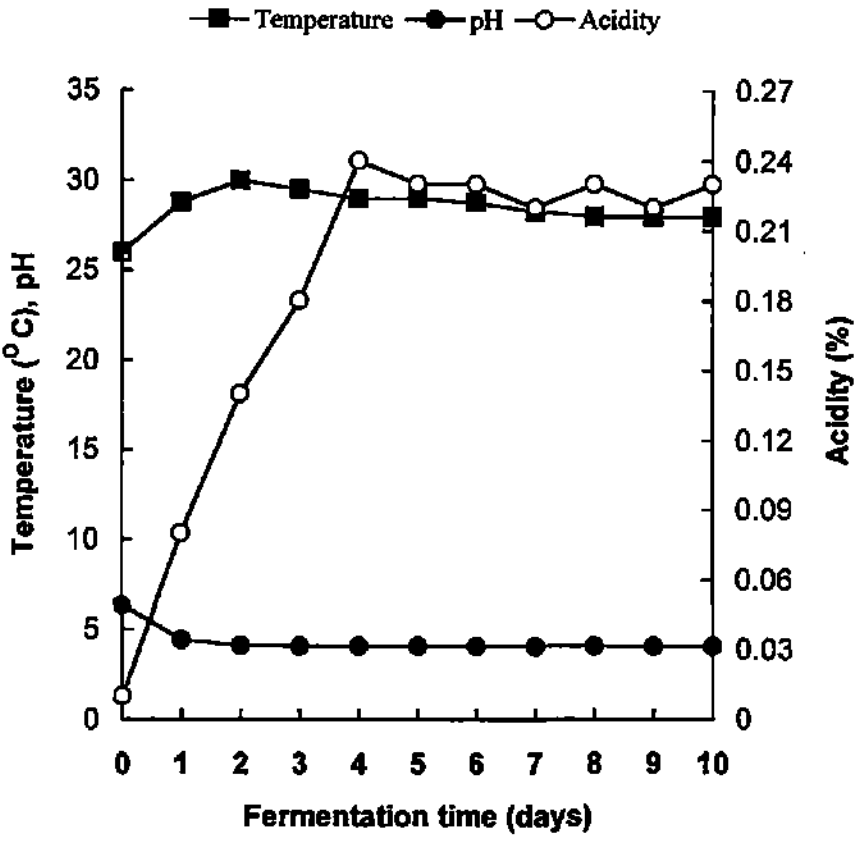


Fig 5. Changes in temperature, pH and acidity during kodo ko jaanr fermentation. Values are the means of three batches of fermentation.

Reducing sugar content was increased significantly ($P<0.05$) up to third day of fermentation and decreased significantly ($P<0.05$) everyday till the end (Table 23). Total sugar contents of fermenting finger millet decreased significantly ($P<0.05$) throughout the fermentation (Fig 6). Maximum activities of α–amylase and glucoamylase was observed on second day of fermentation and decreased significantly ($P<0.05$) till the end (Table 23 and Fig 7).

Table 23. Biochemical and enzymatic changes during kodo ko jaanr fermentation

Fermentation time (days)	Reducing sugar (%)	Total sugar (%)	∝-amylase (U/g)	Glucoamylase (U/mg)
0	0.4 ± 0.16^{k}	85.9 ± 2.45^{a}	6.0 ± 0.16^{h}	33.2 ± 3.70^{f}
1	4.0 ± 0.08^{d}	72.9 ± 3.47^{b}	15.4 ± 0.50^{ef}	121.1 ± 2.11^{c}
2	4.6 ± 0.08^{c}	61.4 ± 1.18^{c}	36.0 ± 1.63^{a}	163.2 ± 4.91^{a}
3	7.0 ± 0.16^{a}	53.6 ± 0.57^{d}	27.1 ± 0.90^{b}	153.9 ± 6.97^{ab}
4	4.8 ± 0.16^{b}	44.3 ± 2.33^{e}	25.6 ± 1.14^{c}	147.8 ± 0.73^{b}
5	3.4 ± 0.08^{e}	40.0 ± 1.72^{f}	21.0 ± 1.30^{d}	147.0 ± 7.30^{b}
6	3.2 ± 0.16^{h}	38.2 ± 1.18^{f}	19.8 ± 1.03^{d}	145.7 ± 4.99^{b}
7	3.0 ± 0.08^{f}	34.2 ± 0.90^{g}	15.8 ± 0.82^{e}	146.5 ± 8.00^{b}
8	2.8 ± 0.08^{g}	31.6 ± 0.61^{gh}	14.3 ± 0.78^{f}	98.5 ± 9.68^{d}
9	1.8 ± 0.16^{i}	30.9 ± 0.57^{hi}	9.1 ± 0.82^{g}	85.1 ± 8.10^{e}
10	1.0 ± 0.08^{j}	28.7 ± 0.61^{i}	8.5 ± 0.41^{g}	34.1 ± 3.31^{f}

Data represent the means ± SD of three batches of fermentation.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

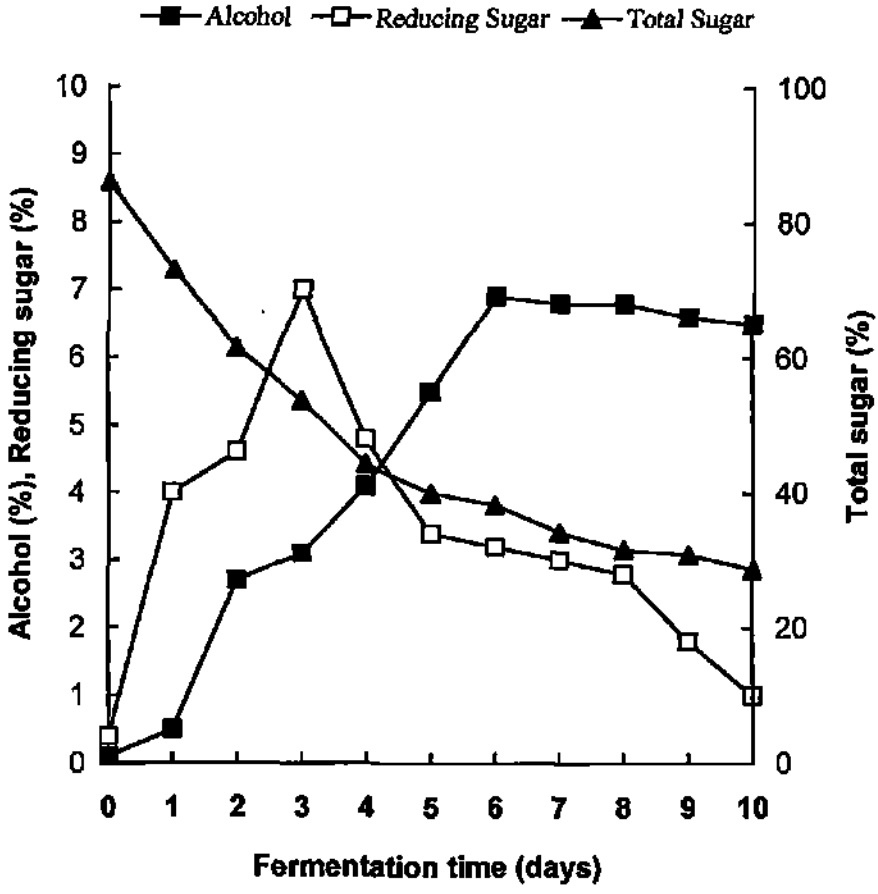


Fig 6. Changes in alcohol, reducing sugar and total sugar contents of finger millet during kodo ko jaanr fermentation. Values are the means of three batches of fermentation.

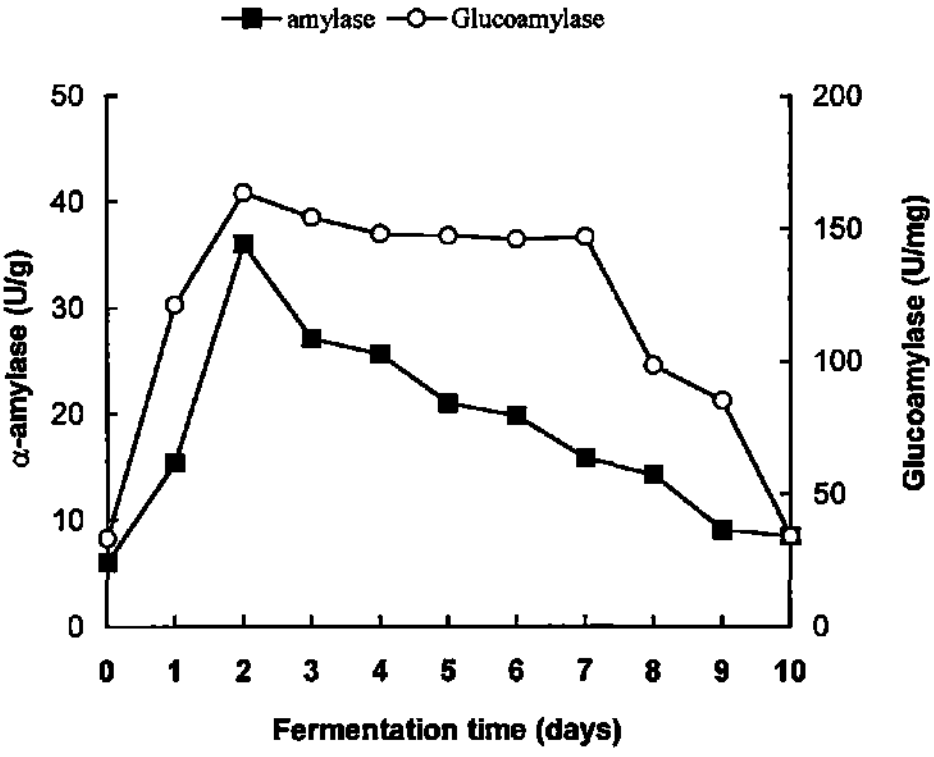


Fig 7. Changes in enzymatic activities in finger millet during kodo ko jaanr fermentation. Values are the means of three batches of fermentation.

4.3.9. Testing of isolates for producing kodo ko jaanr

Sterilised finger millet seeds were allowed to ferment with different combinations of *Rhizopus chinensis* MJ:Rh3 and *Saccharomycopsis fibuligera* MS:YD4, since both of them showed highest glucoamylase and ∝-amylase activities, respectively (Table 12) with other strains of mould and yeast: *Mucor* sp. (close to *M. hiemalis*) MJ:Mu1, *Saccharomyces cerevisiae* MJ:YS2, *Candida glabrata* MS:YC5, *Pichia anomola* MN:YP1, selected on the basis of high amylolytic activities; randomly selected LAB strains *Pediococcus pentosaceus* MA:B2 and *Lactobacillus bifermentans* MA:B5 for testing ability to produce kodo ko jaanr. Table 24 shows changes in pH, reducing sugar and alcohol production by each combination of strain during kodo ko jaanr fermentation. Jaanr prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomycopsis fibuligera* MS:YD4 showed significantly ($P<0.05$) high reducing sugar contents during saccharification period of 2 d with low alcohol content of 1 % on 6 d. Cell suspension mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 produced jaanr with significantly ($P<0.05$) high reducing sugar and high alcohol content of 4.4 % in 6 d than jaanr samples fermented by other strains.

Table 25 shows the sensory evaluation of kodo ko jaanr produced by selected combination of strains. There was no significant ($P<0.05$) difference in aroma attribute of jaanr prepared by a cell suspension mixture of *Rhizopus chinensis* MJ:Rh3 with other strains, except jaanr prepared by a combination of *Saccharomycopsis fibuligera* MS:YD4 with *Saccharomyces cerevisiae* MJ:YS2, *Candida glabrata* MS:YC5, LAB strains and all

strains. There was significant (P<0.05) difference in taste score of jaanr prepared by cell mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 with that of other strains. However, significance (P<0.05) difference in texture and colour scores was observed in some jaanr samples. Jaanr product prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomycopsis fibuligera* MS:YD4 had desirable sweet-sour taste but unpleasant odour due to low alcohol content. Hence based on sensory criteria, jaanr produced by these strains were unacceptable to consumers. Kodo ko jaanr prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 showed significantly (P<0.05) highest score in general acceptability. Kodo ko jaanr prepared by these strains had mild alcoholic-sweet flavour, significantly (P<0.05) acceptable to judges.

Table 24. Changes in pH, reducing sugar contents and alcohol production in fermented finger millet by selected strains

	pH		Reducing sugar (%)		Alcohol (%)	
Cooked millet (unfermented finger millet)	6.37 ± 0.01		0.4 ± 0.16		0.1 ± 0.06	
Strain	**2 days**	**6 days**	**2 days**	**6 days**	**2 days**	**6 days**
Rhizopus chinensis MJ:Rh3 **with**						
Mc	4.62 ± 0.02^{e}	4.82 ± 0.01^{e}	2.10 ± 0.19^{ef}	3.10 ± 0.24^{b}	0.25 ± 0.06^{f}	0.83 ± 0.06^{f}
Sc	4.31 ± 0.01^{f}	4.36 ± 0.01^{h}	4.21 ± 0.05^{c}	3.57 ± 0.05^{a}	2.50 ± 0.07^{a}	4.40 ± 0.13^{a}
Cg	4.32 ± 0.01^{f}	4.30 ± 0.00^{i}	3.95 ± 0.11^{c}	2.00 ± 0.19^{ef}	0.70 ± 0.06^{d}	1.80 ± 0.00^{d}
Pa	4.60 ± 0.00^{e}	4.70 ± 0.01^{f}	2.49 ± 0.08^{def}	3.13 ± 0.45^{b}	0.76 ± 0.00^{c}	2.20 ± 0.06^{c}
Lb + Pp	4.12 ± 0.01^{h}	4.16 ± 0.01^{j}	2.71 ± 0.46^{d}	2.89 ± 0.16^{bc}	1.00 ± 0.06^{b}	2.50 ± 0.06^{b}
Saccharomycopsis fibuligera MS: YD4 **with**						
Rc	4.20 ± 0.02^{g}	4.56 ± 0.02^{g}	6.28 ± 0.14^{a}	2.55 ± 0.05^{cd}	0.40 ± 0.07^{e}	1.00 ± 0.06^{e}
Mc	6.02 ± 0.01^{c}	6.10 ± 0.02^{bc}	4.94 ± 0.04^{b}	2.22 ± 0.05^{de}	0.15 ± 0.00^{g}	0.30 ± 0.00^{i}
Sc	6.08 ± 0.02^{b}	6.13 ± 0.01^{b}	4.74 ± 0.75^{bc}	2.63 ± 0.11^{c}	0.22 ± 0.06^{f}	0.50 ± 0.06^{hi}
Cg	5.95 ± 0.00^{d}	6.00 ± 0.04^{d}	3.07 ± 0.20^{d}	1.65 ± 0.01^{f}	0.15 ± 0.00^{g}	0.45 ± 0.00^{i}
Pa	6.10 ± 0.00^{b}	6.08 ± 0.00^{c}	2.61 ± 0.29^{de}	1.92 ± 0.11^{ef}	0.15 ± 0.06^{g}	0.68 ± 0.06^{g}
Lb + Pp	6.02 ± 0.01^{c}	6.12 ± 0.02^{bc}	2.29 ± 0.08^{def}	2.64 ± 0.04^{c}	0.20 ± 0.06^{g}	0.75 ± 0.00^{fg}
Strains*	6.43 ± 0.02^{a}	6.40 ± 0.00^{a}	1.91 ± 0.41^{f}	1.98 ± 0.02^{ef}	0.05 ± 0.06^{h}	0.15 ± 0.00^{k}

Data represent the means ± SD of three batches of fermentation. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Mc, *Mucor* sp. (close to *M. hiemalis*) MJ:Mu1; **Rc,** *Rhizopus chinensis* MJ:Rh3

Sc, *Saccharomyces cerevisiae* MJ:YS2; **Cg,** *Candida glabrata* MS:YC5; **Pa,** *Pichia anomola* MN:YP1

Lb, *Lactobacillus bifermentans* MA:R5; **Pp,** *Pediococcus pentosaceus* MA:C1.

*Cell mixture of all above mentioned strains.

Table 25. Sensory evaluation of kodo ko jaanr produced by selected strains

Strain	Aroma	Taste	Texture	Colour	General acceptability
Rhizopus chinensis MJ:Rh3 **with**					
Mc	2.00 ± 0.93^{ab}	1.86 ± 0.52^{b}	2.14 ± 0.83^{b}	2.86 ± 0.83^{bcd}	2.29 ± 0.70^{bcd}
Sc	3.43 ± 0.50^{a}	2.79 ± 0.36^{a}	3.43 ± 0.90^{a}	4.29 ± 1.03^{a}	4.43 ± 0.73^{a}
Cg	2.43 ± 0.90^{ab}	1.57 ± 0.73^{b}	2.57 ± 0.73^{ab}	3.00 ± 0.93^{abc}	2.43 ± 0.50^{bc}
Pa	3.43 ± 0.90^{a}	1.86 ± 0.69^{b}	2.43 ± 0.50^{ab}	3.71 ± 0.88^{ab}	2.57 ± 0.50^{b}
Lb + Pp	2.29 ± 0.88^{ab}	1.79 ± 0.53^{c}	2.43 ± 1.18^{ab}	3.14 ± 0.99^{abc}	2.29 ± 0.88^{bcd}
Saccharomycopsis fibuligera MS: YD4 **with**					
Rc	1.86 ± 0.83^{ab}	1.29 ± 0.36^{b}	2.43 ± 0.50^{ab}	1.86 ± 0.64^{cde}	1.57 ± 0.50^{bcdf}
Mc	1.86 ± 0.83^{ab}	1.21 ± 0.36^{b}	2.29 ± 0.88^{ab}	1.86 ± 0.83^{cde}	1.43 ± 0.50^{cdef}
Sc	1.43 ± 0.73^{b}	1.14 ± 0.35^{b}	1.71 ± 0.88^{b}	1.29 ± 0.70^{e}	1.14 ± 0.35^{ef}
Cg	1.43 ± 0.73^{b}	1.21 ± 0.36^{b}	2.00 ± 0.76^{b}	1.29 ± 0.70^{e}	1.29 ± 0.70^{def}
Pa	2.29 ± 0.88^{ab}	1.21 ± 0.30^{b}	2.00 ± 1.07^{b}	1.43 ± 0.73^{e}	1.43 ± 0.73^{cdef}
Lb + Pp	1.57 ± 0.73^{b}	1.29 ± 0.36^{b}	2.29 ± 1.03^{ab}	1.57 ± 0.90^{de}	1.43 ± 0.73^{cdef}
Strains*	1.43 ± 0.73^{b}	1.00 ± 0.00^{b}	1.43 ± 0.73^{b}	1.14 ± 0.35^{e}	1.00 ± 0.00^{f}

Market kodo ko jaanr was used as control; score 1, bad; score 5, good.

Data represent the mean scores ± SD (n = 7). Values bearing different superscripts in each column differ significantly ($P<0.05$).

Mc, *Mucor* sp. (close to *M. hiemalis*) MJ:Mu1; **Rc,** *Rhizopus chinensis* MJ:Rh3

Sc, *Saccharomyces cerevisiae* MJ:YS2; **Cg,** *Candida glabrata* MS:YC5; **Pa,** *Pichia anomola* MN:YP1

Lb, *Lactobacillus bifermentans* MA:R5; **Pp,** *Pediococcus pentosaceus* MA:C1.

*Cell mixture of all above mentioned strains.

4.3.10. Consumers' Preference Trial

The consumers' preference trial showed that kodo ko jaanr prepared in the laboratory by cell suspension mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 as starter was more acceptable than the kodo ko jaanr prepared by conventional marcha. Market jaanr was liked extremely (score, 9) by 10 %, very much (score, 8) by 30 % and moderately (score, 7) by 60 %, the laboratory-made jaanr was liked extremely by 40 %, very much by 50 % and moderately by 10 % of the consumers.

4.4. BHAATI JAANR

Bhaati jaanr is a mild-alcoholic and juicy soft product with distinct sweet aroma, prepared from steamed glutinous rice (Plate 13). Both local and hybrid varieties of rice *(Oryza sativa* L.) are grown in low altitudes of these regions.

4.4.1. Synonym of bhaati jaanr

Bhaati jaanr is Nepali word for fermented rice beverage. Different ethnic people call it by their own dialect such as *tak thee* (Limboo), *kok umaak* (Rai), *kaiyan paa* (Gurung), *kaan chi* (Tamang), *kameshyaabu* (Sunwar), *chho haan* (Magar), *ja thon* (Newar), *dacchhang* (Sherpa), *laayakaa chhyaang* (Bhutia), and *jo chee* (Lepcha).

4.4.2. Traditional method of preparation

During traditional method of bhaati jaanr preparation, rice mainly glutinous, is cooked for about 15 min in an open cooker. Excess water is drained off and spread on a bamboo mat called mandro for cooling (~ 40° C). Powdered marcha (1 to 2 %) is sprinkled over cooked rice, mixed well and kept in a vessel or an earthen pot for 1-2 days at room temperature for saccharification. During saccharification sweet aroma is emitted out. After saccharification, the vessel is made airtight and fermented for 2-3 days in summer and 7-8 days in winter (Fig 8).

Plate 13. Rice and its fermented product bhaati jaanr

Plate 14. Maize and its fermented product makai ko jaanr

Plate 15. Wheat and its fermented product gahoon ko jaanr

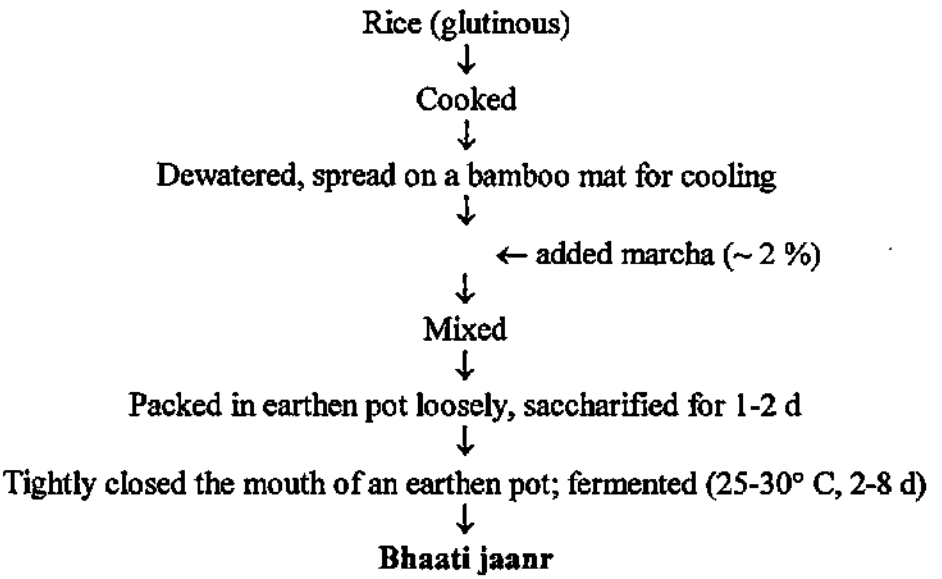


Fig 8. Flow sheet of bhaati jaanr preparation in South Sikkim

4.4.3. Mode of consumption

Bhaati jaanr is made into a thick paste by stirring the fermented mass with the help of a hand-driven wooden or bamboo-made stirrer. It is consumed directly. Sometimes, bhaati jaanr is stored in an earthenware crock for a week or more after desired fermentation is completed to make yellowish-white supernatant liquor called nigaar, collected at the bottom of the earthenware crock. Nigaar is drunk directly with or without addition of water. It is more alcoholic and slightly acidic in taste. It is a traditional diet for new mothers in villages who believe that it helps them to regain their strength.

Feeding frequency of bhaati jaanr is presented in Table 26. Only 5 % of people consume bhaati jaanr daily in rural areas of the Sikkim Himalayas. Per capita daily consumption of bhaati jaanr in the Darjeeling hills and Sikkim is 252.5 g and 323.5 g, respectively.

Table 26. Feeding frequency and consumption of bhaati jaanr

	The Darjeeling hills	Sikkim
	Feeding frequency (%)	
Daily	5	5
Weekly	20	25
Monthly	10	-
Occasional	20	10
Consumption *(g/capita/day)*	252.5 (0-893.0)	323.5 (0-1428.5)

Weekly means twice in a week. Occasional means every three months.

Values are the means of 100 households each in rural areas of the Darjeeling hills and Sikkim, respectively. Ranges are given in parentheses.

4.4.4. Similar product

Bhaati jaanr is similar to other fermented rice products of Asia such as *tapé ketan* (Indonesia), *lao-chao* (Cantonese in China), *tien-chiu-niang* (Mandarin in China), *yakju* (Korea) and *khao-maak* (Thailand).

4.4.5. Microorganisms

Twenty-four samples of bhaati jaanr were collected from different places of the Darjeeling hills and Sikkim and were analysed. Yeasts population was found higher than that of lactic acid bacteria (Table 27). Moulds were not recovered in any bhaati jaanr product analysed. Out of 127 strains of microorganisms isolated from bhaati jaanr samples, 69 isolates were yeasts and 58 isolates were lactic acid bacteria.

Table 27. Microbial load of bhaati jaanr

Source	$\times 10^7$cfu/g fresh weight		
	Yeast	LAB	Total Viable Count
Rongli	5.0 (1.5-8.1)	0.8 (0.7-1.0)	5.6 (3.1-7.5)
Namchi	7.7 (6.8-8.7)	2.3 (2.0-2.4)	9.2 (7.5-11.5)
Aho	2.1 (1.6-2.6)	0.2 (0.07-0.3)	2.4 (1.4-4.1)
Kalimpong	0.2 (0.1-0.3)	0.01 (0.003-0.03)	0.2 (0.1-0.5)

Data represent the means of 6 samples from each source. Ranges are given in parentheses.

4.4.5.1. Characterisation and identification of yeasts

Representative strains of yeasts were selected on the basis of colony, cell morphology, vegetative reproduction and type of ascospores (Table 28). Only two types of yeasts were recovered from bhaati jaanr samples. Representative strains BR:YP1 and BN:YP1 were identified as *Pichia anomala* (E.C. Hansen) Kurtzman, and representative strains BA:YS1 and BK:YS2 as *Saccharomyces cerevisiae* Meyen ex Hansen (Table 29).

Table 28. Selection of representative strains of yeasts isolated from bhaati jaanr samples[a]

Source	Number of strains isolated	Colony	Cell shape	Mycelium	Ascospore	Grouped strains	Representative strains
Rongli	18	Ss	O-E	Pseudo	Hat-shaped	12	BR:YP1
		Ss	O-E	Pseudo	Globose	6	BR:YS1
Namchi	20	Ss	O-E	Pseudo	Hat-shaped	12	BN:YP1
		Ss	O-E	Pseudo	Globose	8	BN:YS2
Aho	15	Ss	O-E	Pseudo	Hat-shaped	8	BA:YP3
		Ss	O-E	Pseudo	Globose	7	BA:YS1
Kalimpong	16	Ss	O-E	Pseudo	Hat-shaped	8	BK:YP1
		Ss	O-E	Pseudo	Globose	8	BK:YS2

[a]Number of samples was 6 from each source. All isolates reproduced by multilateral budding.

Ss, smooth surface; O-E, Oval to ellipsoidal.

Table 29. Characteristics of representative strains of yeasts isolated from bhaati jaanr

Parameter	BR:YP1	BN:YP1	BA:YS1	BK:YS2
Cell width (µm)	1.1-3.1	1.3-3.0	1.4-3.0	1.5-3.8
Cell length (µm)	1.6-3.5	1.8-4.8	1.6-5.0	1.9-4.5
Nitrate reduction	+	+	–	–
Growth at 37°C	+	+	+	+
Sugar fermentation:				
Glucose	+	+	+	+
Galactose	–	–	+	+
Lactose	–	–	–	–
Maltose	+	+	+	+
Raffinose	+	+	+	+
Sucrose	+	+	+	+
Starch	–	–	+	+
Trehalose	+	+	–	–
Sugar assimilation:				
Arabinose	+	$+_w$	–	–
Cellobiose	+	+	–	–
Galactose	+	+	+	+
Glycerol	–	–	–	–
Inositol	–	–	–	–
Lactose	–	–	–	–
Maltose	+	+	+	+
Melibiose	–	–	+	+
Mannitol	+	+	–	–
Rhamnose	–	–	–	–
Raffinose	+	+	+	+
Sucrose	+	+	+	+
Starch	+	+	+	+
Trehalose	+	+	+	+
Xylose	–	+	–	–
Identification	*Pichia*		*Saccharomyces*	

4.4.5.2. Characterisation and identification of bacteria

Out of 58 strains of lactic acid bacteria, isolated from bhaati jaanr samples, 31 strains were coccii-tetrads and 27 strains were non-sporeforming rods (Table 30). Species of lactic acid bacteria were identified as *Pediococcus pentosaceus* Mees and *Lactobacillus bifermentans* Kandler, Schillinger and Weiss (Table 31a & b).

Table 30. Selection of representative strains of LAB isolated from bhaati jaanr[a]

Source	Number of strains isolated	Cell shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Rongli	16	Coccus	–	+	8	BR:C1
		Rod	+	–	8	BR:R1
Namchi	12	Coccus	–	+	6	BN:C1
		Rod	+	–	6	BN:R2
Aho	16	Coccus	–	+	10	BA:C1
		Rod	+	–	6	BA:R2
Kalimpong	14	Coccus	–	+	7	BK:C1
		Rod	+	–	7	BK:R3

[a]Number of samples was 6 from each source

[b]All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile

Table 31a. Phenotypic characters of representative strains of LAB isolated from bhaati jaanr

Parameter	BR:C1	BN:C1	BA:C1	BK:C1	BR:R1	BN:R2	BA:R2	BK:R3
Cell shape	Ct	Ct	Ct	Ct	R	R	R	R
Cell diameter (μm)	0.2-0.7	0.2-0.7	0.2-0.5	0.4-0.7				
Cell width (μm)					0.2-0.3	0.2-0.3	0.2-0.4	0.2-0.3
Cell length (μm)					0.8-2.2	0.8-2.2	1.0-2.2	1.0-2.2
Anaerobic growth	+	+	+	+	+	+	+	+
Hydrolysis of:								
Casein	–	–	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–	–	–
Arginin	+	+	+	+	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–	–	–
Growth in NaCl:								
4.0 %	+	+	+	+	+	+	+	+
6.5 %	+	+	+	+	+	$+_w$	+	+
10.0 %	+	+	+	+	+	$+_w$	+	+
18.0 %	–	–	–	–	–	–	–	–
Growth in pH:								
4.2	+	+	+	+	+	+	+	+
7.5	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+
Growth at:								
15° C	+	+	+	+	+	+	+	+
45° C	–	–	–	–	–	–	–	–

Ct, coccus, tetrad; R, rod in chain with 2-3 cells

Table 31b. Sugar fermentation of LAB strains using API 50 CHL system

Parameter	BR:C1	BN:C1	BA:C1	BK:C1	BR:R1	BN:R2	BA:R2	BK:R3
Glycerol	+	+	–	–	–	–	+	–
Erythritol	+	+	–	–	–	–	–	–
D-Arabinose	+	+	–	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+
L-Xylose	–	–	–	–	–	–	–	$+_w$
Adonitol	+	+	–	–	–	–	+	–
β-Methyl-D-Xyloside	–	–	–	–	–	–	–	–
Galactose	+	+	+	+	+	$+_w$	–	+
D-Glucose	–	–	+	+	+	–	+	+
D-Fructose	–	–	+	+	+	+	+	+
D-Mannose	–	–	+	+	–	–	+	–
L-Sorbose	+	–	–	–	–	–	–	–
Rhamnose	+	+	–	–	–	–	+	–
Dulcitol	+	+	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	$+_w$
Sorbitol	+	+	–	–	–	–	–	–
α-Methyl-D-Mannoside	+	+	–	–	–	–	+	–
α-Methyl-D-Glucoside	–	–	–	–	$+_w$	$+_w$	+	+
N-Acetyl-Glucosamine	+	+	+	+	$+_w$	$+_w$	+	+
Amygdalin	+	+	+	+	–	–	–	–
Arbutin	–	–	–	–	–	–	–	–
Esculin	–	–	–	–	–	–	–	–
Salicin	$+_w$	–	–	–	–	–	–	–
Cellobiose	+	+	+	+	–	–	–	–
Maltose	+	+	+	+	+	+	+	+

Parameter	BR:C1	BN:C1	BA:C1	BK:C1	BR:R1	BN:R2	BA:R2	BK:R3
Lactose	–	–	–	+	–	–	–	–
Melibiose	–	–	–	–	$+_w$	$+_w$	+	$+_w$
Sucrose	–	–	–	–	–	–	–	–
Trehalose	+	+	+	+	–	–	–	–
Inulin	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–
Gentiobiose	+	+	+	+	–	–	–	–
D-Turanose	–	–	–	–	–	–	–	–
D-Lyxose	–	–	–	–	–	–	–	–
D-Tagatose	+	+	+	+	–	–	–	–
D-Fucose	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	–	–	–	–
L-Arabitol	–	–	–	–	–	–	–	–
Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
2-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	–
5-Keto-Gluconate	–	–	–	–	$+_w$	$+_w$	–	$+_w$
Identification	*Pediococcus*				*Lactobacillus*			

4.4.6. Proximate composition

Proximate composition of bhaati jaanr is presented in Table 32. Average pH of the product was 3.5, acidity and alcohol contents were 0.24 % and 5.9 %, respectively. Fat, protein and calorie contents remained same as the substrate. Considerable increase in calcium, manganese, iron, zinc,

sodium, potassium and phosphorous was observed in bhaati jaanr over the substrate (Table 33).

Table 32. Proximate composition of cooked rice and bhaati jaanr

Parameter	Unfermented	Fermented (Bhaati jaanr)			
	Cooked rice	Rongli	Namchi	Aho	Kalimpong
pH	6.01 (6.0-6.02)	3.5 (3.3-3.7)	3.4 (3.2-3.6)	3.3 (3.1-3.5)	3.7 (3.5-4.0)
Moisture (%)	67.2 (62.4-68.2)	82.8 (80.9-83.9)	85.2 (83.6-86.8)	82.4 (79.3-89)	83.0 (78.4-85.0)
Acidity (%)	0.01 (0.01-0.02)	0.20 (0.10-0.30)	0.25 (0.2-0.3)	0.25 (0.2-0.3)	0.27 (0.2-0.34)
Alcohol (%)	0.0 (0.0-0.0)	6.6 (5.3-7.4)	5.0 (4.0-6.0)	5.3 (4.0-8.3)	6.8 (6.6-6.9)
Ash (% DM)	0.6 (0.5-1.2)	1.7 (1.1-2.0)	2.0 (1.8-2.2)	0.8 (0.7-1.0)	2.1 (1.5-2.3)
Fat (% DM)	2.4 (1.9-2.9)	2.9 (1.9-3.2)	2.0 (1.5-2.5)	1.2 (1.0-2.0)	1.8 (1.2-2.6)
Protein (% DM)	9.5 (8.7-10.3)	9.4 (8.0-10.1)	9.8 (8.2-10.4)	9.3 (8.3-9.7)	9.5 (8.5-9.8)
Crude fibre (% DM)	0.6 (0.4-0.8)	1.5 (1.4-1.7)	ND	1.4 (1.2-1.7)	ND
Carbohydrate (% DM)	87.5 (85.6-88.9)	86.0 (84.7-89.0)	86.2 (84.9-88.5)	88.7 (87.2-90.0)	86.6 (85.3-88.8)
Energy (MJ/100g DM)	409.6 (394.3-422.9)	407.7 (387.9-425.2)	402.0 (385.9-418.1)	402.8 (391.0-416.8)	400.6 (386.0-417.8)

Data represent the means of 5 samples from each source. % DM, percentage on dry matter basis. Ranges are given in parentheses. ND, not determined.

Table 33. Mineral contents of raw and fermented rice

Mineral	mg/100 g dry matter	
	Rice	Bhaati jaanr
Calcium	2.5	12.8
Magnesium	22	50
Manganese	0.4	1.4
Copper	0.5	1.4
Iron	2.2	7.7
Zinc	0.6	2.7
Sodium	5.3	24.7
Potassium	57	146
Phosphorus	156	595

Data represent the means of 2 samples.

4.4.7. Successional studies during bhaati jaanr fermentation

Bhaati jaanr was prepared in the laboratory following the traditional method by using marcha, collected from Aho village, as mentioned in 3.3.6. Successional studies were carried at every 1 day interval within a range of 0-10 days.

4.4.7.1. Microbial changes

Loads of moulds decreased significantly ($P<0.05$) during fermentation and disappeared after the fifth day of fermentation (Table 34). Population of yeasts increased significantly ($P<0.05$) from 10^5 cfu/g to 10^8 cfu/g within 2 d, and decreased to a level of 10^5 cfu/g in 10 d (Fig 9). Exponential increase in load of lactic acid bacteria was significant ($P<0.05$) till second day of fermentation, and then declined slowly. However, the decrease was not significant till 6 d. Total viable count

increased significantly ($P<0.05$) from 0 d to 2 d, and decreased significantly ($P<0.05$) in every interval of 1 d.

Table 34. Microbial changes during bhaati jaanr fermentation

Fermentation time (days)	Log cfu/g			
	Mould	Yeast	LAB	Total Count
0	4.3 ± 0.13^{a}	5.9 ± 0.08^{h}	6.0 ± 0.13^{bc}	6.3 ± 0.08^{h}
1	3.5 ± 0.29^{b}	7.5 ± 0.13^{d}	6.4 ± 0.33^{ab}	7.5 ± 0.08^{d}
2	1.7 ± 0.08^{c}	8.0 ± 0.13^{a}	7.0 ± 0.08^{a}	8.1 ± 0.08^{a}
3	0.4 ± 0.26^{d}	7.9 ± 0.08^{b}	6.9 ± 0.08^{a}	8.0 ± 0.08^{b}
4	<DL	7.8 ± 0.08^{c}	6.6 ± 0.08^{a}	7.8 ± 0.08^{c}
5	0	7.5 ± 0.16^{d}	6.6 ± 0.08^{a}	7.5 ± 0.08^{d}
6	0	6.8 ± 0.08^{e}	6.6 ± 0.08^{a}	7.0 ± 0.08^{e}
7	0	6.5 ± 0.08^{f}	5.9 ± 0.13^{bc}	6.6 ± 0.13^{f}
8	0	6.4 ± 0.08^{g}	5.7 ± 0.13^{cd}	6.5 ± 0.16^{g}
9	0	5.8 ± 0.08^{i}	5.2 ± 0.13^{d}	6.3 ± 0.08^{h}
10	0	5.6 ± 0.08^{j}	5.2 ± 0.16^{d}	6.0 ± 0.08^{i}

Data represent the means ± SD of three batches of fermentation. Data were transformed into logarithmic values. DL, detection limit (10 cfu/g).

Values bearing different superscripts in each column differ significantly ($P<0.05$).

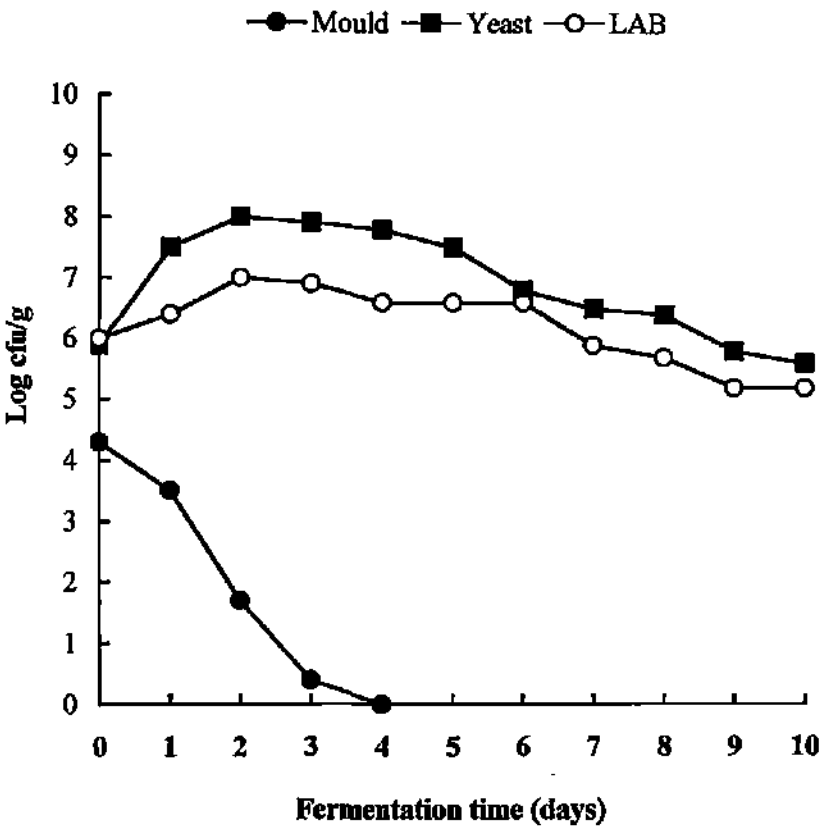


Fig 10. Changes in microbial load during bhaati jaanr fermentation. Values are the means of three batches of fermentation. LAB, lactic acid bacteria.

4.4.7.2. Physico-chemical and enzymatic changes

Temperature of fermenting rice remained relatively constant in between 28° C to 30° C throughout the fermentation after first day. During fermentation, pH decreased significantly ($P<0.05$) from 6.27 to 3.21 within 2 d and slightly increased to 3.96 at the end (Fig 10). Titratable acidity increased significantly ($P<0.05$) from 0.01 % to 0.20 % till 4 d and remained at a level of 0.17 % till end (Table 35). Alcohol content increased significantly ($P<0.05$) during fermentation (Fig 11).

Table 35. Physico-chemical changes during bhaati jaanr fermentation

Fermentation time (days)	Temperature (°C)	pH	Acidity (%)	Alcohol (%)
0	26.0 ± 0.00^{f}	6.27 ± 0.01^{a}	0.01 ± 0.00^{g}	0.1 ± 0.04^{e}
1	31.0 ± 0.00^{a}	3.36 ± 0.01^{i}	0.11 ± 0.01^{f}	0.4 ± 0.08^{e}
2	30.0 ± 0.00^{b}	3.21 ± 0.07^{j}	0.19 ± 0.01^{b}	2.8 ± 0.13^{d}
3	30.0 ± 0.00^{b}	3.37 ± 0.00^{i}	0.19 ± 0.02^{b}	3.6 ± 0.08^{d}
4	29.2 ± 0.13^{c}	3.45 ± 0.01^{h}	0.20 ± 0.00^{a}	6.8 ± 1.03^{c}
5	28.5 ± 0.00^{d}	3.51 ± 0.01^{g}	0.18 ± 0.01^{c}	7.3 ± 0.49^{bc}
6	28.5 ± 0.00^{d}	3.71 ± 0.01^{f}	0.18 ± 0.02^{c}	7.8 ± 0.29^{b}
7	28.5 ± 0.00^{d}	3.78 ± 0.01^{e}	0.18 ± 0.01^{c}	9.3 ± 0.29^{a}
8	28.3 ± 0.08^{e}	3.93 ± 0.01^{c}	0.18 ± 0.01^{c}	9.6 ± 0.29^{a}
9	28.3 ± 0.05^{e}	3.89 ± 0.01^{d}	0.18 ± 0.01^{c}	9.8 ± 0.13^{a}
10	28.2 ± 0.05^{e}	3.96 ± 0.01^{b}	0.17 ± 0.01^{d}	10.1 ± 0.29^{a}

Data represent the means ± SD of three batches of fermentation.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

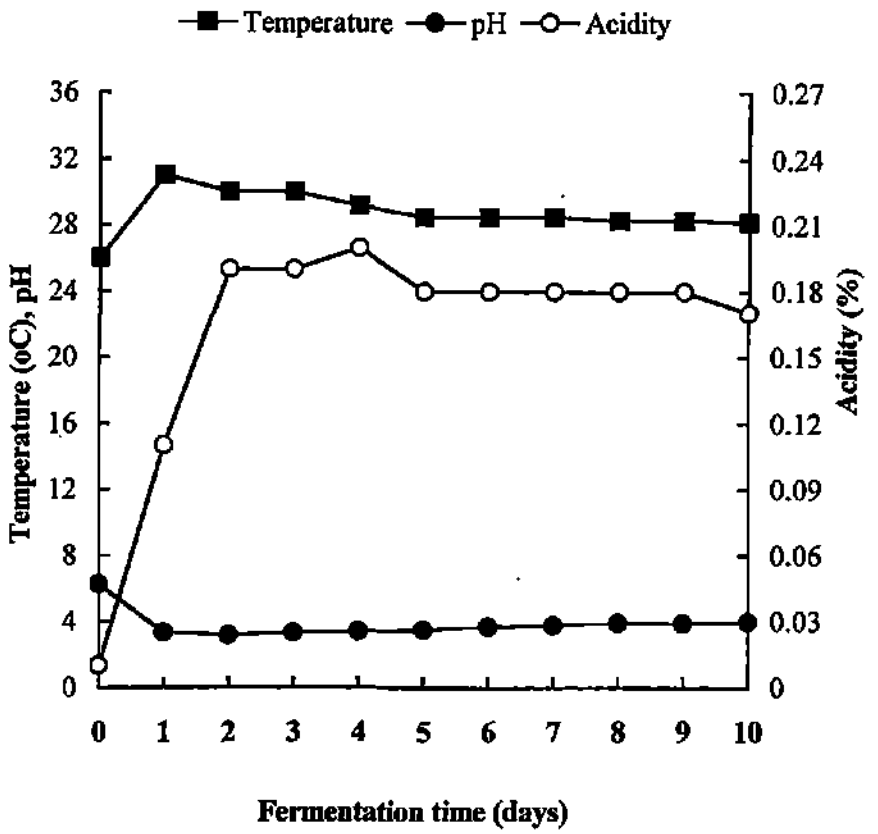


Fig. 11. Changes in temperature, pH and acidity during bhaati jaanr fermentation. Values are the means of three batches of fermentation.

Reducing sugar contents increased significantly ($P<0.05$) from 0.01 % to 12.6 % within 3 d, and declined till end (Table 36). Total sugar contents decreased significantly ($P<0.05$) throughout the fermentation. Maximum activities of α–amylase and glucoamylase was observed on third day of fermentation and decreased significantly ($P<0.05$) till the end (Table 36 and Fig 12).

Table 36. Biochemical and enzymatic changes during bhaati jaanr fermentation

Fermentation time (days)	Reducing sugar (%)	Total sugar (%)	∝-amylase (U/g)	Glucoamylase (U/mg)
0	0.01 ± 0.00^{g}	64.1 ± 1.02^{a}	12.5 ± 1.23^{h}	61.7 ± 9.55^{e}
1	2.2 ± 0.41^{d}	61.6 ± 2.49^{a}	37.6 ± 0.65^{c}	181.1 ± 13.15^{c}
2	3.8 ± 0.21^{b}	49.1 ± 2.94^{b}	41.8 ± 0.82^{b}	227.9 ± 6.45^{b}
3	12.6 ± 0.13^{a}	39.3 ± 0.98^{c}	45.3 ± 0.25^{a}	510.9 ± 8.90^{a}
4	2.9 ± 0.21^{c}	30.9 ± 0.94^{d}	34.9 ± 0.74^{d}	115.5 ± 4.33^{d}
5	0.6 ± 0.08^{e}	27.7 ± 0.65^{e}	20.1 ± 0.08^{e}	41.3 ± 0.45^{e}
6	0.6 ± 0.05^{e}	27.6 ± 0.21^{e}	16.6 ± 0.49^{f}	44.4 ± 1.92^{e}
7	0.5 ± 0.05^{ef}	22.2 ± 0.94^{f}	15.5 ± 0.82^{g}	52.6 ± 1.18^{e}
8	0.4 ± 0.05^{ef}	18.1 ± 0.50^{g}	12.6 ± 0.33^{h}	40.3 ± 0.86^{e}
9	0.3 ± 0.08^{efg}	16.2 ± 0.05^{g}	8.8 ± 0.41^{i}	35.1 ± 3.39^{e}
10	0.2 ± 0.05^{fg}	13.4 ± 1.47^{h}	7.2 ± 0.16^{j}	33.5 ± 1.35^{e}

Data represent the means ± SD of three batches of fermentation.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

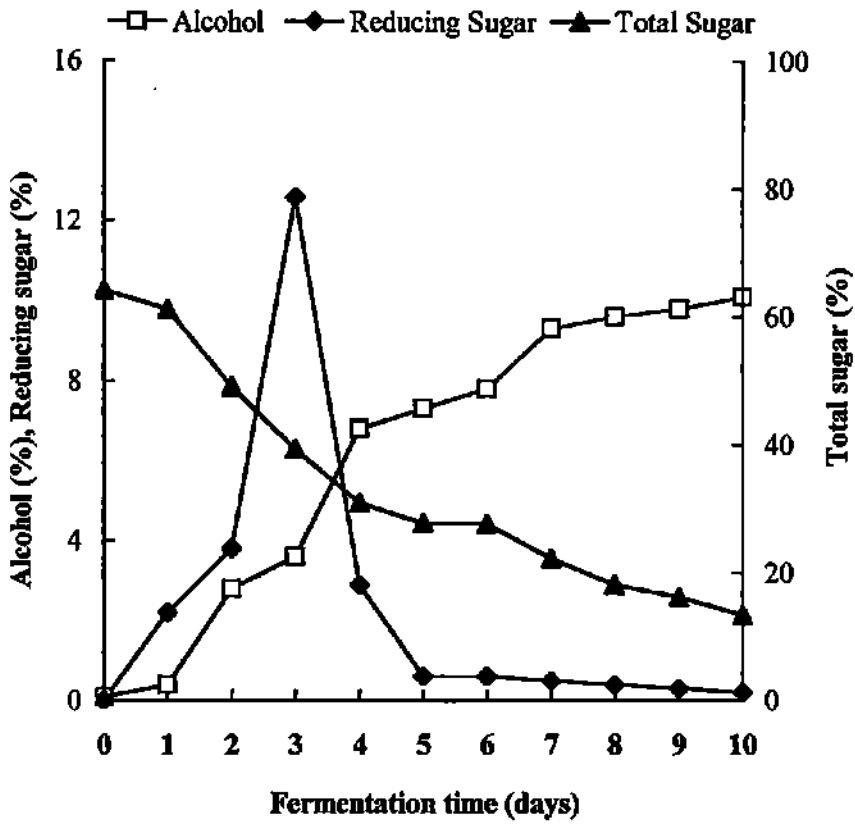


Fig. 12. Changes in alcohol, reducing sugar and total sugar contents of rice during bhaati jaanr fermentation. Values are the means of three batches of fermentation.

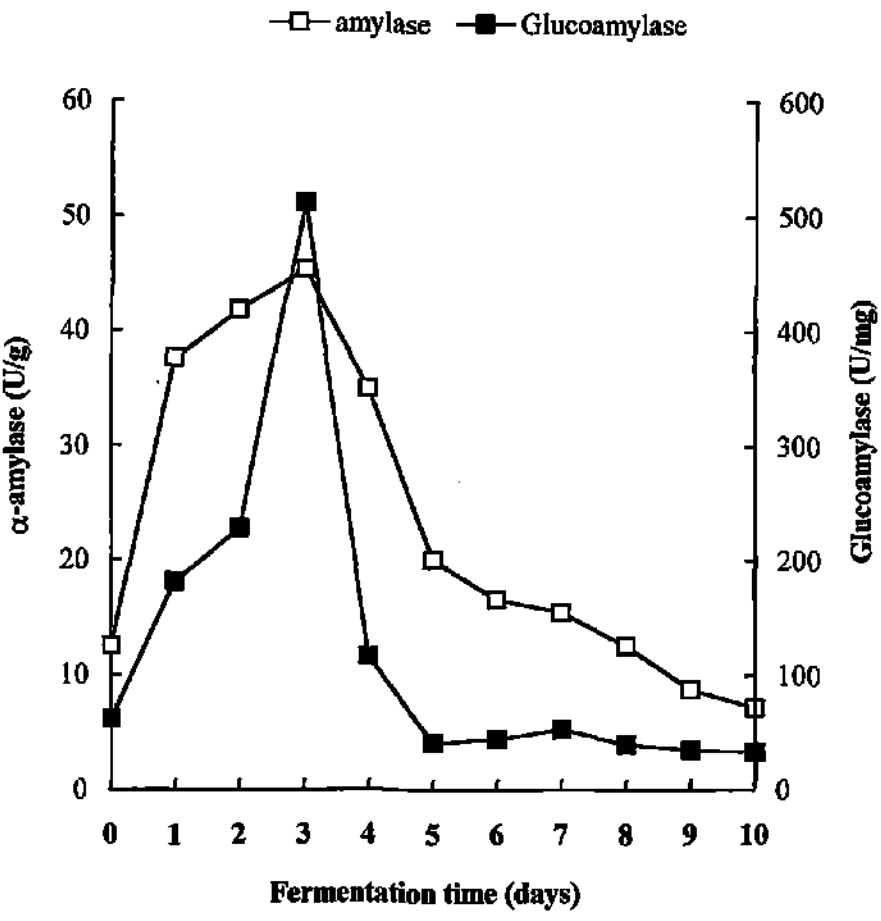


Fig. 13. Changes in enzymatic activities of rice during bhaati jaanr fermentation. Values are the means of three batches of fermentation.

4.5. MAKAI KO JAANR

Makai ko jaanr is a viscous, slightly bitter, mild-alcoholic beverage, fermented from maize (Plate 14). Preparation and consumption of makai ko jaanr are confined to few places of the Darjeeling hills and Sikkim.

4.5.1. Synonym of makai ko jaanr

Common name for the maize is makai in Nepali language. Different ethnic gropus call it such as *makai thee* by Limboo, *yobbhacha umaak* by Rai, *makhain paa* by Gurung, *maagnila jheen* by Tamang, *aakan shyaabu* by Sunwar, *makai haan* by Magar, *kahni thon* by Newar, *lichee chhyaang* by Sherpa, *kinya chhyaang* by Bhutia and *kanchung chee* by Lepcha.

4.5.2. Method of preparation

During traditional method of preparation of makai ko jaanr, dry seeds of maize *(Zea mays* L.) are grinded and dehuksed. Bigger grinded granules of maize called *chekhla* are selected for preparation of makai ko jaanr. Chekhla are washed, cooked to a thick porridge, cooled and inoculated with powdered marcha (1.0-2.0 %). Saccharification and fermentation method of makai ko jaanr are same as bhaati jaanr (Fig 13).

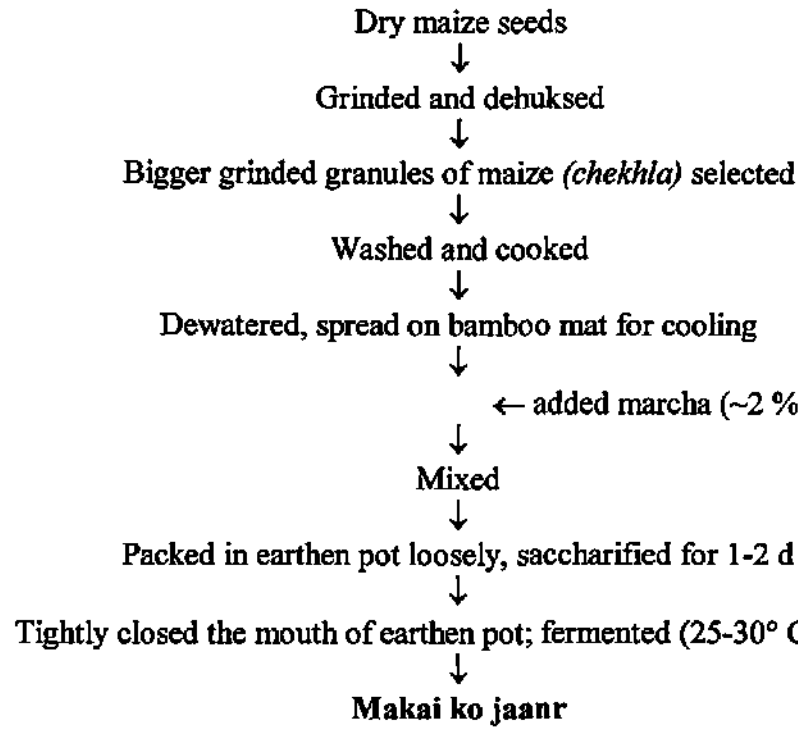


Fig 15. Flow sheet of makai ko jaanr preparation at Algarah in Kalimpong

4.5.3. Mode of consumption

Fermented porridge is mashed, filtered and desirable amount of lukewarm water is added. Extract of makai ko jaanr is drunk directly. It is slightly bitter, mild alcoholic beverage. Feeding frequency of makai ko jaanr in rural areas of the Sikkim Himalayas is shown in Table 37.

Table 37. Feeding frequency and consumption of makai ko jaanr

	The Darjeeling hills	Sikkim
Feeding frequency (%)		
Daily	20	-
Weekly	10	15
Monthly	5	5
Occasional	10	15
Consumption *(g/capita/day)*	58.2 (0-285.7)	323.5 (0-476.2)

Weekly means twice in a week. Occasional means every three months.

Values are the means of 100 households each in rural areas of the Darjeeling hills and Sikkim, respectively. Ranges are given in parentheses.

4.5.4. Microorganisms

Load of yeasts was found 10 times higher than that of lactic acid bacteria in makai ko jaanr samples (Table 38). Moulds were not recovered in the final product.

Table 38. Microbial load of makai ko jaanr

Source	x 10^7cfu/g fresh weight		
	Yeast	LAB	Total Viable Count
Barnyak	2.5 (2.2-2.8)	0.4 (0.02-0.7)	7.4 (5.0-9.8)
Kalimpong	0.5 (0.3-0.7)	0.02 (0.01-0.03)	0.6 (0.5-0.7)

Data represent the means of 6 samples from each source. Ranges are given in parentheses.

Out of 100 strains of microorganisms isolated from twelve samples of makai ko jaanr, 54 isolates were yeasts and 46 isolates were lactic acid bacteria.

4.5.4.1. Characterisation and identification of yeasts

Table 39 shows selection of representative strains of yeasts, isolated from twelve samples of makai ko jaanr. Out of 54 strains of yeasts, 30 were oval with hat-shaped ascopres and 24 strains were oval with globose-shaped ascopores. The representative strains with hat-shaped ascospores MJB:YP1 and MJK:YP5 were identified as *Pichia anomala* (E.C. Hansen) Kurtzman; representative strains with globose-shaped ascospores MJB:YS4 and MJK:YS7 were identified *Saccharomyces cerevisiae* Meyen ex Hansen (Table 40).

Table 39. Selection of representative strains of yeasts isolated from makai ko jaanr samples[a]

Source	Number of strains isolated	Colony	Cell shape	Mycelium	Ascospore	Grouped strains	Representative strains
Barnyak	24	Ss	O-E	Pseudo	Hat-shaped	12	MJB:YP1
		Ss	O-E	Pseudo	Globose	12	MJB:YS4
Kalimpong	30	Ss	O-E	Pseudo	Hat-shaped	18	MJK:YP5
		Ss	O-E	Pseudo	Globose	12	MJK:YS7

[a]Number of samples was 6 from each source. All isolates reproduced by multilateral budding.
Ss, smooth surface; O-E, Oval to ellipsoidal.

Table 40. Characteristics of representative strains of yeasts isolated from makai ko jaanr

Parameter	MJB:YP1	MJK:YP5	MJB:YS4	MJK:YS7
Cell width (µm)	1.0-3.0	1.1-3.0	1.5-3.2	1.7-3.5
Cell length (µm)	1.6-4.6	1.6-4.4	1.6-5.1	1.9-4.9
Nitrate reduction	+	+	–	–
Growth at 37°C	+	+	+	+
Sugar fermentation:				
Glucose	+	+	+	+
Galactose	–	–	+	+
Lactose	–	–	–	–
Maltose	+	+	+	+
Raffinose	+	+	+	+
Sucrose	+	+	+	+
Starch	–	–	+	+
Trehalose	+	+	–	–
Sugar assimilation:				
Arabinose	+	+	–	–
Cellobiose	+	+	–	–
Galactose	+	+	+	+
Glycerol	–	–	–	–
Inositol	–	–	–	–
Lactose	–	–	–	–
Maltose	+	+	+	+
Melibiose	–	–	+	+
Mannitol	+	+	–	–
Rhamnose	–	–	–	–
Raffinose	+	+	+	+
Sucrose	+	+	+	+
Starch	+	+	+	+
Trehalose	+	+	+	+
Xylose	–	+	–	–
Identification	*Pichia*		*Saccharomyces*	

4.5.4.2. Characterisation and identification of bacteria

Representative strains of lactic acid bacteria were selected (Table 41). Out of 46 LAB strains isolated from makai ko jaanr, 28 strains were coccii-tetrads and 18 strains were non-sporeforming rods. Representative strains of coccii-tetrads MJB:C7 and MJK:C6 were identified as *Pediococcus pentosaceus* Mees and representative strains of rod-shaped isolates MJB:R4 and MJK:R5 were identified *Lactobacillus bifermentans* Kandler, Schillinger and Weiss (Table 42 a & b).

Table 41. Selection of representative strains of LAB isolated from makai ko jaanr[a]

Source	Number of strains isolated	Cell shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Barnyak	20	Coccus	–	+	12	MJB:C7
		Rod	+	–	8	MJB:R4
Kalimpong	26	Coccus	–	+	16	MJK:C6
		Rod	+	–	10	MJK:R5

[a]Number of samples was 6 from each source

[b]All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile

Table 42a. Phenotypic characteristics of representative strains of LAB isolated from makai ko jaanr

Parameter	MJB:C7	MJK:C6	MJB:R4	MJK:R5
Cell shape	Coccus, tetrads	Coccus, tetrads	Rod	Rod
Cell diameter (μm)	0.2-0.5	0.2-0.6		
Cell width (μm)			0.2-0.3	0.2-0.3
Cell length (μm)			1.0-2.2	1.0-2.3
Anaerobic growth	+	+	+	+
Hydrolysis of:				
Casein	–	–	–	–
Gelatin	–	–	–	–
Arginine	+	+	–	–
Starch	–	–	–	–
Indole production	–	–	–	–
Nitrate reduction	–	–	–	–
Growth in NaCl:				
4.0 %	+	+	+	+
6.5 %	+	+	+	+
10.0 %	+	+	+	+
18.0 %	–	–	–	–
Growth in pH:				
4.2	+	+	+	+
7.5	+	+	+	+
8.5	+	+	+	+
Growth at:				
15° C	+	+	+	+
45° C	–	–	–	–

Table 42 b. Sugar fermentation of LAB strains using API 50 CHL system

Parameter	MJB:C7	MJK:C6	MJB:R4	MJK:R5
Glycerol	+	+	–	+
Erythritol	+	+	–	–
D-Arabinose	+	+	–	–
L-Arabinose	+	+	+	+
Ribose	+	+	+	+
D-Xylose	+	+	+	+
L-Xylose	–	–	–	–
Adonitol	+	+	+	–
β-Methyl-D-Xyloside	–	–	–	–
Galactose	+	+	–	$+_w$
D-Glucose	–	–	+	+
D-Fructose	–	–	+	+
D-Mannose	–	$+_w$	+	–
L-Sorbose	$+_w$	–	–	–
Rhamnose	+	+	+	–
Dulcitol	+	+	–	–
Inositol	–	–	–	–
Mannitol	–	–	–	$+_w$
Sorbitol	+	+	–	–
α-Methyl-D-Mannoside	+	+	–	$+_w$
α-Methyl-D-Glucoside	–	–	+	+
N-Acetyl-Glucosamine	+	+	+	$+_w$
Amygdalin	+	+	–	–
Arbutin	–	–	–	–
Esculin	–	–	–	–
Salicin	$+_w$	–	–	–
Cellobiose	+	+	–	–
Maltose	+	+	+	$+_w$

Parameter	MJB:C7	MJK:C6	MJB:R4	MJK:R5
Lactose	–	–	–	–
Melibiose	–	–	+	+
Sucrose	–	–	–	–
Trehalose	+	+	–	–
Inulin	–	–	–	–
Melezitose	–	–	–	–
Raffinose	–	–	–	–
Starch	–	–	–	–
Glycogen	–	–	–	–
Xylitol	–	–	–	–
Gentiobiose	+	+	–	–
D-Turanose	–	–	–	–
D-Lyxose	–	–	–	–
D-Tagatose	+	+	–	–
D-Fucose	–	–	–	–
L-Fucose	–	–	–	–
D-Arabitol	–	–	–	–
L-Arabitol	–	–	–	–
Gluconate	–	–	–	–
2-Keto-Gluconate	–	–	–	–
5-Keto-Gluconate	–	–	$+_w$	$+_w$
Identification	*Pediococcus*		*Lactobacillus*	

4.5.5. Proximate composition

Proximate composition of makai ko jaanr is shown in Table 43. Mean average pH, acidity and alcohol content of the product was 3.3, 0.38 % and 2.5 %, respectively. Alcohol content was comparatively less in makai ko jaanr than that of other cereal-based jaanr products. Increase in crude fibre content was observed in makai ko jaanr. Calorie value

remained almost same in both substrate and the product. Remarkable increase in iron, potassium and phosphorous was observed in makai ko jaanr (Table 44).

Table 43. Proximate composition of cooked maize and makai ko jaanr

Parameter	Unfermented	Fermented (Makai ko jaanr)	
	Cooked maize	Barnyak	Kalimpong
PH	6.0 (5.9-6.2)	3.2 (3.1-3.5)	3.4 (3.1-3.8)
Moisture (%)	65.2 (63.0-68.2)	82.4 (79.2-84)	81.4 (79.0-83.1)
Acidity (%)	0.01 (0.01-0.01)	0.40 (0.34-0.62)	0.35 (0.31-0.56)
Alcohol (%)	0	2.0 (1.8-2.1)	3.0 (2.8-3.6)
Ash (% DM)	1.4 (1.2-2.2)	2.2 (1.5-2.7)	2.0 (1.5-2.5)
Fat (% DM)	3.7 (3.0-3.9)	3.0 (2.7-3.5)	3.2 (2.8-3.7)
Protein (% DM)	13.8 (11.0-14.5)	12.9 (10.5-14.0)	13.2 (10.8-14.3)
Crude fibre (% DM)	1.4 (1.0-2.8)	2.9 (2.0-3.8)	2.7 (2.3-3.2)
Carbohydrate (% DM)	81.1 (79.4-84.8)	81.9 (79.8-85.3)	81.6 (79.5-84.9)
Energy (MJ/100g DM)	412.9 (388.6-432.3)	406.2 (385.5-428.7)	408.0 (386.4-430.1)

Data represent the means of 5 samples from each source.

% DM, percentage on dry matter basis. Ranges are given in parentheses.

Table 44. Mineral contents of raw and fermented maize

Mineral	mg/100 g dry matter	
	Maize	Makai ko jaanr
Calcium	2.3	5.2
Magnesium	53	70
Manganese	0.3	0.5
Copper	0.4	0.9
Iron	5.5	17
Zinc	0.7	1.2
Sodium	12.3	21.5
Potassium	153	227
Phosphorus	342	538

Data represent the means of 2 samples.

4.5.6. Successional studies during makai ko jaanr fermentation

Makai ko jaanr was prepared in the laboratory following the traditional method by using marcha, collected from Aho village, as mentioned in 3.3.6. Successional studies were carried at every 1day interval within a range of 0-10 days.

4.5.6.1. Microbial changes

The load of moulds declined significantly ($P<0.05$) during makai ko jaanr fermentation and disappeared after the 4th day (Table 45). The load of yeasts increased significantly ($P<0.05$) from 0 day to 2 day and remained constant till 8th day, and decreased significantly ($P<0.05$) till an end of fermentation. Load of lactic acid bacteria increased significantly ($P<0.05$) from 0 day to 2 day and declined gradually till the end (Fig 14). Total viable counts increased significantly ($P<0.05$) from

10^6 to 10^7 cfu/g within second day and remained constant till 8 d, and then decreased significantly ($P<0.05$) to 10^6 cfu/g on 10 d.

Table 45. Microbial changes during makai ko jaanr fermentation

Fermentation time (days)	Log cfu/g			
	Mould	Yeast	LAB	Total Count
0	4.2 ± 0.08^{a}	6.9 ± 0.08^{d}	5.0 ± 0.17^{g}	6.7 ± 0.34^{de}
1	3.2 ± 0.05^{b}	7.6 ± 0.22^{b}	5.8 ± 0.29^{ef}	7.6 ± 0.08^{b}
2	1.5 ± 0.16^{c}	7.7 ± 0.08^{a}	7.1 ± 0.08^{ab}	7.8 ± 0.08^{ab}
3	< DL	7.8 ± 0.08^{a}	7.5 ± 0.08^{a}	7.9 ± 0.08^{a}
4	0	7.8 ± 0.08^{a}	6.9 ± 0.08^{bc}	7.8 ± 0.08^{ab}
5	0	7.8 ± 0.08^{a}	6.8 ± 0.08^{bc}	7.9 ± 0.08^{a}
6	0	7.8 ± 0.08^{a}	6.7 ± 0.08^{bcd}	7.9 ± 0.08^{a}
7	0	7.8 ± 0.08^{a}	6.6 ± 0.08^{cd}	7.8 ± 0.08^{ab}
8	0	7.7 ± 0.13^{a}	6.4 ± 0.08^{de}	7.8 ± 0.08^{ab}
9	0	7.2 ± 0.08^{c}	5.8 ± 0.08^{ef}	7.1 ± 0.08^{c}
10	0	6.5 ± 0.13^{e}	6.0 ± 0.54^{f}	6.6 ± 0.08^{e}

Data represent the means ± SD of three batches of fermentation. Data were transformed into logarithmic values. DL, detection limit (10 cfu/g).

Values bearing different superscripts in each column differ significantly ($P<0.05$).

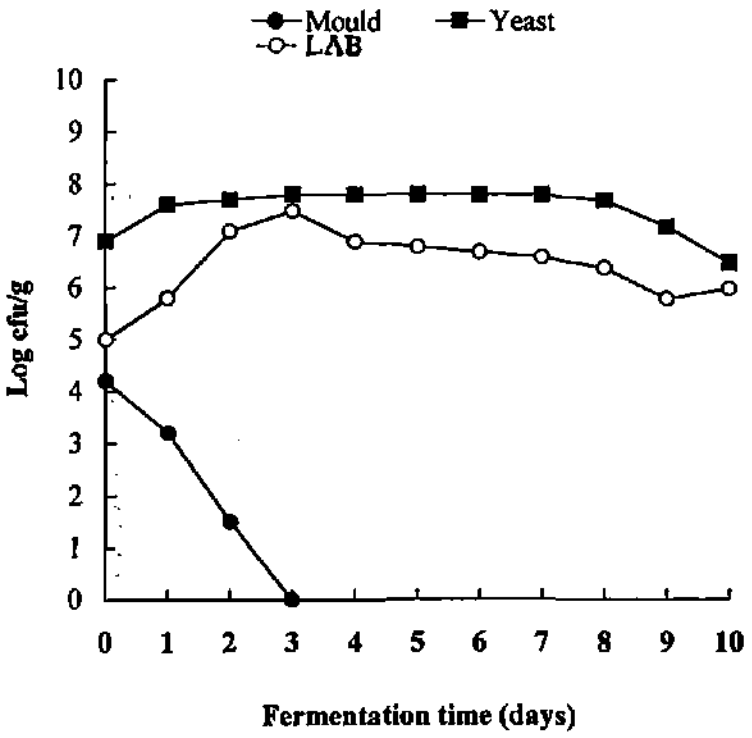


Fig 14. Changes in microbial load during makai ko jaanr fermentation. Values are the means of three batches of fermentation. LAB, lactic acid bacteria.

4.5.6.2. Physico-chemical and enzymatic changes

Temperature of fermenting maize remained relatively constant throughout the fermentation after third day (Table 46). During fermentation, pH dropped significantly ($P<0.05$) from 6.24 to 3.89 within 10 days. Acidity increased significantly ($P<0.05$) on each day till the sixth day, after which there was no significant change (Fig 15). The mean alcohol content increased significantly ($P<0.05$) during fermentation (Fig 16).

Table 46. Physico-chemical changes during makai ko jaanr fermentation

Fermentation time (days)	Temperature (°C)	pH	Acidity (%)	Alcohol (%)
0	24.0 ± 0.00^{f}	6.24 ± 0.00^{a}	0.01 ± 0.00^{g}	0^{j}
1	29.5 ± 0.41^{a}	3.14 ± 0.00^{j}	0.11 ± 0.01^{f}	0.5 ± 0.08^{i}
2	29.0 ± 0.41^{b}	3.18 ± 0.02^{i}	0.16 ± 0.01^{e}	2.9 ± 0.13^{h}
3	28.5 ± 0.00^{cd}	3.24 ± 0.01^{h}	0.17 ± 0.01^{d}	4.2 ± 0.08^{g}
4	28.3 ± 0.00^{de}	3.38 ± 0.01^{g}	0.22 ± 0.01^{a}	5.1 ± 0.08^{f}
5	28.2 ± 0.08^{de}	3.52 ± 0.01^{f}	0.21 ± 0.01^{b}	5.7 ± 0.25^{e}
6	28.0 ± 0.00^{e}	3.58 ± 0.01^{e}	0.18 ± 0.01^{c}	6.9 ± 0.08^{d}
7	28.0 ± 0.00^{e}	3.72 ± 0.01^{d}	0.17 ± 0.01^{d}	7.1 ± 0.13^{d}
8	28.0 ± 0.00^{e}	3.86 ± 0.01^{c}	0.17 ± 0.01^{d}	7.7 ± 0.21^{c}
9	28.0 ± 0.00^{e}	3.89 ± 0.01^{b}	0.17 ± 0.02^{d}	8.9 ± 0.13^{b}
10	28.0 ± 0.00^{e}	3.89 ± 0.02^{b}	0.17 ± 0.01^{d}	9.9 ± 0.21^{a}

Data represent the means ± SD of three batches of fermentation.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

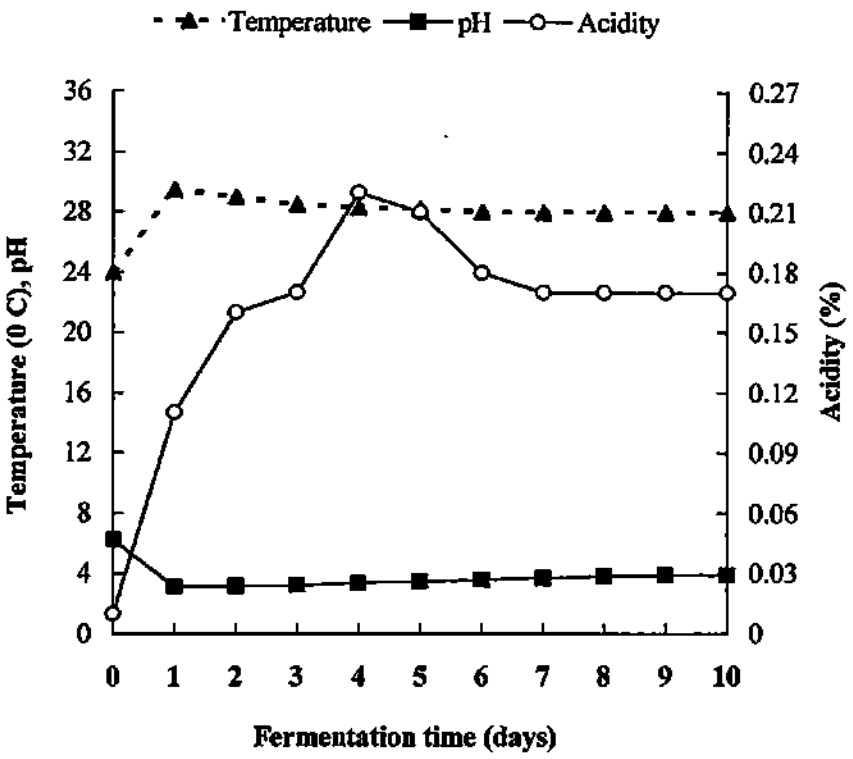


Fig 15. Changes in temperature, pH and acidity in fermenting maize during makai ko jaanr fermentation. Values are the means of three batches of fermentation.

Reducing sugar increased significantly ($P<0.05$) from 0 d to 1 d and then gradually reduced until 10 d (Table 16). Total sugar content decreased significantly ($P<0.05$) till 5 d, and the level decreased slowly towards the end of fermentation (Fig 17). Maximum ∝-amylase activities of fermenting millets was observed on 3 d and whereas that of glucoamylase activities was observed on 1 d (Table 47 and Fig 17).

Table 47. Biochemical and enzymatic changes during makai ko jaanr fermentation

Fermentation time (days)	Reducing sugar (%)	Total sugar (%)	∝-amylase (U/g)	Glucoamylase (U/mg)
0	0.01 ± 0.01^{i}	70.0 ± 6.61^{a}	18.2 ± 0.33^{e}	26.3 ± 1.88^{e}
1	4.1 ± 0.03^{a}	58.8 ± 4.41^{b}	34.9 ± 0.74^{d}	78.0 ± 9.10^{a}
2	3.9 ± 0.08^{a}	49.8 ± 4.33^{c}	47.7 ± 0.57^{b}	58.8 ± 4.74^{b}
3	1.5 ± 0.25^{bc}	38.4 ± 4.57^{d}	48.6 ± 1.10^{a}	58.3 ± 15.31^{b}
4	1.3 ± 0.08^{cd}	24.6 ± 4.90^{e}	47.2 ± 0.33^{b}	54.7 ± 5.47^{b}
5	1.2 ± 0.08^{de}	20.2 ± 2.29^{f}	45.9 ± 0.74^{c}	50.7 ± 7.69^{bc}
6	1.1 ± 0.08^{de}	18.8 ± 3.18^{fg}	18.1 ± 0.65^{e}	49.6 ± 0.49^{bc}
7	1.0 ± 0.25^{ef}	15.7 ± 2.53^{gh}	12.8 ± 0.65^{f}	42.0 ± 4.25^{cd}
8	0.8 ± 0.16^{f}	13.4 ± 2.21^{hi}	9.5 ± 0.41^{g}	37.5 ± 4.16^{de}
9	0.4 ± 0.08^{gh}	13.0 ± 2.61^{hi}	7.6 ± 0.25^{h}	35.0 ± 4.08^{de}
10	0.2 ± 0.08^{hi}	10.5 ± 2.29^{i}	7.0 ± 0.33^{h}	30.0 ± 1.63^{e}

Data represent the means ± SD of three batches of fermentation.

Values bearing different superscripts in each column differ significantly ($P<0.05$).

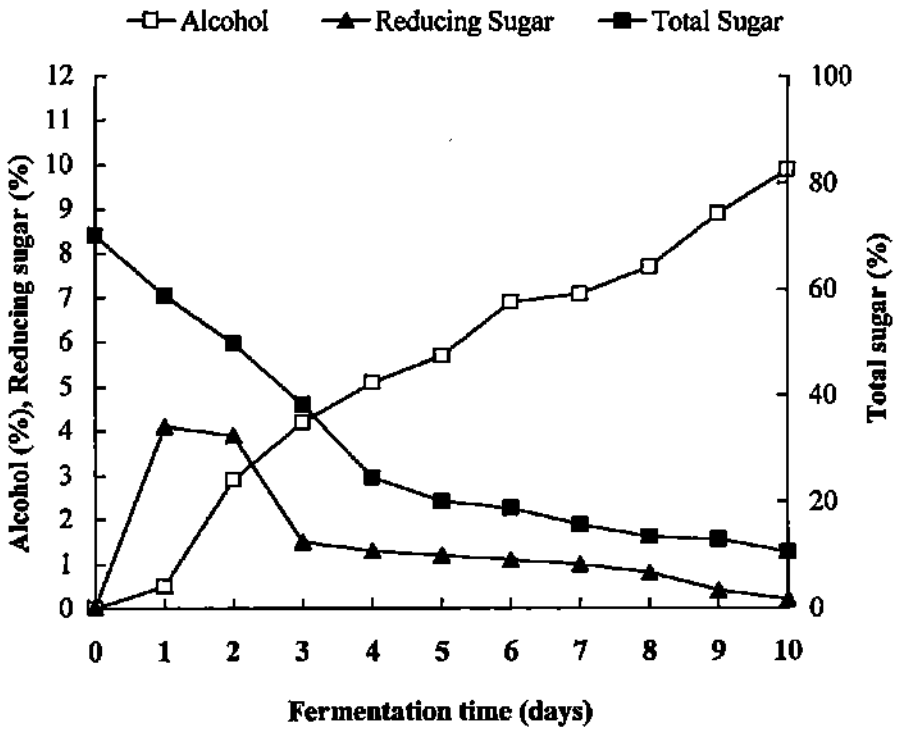


Fig 16. Changes in alcohol, reducing sugar and total sugar contents of fermenting maize during makai ko jaanr fermentation. Values are the means of three batches of fermentation.

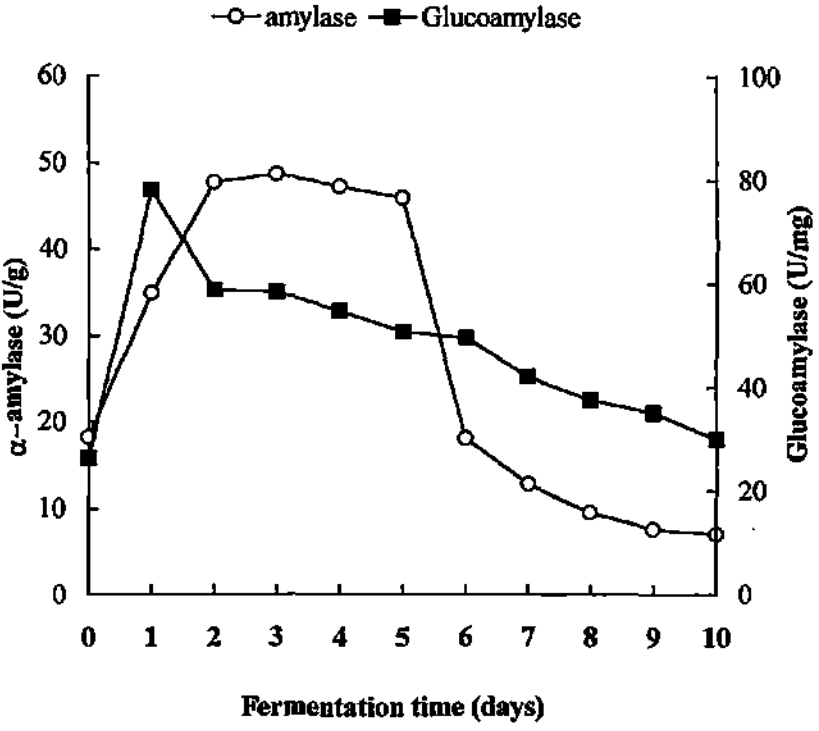


Fig 17. Changes in enzymatic activities of fermenting maize during makai ko jaanr fermentation. Values are the means of three batches of fermentation.

4.6. GAHOON KO JAANR

Gahoon ko jaanr is an alcoholic beverage, prepared from wheat (*Triticum aestivum* L.) (Plate 15). Method of preparation of gahoon ko jaanr is same as kodo ko jaanr (Fig 18). It is drunk directly by filtering the fermented grits. Sometimes, gahoon ko jaanr is mixed with kodo ko jaanr and filled up in toongbaa and consumed. Gahoon ko jaanr is mostly used for distillation to get raksi, clear distilled liquor.

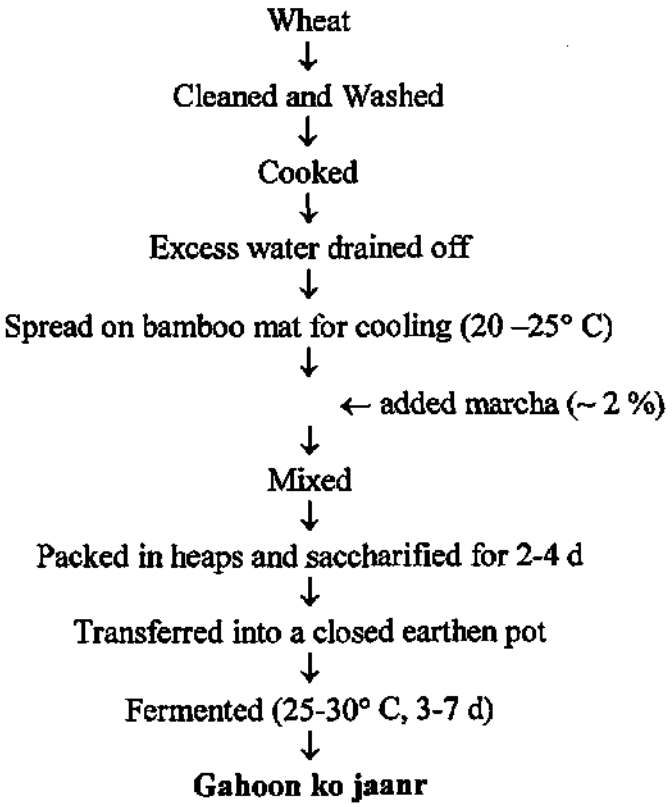


Fig 18. Flow sheet of gahoon ko jaanr preparation in East Sikkim

Feeding frequency of gahoon ko jaanr is shown in Table 48. Daily per capita consumption of gahoon ko jaanr is 13.5 g and 91.5 g in the Darjeeling hills and Sikkim, respectively.

Table 48. Feeding frequency and consumption of gahoon ko jaanr

	The Darjeeling hills	Sikkim
Feeding frequency (%)		
Daily	0	5
Weekly	15	10
Monthly	0	5
Occasional	0	5
Consumption *(g/capita/day)*	13.5 (0-142.3)	91.5 (0-357.1)

Weekly means twice in a week. Occasional means every three months.

Values are the means of 100 households each in rural areas of the Darjeeling hills and Sikkim, respectively. Ranges are given in parentheses.

4.6.1. Microorganisms

Ten samples of gahoon ko jaanr were collected from Aho village in East Sikkim and Algarah in Kalimpong, and were analysed for microbial load (Table 49). Yeasts population was 10 times higher than that of lactic acid bacteria. Moulds were not recovered in the final product.

Table 49. Microbial load of gahoon ko jaanr

Source	x 10^7cfu/g fresh weight		
	Yeast	LAB	Total Viable Count
Aho	4.8 (3.6-7.8)	0.4 (0.3-0.5)	5.6 (2.8-9.1)
Kalimpong	0.6 (0.2-1.0)	0.04 (0.03-0.05)	6.7 (5.0-10.0)

Data represent the means of 5 samples from each source. Ranges are given in parentheses.

4.6.1.1. Identification of yeasts and bacteria

Out of 47 strains of microorganisms isolated from ten samples of gahoon ko jaanr, 25 isolates were yeasts (Table 50) and 22 strains were lactic acid bacteria (Table 51). Representative strains of yeasts GJA:YP3 and GJK:YP2 were identified as *Pichia anomala* (E.C. Hansen) Kurtzman, and representative strains GJA:YS1 and GJK:YS3 were identified as *Saccharomyces cerevisiae* Meyen ex Hansen. Representative strains of lactic acid bacteria GJA:C1 and GJ:C2 were identified as *Pediococcus pentosaceus* Mees and GJA:R1 and GJK:R2 as *Lactobacillus bifermentans* Kandler, Schillinger and Weiss.

Table 50. Selection of representative strains of yeasts isolated from gahoon ko jaanr samples[a]

Source	Number of strains isolated	Colony	Cell shape	Mycelium	Ascospore	Grouped strains	Representative strains
Aho	15	Ss	O-E	Pseudo	Hat-shaped	9	GJA:YP3
		Ss	O-E	Pseudo	Globose	6	GJA:YS1
Kalimpong	10	Ss	O-E	Pseudo	Hat-shaped	5	GJK:YP2
		Ss	O-E	Pseudo	Globose	5	GJK:YS3

[a]Number of samples was 6 from each source. All isolates reproduced by multilateral budding.

Ss, smooth surface; O-E, Oval to ellipsoidal.

Table 51. Selection of representative strains of LAB isolated from gahoon ko jaanr[a]

Source	Number of strains isolated	Cell shape	Gas from glucose	NH_3 from arginine	Grouped strains	Representative strains
Aho	12	Coccus	–	+	6	GJA:C1
		Rod	+	–	6	GJA:R1
Kalimpong	10	Coccus	–	+	5	GJK:C2
		Rod	+	–	5	GJK:R2

[a]Number of samples was 6 from each source

[b]All isolates were Gram-positive, catalase-negative, non-sporeformers and non-motile

4.6.2. Proximate composition

Proximate composition of gahoon ko jaanr is presented in Table 52. The mean pH, acidity and alcohol content of the product was 3.9, 0.35 % and 3.1 %, respectively. Remarkable increase in crude fibre content was observed in gahoon ko jaanr. Calorie content remained same in the product. Magnesium, iron, sodium, potassium and phosphorous contents increased in finish product (Table 53).

Table 52. Proximate composition of cooked wheat and gahoon ko jaanr

Parameter	Unfermented	Fermented (Gahoon ko jaanr)	
	Cooked wheat	Aho	Kalimpong
pH	6.7 (6.6-6.7)	3.9 (3.4-4.0)	3.8 (3.6-4.0)
Moisture (%)	53.2 (52.0-55.0)	73.4 (70.0-75.5)	74.0 (67.0-81.0)
Acidity (%)	0.01 (0.01-0.01)	0.40 (0.34-0.62)	0.34 (0.25-0.42)
Alcohol (%)	0	2.6 (1.2-5.1)	3.5 (3.3-3.6)
Ash (% DM)	1.5 (1.3-1.8)	2.3 (1.5-2.8)	2.6 (1.8-2.9)
Fat (% DM)	0.6 (0.4-0.7)	0.6 (0.4-0.8)	0.5 (0.3-0.7)
Protein (% DM)	12.3 (11.8-12.7)	12.8 (11.0-13.0)	11.8 (11.0-12.0)
Crude fibre (% DM)	5.0 (3.3-6.0)	10.5 (9.2-11.9)	10.4 (9.5-11.0)
Carbohydrate (% DM)	85.6 (84.8-86.5)	84.3 (83.4-87.1)	85.1 (84.4-86.9)
Energy (MJ/100g DM)	397.0 (390.0-403.1)	393.8 (381.2-407.6)	392.1 (384.3-401.9)

Data represent the means of 5 samples from each source.

% DM, percentage on dry matter basis. Ranges are given in parentheses.

Table 53. Mineral contents of raw and fermented wheat

Mineral	mg/100 g dry matter	
	Wheat	Gahoon ko jaanr
Calcium	11.6	18.3
Magnesium	54	102
Manganese	1.7	2.9
Copper	0.8	1.0
Iron	4.7	13.6
Zinc	1.1	1.6
Sodium	13.1	26.7
Potassium	182	300
Phosphorous	392	763

Data represent the means of 2 samples.

4.7. RAKSI

Raksi is a clear distilled wine with characteristic aroma prepared from fermented cereal beverages such as kodo ko jaanr, bhaati jaanr, makai ko jaanr, gahoon ko jaanr, etc. Fermented masses of buckwheat, potato, canna, cassava roots are also distilled to get raksi.

4.7.1. Synonym of raksi

Raksi is a common term in Nepali meaning alcoholic drink. The Limboo calls it *sijongwaa aara*, Rai calls it *aarakha/hemma*, Gurung calls it *paa*, Tamang calls it *aaerak*, Sunwar calls it *rindho*, Newar calls it *aayala*, Magar calls it *dhise*, Sherpa calls it *aarak*, Bhutia calls it *aarak* and Lepcha calls it *aarok*.

4.7.2. Method of preparation

Fermented cereal beverages are distilled in a big cylindrical metallic vessel continuously for 2-3 h in an earthen-oven over firewood (Plate 16). At the top of the distilling vessel, cold water is kept in a metallic container used as condenser, water is replaced for 3-5 times after it gets boiled. Condensed raksi is collected in a small collecting metallic vessel called *poini*. Raksi prepared after replacing condensing water for 3 times is known as *theen pani raksi* which contains high alcohol and traditionally prepared for religious purposes. Raksi prepared after replacing the condensing water for 5 times is known as *panch pani raksi* which is a common alcoholic drink. Raksi is usually stored in bottle capped with piece of dry corncob (Plate 17).

Sometimes, petals of *Rhododendron* spp. are mixed during distillation to give distinct aroma in raksi. This type of raksi is

commonly prepared in Rimbik of Darjeeling and few places in West Sikkim.

4.7.3. Mode of consumption

Raksi is drunk directly without addition of water along with fried meat or side dish. Average daily per capita consumption of raksi is 91.5 ml and 66.9 ml in rural areas of the Darjeeling hills and Sikkim, respectively (Table 54).

The 100 ml of raksi costs about Rs.1.70 to Rs.2.00 per 100 ml in villages of the Sikkim Himalayas.

Table 46. Feeding frequency and consumption of raksi

	The Darjeeling hills	Sikkim
Feeding frequency (%)		
Daily	55	20
Weekly	15	20
Monthly	-	-
Occasional	15	20
Consumption *(ml/capita/day)*	91.5 (0-357.1)	66.9 (0-267.9)

Weekly means twice in a week. Occasional means every three months.

Values are the means of 100 households each in rural areas of the Darjeeling hills and Sikkim, respectively. Ranges are given in parentheses.

4.7.4. Equipment used

The traditional raksi distillation apparatus is made up of a metallic vessel (Plate 18). In main cylindrical metallic vessel measuring 40 cm × 30 cm × 25 cm, fermented grits (kodo ko jaanr or bhaati jaanr or gahoon ko jaanr) are steamed continuously for 2-3 h over firewood. Above main cylindrical vessel, a perforated container called *phunga* is placed. Inside *phunga*, a small metallic collector called *poini* is placed on iron-made tripod called *odhan* to collect distillate (raksi). Avove *phunga*, metallic vessel with cold water used as condenser is placed. Bottom of the condenser vessel is plastered by mud with the tip of *phunga* to prevent excess ventilation during distillation. This apparatus can distil 2-4 kg of *jaanr* to get 1-2 L of raksi after replacing condensing water 3 times.

4.7.5. Ethnical importance

Ethnical importance and essence of raksi in social activities by different ethnic communities was documented and noted in discussion chapter.

4.7.6. Proximate composition

Average pH, acidity and alcohol content of raksi, collected from different places of the Darjeeling hills and Sikkim, was 3.6, 0.06 % and 22.9 %, respectively (Table 55). Raksi distilled from bhaati jaanr mixed with few petals of *Rhododendron* showed highest alcohol content (~ 27 %) comparable to raksi prepared from other fermented cereals (Table 55).

Plate 16. *Raksi* distillation

Plate 17. *Raksi* in bottle capped with corncob

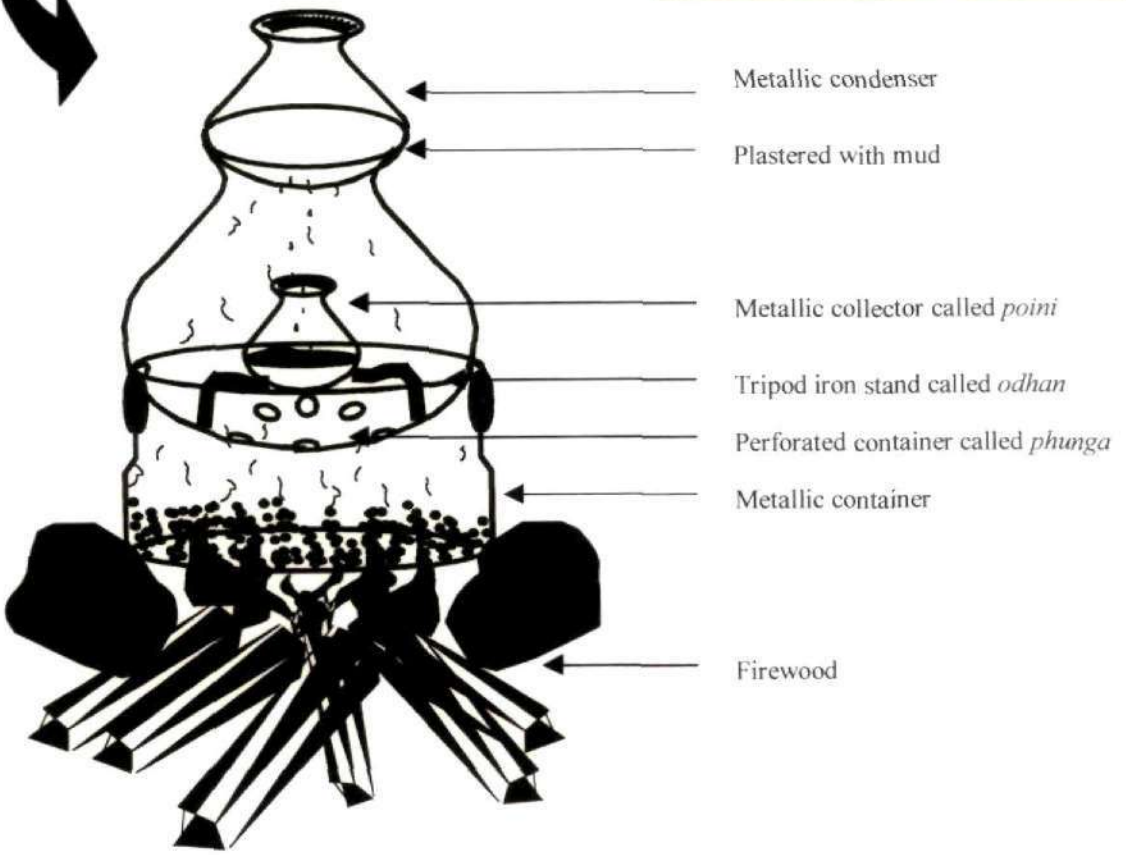


Plate 18. Internal diagrammatic view of *raksi* distillation apparatus

Table 55. Proximate composition of raksi

Parameter	RA	RRB	RR	RK	RN
pH	3.6 (3.2-3.8)	3.5 (3.3-3.7)	3.6 (3.3-3.8)	3.5 (3.4-3.6)	3.6 (3.5-3.7)
Acidity (%)	0.08 (0.03-0.1)	0.1 (0.1-0.1)	0.04 (0.03-0.04)	0.05 (0.04-0.06)	0.04 (0.04-0.05)
Alcohol (%)	22.6 (22.5-23.3)	24.0 (22.9-26.5)	22.8 (22.7-22.9)	22.7 (22.5-22.8)	22.5 (22.5-22.5)

RA: raksi (distilled from gahoon ko jaanr) collected from Aho

RRB: raksi (distilled from bhaati jaanr mixed with *Rododendron* petals) collected from Rimbik in Darjeeling

RR: raksi (distilled from bhaati jaanr) collected from Rongli

RK: raksi (distilled from makai ko jaanr) collected from Kalimpong

RN: raksi (distilled from kodo ko jaanr) collected from Namchi.

Data represent the means of 5 samples from each source. Ranges are given in parentheses.

5. DISCUSSION

5.1. SURVEY ON FERMENTED BEVERAGES

Common traditional fermented cereal-based beverages prepared and consumed by the different ethnic groups people in the Darjeeling hills and Sikkim are kodo ko jaanr, bhaati jaanr, makai ko jaanr and gahoon ko jaanr; and lesser-known alcoholic beverages are simal tarul ko jaanr, jao ko jaanr and faapar ko jaanr. Raksi, a wine distilled from fermented starchy materials is common alcoholic drink. Survey data indicated that in rural areas production of the traditional fermented beverages was mostly done at the individual household level for home consumption.

5.2. ETHNICAL IMPORTANCE

Traditional alcoholic beverages have strong ritual importance and are deep-rooted in the cultural heritage of the various ethnic groups of people in the Darjeeling hills and Sikkim. In these regions social activities require provision and consumption of appreciable quantities of alcoholic beverages by the 'matwali' castes meaning alcohol drinkers of the non-Brahmin Nepali community mostly Limboo, Rai, Gurung, Magar, Tamang, Sunwar, Newar and Sherpa; the Bhutia and the Lepcha tribes. Jaanr and raksi are essential to solemnize marriage ceremony of non-Brahmin Hindu Nepalis, the Buddhist tribes. Fermented beverages are offered to perform *the pitri puja* or *kul puja,* the religious practice to pray family Gods and Goddesses. Among the Lepcha, mong chee or kodo ko jaanr is essential to perform various cultural functions such as *lirum, sejum* and *namsung*. Mandokpenaa thea or kodo ko jaanr, filled in toongbaa and rice-made raksi are among the

important materials to perform the ritual practice of the Limboo called *tonsin mundhum*. In mourning, callers for condolences gathered to perform a memorial service are served traditional alcoholic beverages, mostly seen among the Sherpa and the Bhutia tribes. Spirit possession by the Limboo priests called *phedangma* and *bijuwa* need freshly distilled raksi.

5.3. MARCHA

Marcha is not a food but is the mixed dough inocula used as a starter culture for preparation of various indigenous alcoholic beverages. Marcha is produced at home in few villages in the Sikkim Himalayas. These marcha-making villages have been identified: Nor busty in Darjeeling (Bijanbari block), Kashyong and Mangzing in Kalimpong, Jhosing and Tibuk in North Sikkim, Chhejo and Lingchom in West Sikkim, Salghari and Barnyak in South Sikkim, Aho and Kopchey in East Sikkim. Marcha is exclusively prepared by the rural women belonging to the Limboo and Rai castes of the Nepali and the Lepcha tribes. This art of technology is protected as hereditary trade and passes from mother to daughters. Some local people are economically dependent upon this product. The marcha-making villages have linkages to nearby markets where marcha-makers can sell the products once or twice in a week. Earnings out of selling marcha supplement the domestic expenses. Among Rai, marital status is very strong determinant, only widows or spinsters are allowed to make marcha. Both Rai and Limboo consider marcha as an indispensable item for performing their ritual rites.

Unlike mixed culture starters of the other Asian countries, marcha is usually prepared by wrapping kneaded dough cakes in fern fronds with the fertile side touching them. This may be due to abundance of ferns locally called 'pire uneu' {*Glaphylopteriolopsis erubescens* (Wall ex Hook.) Ching} in the Sikkim Himalayas. Probably, germination of spores in sori helps to maintain the warmth of the fermenting mass in cold climates. Preparation of marcha is similar to those of other starter cultures of Asia. Marcha makers believe that addition of wild herbs give more sweetness to the product and they also believe that addition of chillies and ginger during marcha preparation is to get rid of devils that may spoil the product. This is actually to check growth of undesirable microorganisms that may inhibit growth of indigenous microflora of marcha. Studies of Soedarsono (1972) in ragi, an Indonesian rice-based starter culture, reveal that certain spices inhibit many undesirable microorganisms at the time of fermentation. Hesseltine (1983) has speculated that the spices, which are known to be inhibitory to many bacteria and moulds, are the agents that select the right population of microorganisms for fermentation. Marcha making technology reflects the traditional method of sub-culturing desirable inocula from previous batch to new culture using rice as base substrates. This technique preserves the microbial diversity essential for beverages production. Marcha retains its potency *in situ* for over a year or more.

Like other starter cultures of South-east Asia, marcha showed coexistence of mixed population of filamentous moulds, yeasts and lactic acid bacteria. The microbial analysis of marcha revealed that load of yeasts was higher than that of moulds and lactic acid bacteria. Out of 733 strains

of microorganisms isolated from sixty-six samples of marcha, 152 isolates were filamentous moulds, 321 were yeasts and 260 were lactic acid bacteria.

Following the taxonomical keys of Schipper (1976, 1984) and Hesseltine (1991), species of filamentous moulds were identified as *Mucor circinelloides* forma *circinelloides* van Tieghem, *Mucor* sp. close to *M. hiemalis* sensu lato, *Rhizopus chinensis* Saito, and *Rhizopus stolonifer* variety *lyococcus* (Ehrenb.) Stalp. & Schipper. Species of *Mucor* were more prevalent than species of *Rhizopus* in marcha samples anlaysed. *Aspergillus, Penicillium, Amylomyces* and *Actinomucor* were not recovered in marcha. According to Tanaka and Okazaki (1982), *Rhizopus* grows well on non-steamed grains, but its specific growth rate is decreased remarkably by steaming rice grain. The decrease is due to the heat denaturation of proteins in rice grain. *Rhizopus* and *Mucor* have also been reported in other similar rice-based cultures, such as ragi and chiuyueh (Hesseltine *et al.*, 1988), bubod (Tanimura *et al.*, 1977) and loogpang (Pichyangkura and Kulprecha, 1977).

Following the taxonomical keys described by Kreger-van Rij (1984) and Kurtzman and Fell (1998), four different genera of yeasts were isolated from marcha samples and identified as *Saccharomycopsis fibuligera* (Lindner) Klöcker, *Pichia anomala* (E.C. Hansen) Kurtzman, *Saccharomyces cerevisiae* Meyen ex Hansen and *Candida glabrata* (Anderson) Meyer et Yarrow. The nomenclature of *Endomyces fibuligera* Lindner (= *Endomyces lindneri* Saito) and *Hansenula anomala* (Hansen) Sydow and Sydow are now changed to *Saccharomycopsis fibuligera*

(Lindner) Klöcker and *Pichia anomala* (Hansen) Kurtzman, respectively (Hesseltine and Kurtzman, 1990). All strains of yeasts were present in marcha samples except *Candida glabrata* which showed only 45 % prevalence in sixty-six samples of marcha analysed. Among yeasts, *Saccharomycopsis fibuligera* was most dominant in marcha. *Saccharomycopsis fibuligera* is typically found growing on cereal products (Hesseltine and Kurtzman, 1990). *Saccharomycopsis malanga*, a phenotypically similar species that also produces amylase, was found in Chinese yeasts and ragi but not in bubod, probably due to geographical distribution of the species (Hesseltine and Kurtzman, 1990). In this finding, presence of *Saccharomyces cerevisiae* and *Candida glabrata* in marcha has been reported for the first time.

All isolates of lactic acid bacteria were Gram-positive, non-sporeforming, non-motile, catalase negative and facultative anaerobes and were grouped into coccii-tetrads and rods. Following the taxonomical keys of Sneath *et al.* (1986) and Wood and Holzapfel (1995), species of coccii-tetrads were identified as *Pediococcus pentosaceus* Mees. and heterofermentative rods were identified as *Lactobacillus bifermentans* Kandler, Schillinger and Weiss. Among lactic acid bacteria recovered *Pediococcus pentosaceus* was more predominant than *Lactobacillus bifermentans* in marcha. Hesseltine and Ray (1988) and Tamang and Sarkar (1995) reported only the presence of *Pediococcus pentosaceus* in marcha. Recovery of *Lactobacillus bifermentans* in marcha samples has been reported for the first time in this dissertation. The inability to utilize starch by these species indicates that they are not significant contributors to the

breakdown of starch of substrates during preparation of marcha itself or any beverage, probably their role is to give flavour and impart mild sour-taste, typical to jaanr. Role of lactic acid bacteria in the oriental starter cultures is likely to give flavour to the product with a pleasant taste (Hesseltine, 1983).

Marcha contains only 14 % moisture due to sun drying after the fermentation. The acidic nature (pH 5.58 with 0.01 % acidity) of marcha was due to the presence of high population of lactic acid bacteria. Due to low moisture content and acidic in nature, the shelf-life of marcha is long and can be stored at room temperature for a year or more.

The use of the API-zym technique has been reported (Arora *et al.*, 1990) as a rapid and simple means of evaluating and localizing 19 different hydrolases of microorganisms associated with dairy fermentations, mostly for selection of strains as potential starter cultures on the basis of superior enzyme profiles. We tried to use this API-zym technique to know the enzyme profiles of marcha isolates. Isolates of marcha showed relatively weak esterase and lipase activities (except by *Pichia anomala*) as compared with phosphatase activities. Yeasts strains mainly *Saccharomycopsis fibuligera* and *Pichia anomala* showed strong phosphatase and peptidase (leucine arylamidase) activities. Absence of proteinases (trypsin and chymotrypsin) and presence of high peptidase (leucine-arylamidase) and low esterase-lipase (C4 and C8) activities produced by the predominant organisms isolated from marcha are trades of desirable quality for their use in production of typical jaanr flavour.

Preliminary screenings of amylolytic activities of all isolates of marcha were tested in starch agar plates and strains were selected

accordingly. All strains of moulds and yeasts showed the amylolytic activities. None of the lactic acid bacteria showed amylolytic activity indicating they have no role in saccharification and liquefaction of substrates during jaanr fermentation. *Saccharomycopsis fibuligera* MS:YD4 showed highest liquefying activity (α-amylase) and *Rhizopus chinensis* MJ:Rh3 showed highest saccharifying activity (glucoamylase). It showed that *Rhizopus* played main roles in saccharification whereas *Saccharomycopsis fibuligera* played important role in liquefaction of substrate during jaanr fermentation. However, both of them played the main role in amylase production in starter cultures. Suprianto *et al.* (1989) reported the similar results in tapé that *Saccharomycopsis fibuligera* produced mainly α-amylase and *Rhizopus* sp. produced glucoamylase. Earlier reports showed that *Saccharomycopsis fibuligera* played the main roles in amylase production in starters of South-east Asia, and *Rhizopus* seemed to supplement the saccharification (Sukhumavasi *et al.*, 1975; Cronk *et al.*, 1977; Wei and Jong, 1983; Uchimura *et al.*, 1990; Yokotsuka, 1991). *Rhizopus* is known to produce good amount of glucomaylase (Ueda and Kano, 1975; Selvakumar *et al.*, 1996).

5.4. KODO KO JAANR

Kodo ko jaanr, prepared from dry seeds of finger millets, is one of the most popular fermented beverages consumed by 70 % of rural people in the Sikkim Himayalas. Milky white extract of kodo ko jaanr is sipped through a narrow bamboo straw called pipsing in a bamboo-or wood-made vessel called toongbaa which is a distinct feature of alcohol-drinking culture of the

mountain people. Consumption of fermented finger millets beverages in exclusively decorated bamboo or wood-made vessel called toongbaa is uncommon among other ethnic communities in Asia or elsewhere. This type of alcohol consumption or drinking is restricted only in the Himalayan regions. Guests are served with toongbaa along with fried meat or pickles. Description of alcohol-drinking custom in the Sikkim Himalayas was cited in some historical documents (Hooker, 1854, O'Malley, 1907; Risley, 1928). Kodo ko jaanr liquor is believed to be good tonic for ailing persons and post-natal women. After consumption, grits of kodo ko jaanr are used as fodder for pigs and cattle. This is a good example of total utilization of finger millet kodo as food beverage and fodder.

Population of yeasts was detected at the level of 10^7 cfu/g whereas that of lactic acid bacteria was comparatively less (~10^5 cfu/g) in forty samples of kodo ko jaanr, collected from different places of the Darjeeling hills and Sikkim. Filamentous moulds were not recovered in any finish product of kodo ko jaanr indicating that moulds have roles only in the initial phase of fermentation mostly in saccharification of the substrates. Out of 161 strains of microorganisms isolated, 81 isolates were yeasts and 80 isolates were lactic acid bacteria. These representative strains of yeasts were identified as *Pichia anomala* (E.C. Hansen) Kurtzman, *Saccharomyces cerevisiae* Meyen ex Hansen and *Candida glabrata* (Anderson) Meyer et Yarrow. *Saccharomycopsis fibuligera* was not recovered in finish product. Since it showed high amylolytic activities on the initial stage of fermentation, breaking starch into glucose, and on later stage, the population declined and disappeared. *Pichia anomala* and

Saccharomyces cerevisiae were present in all samples whereas *Candida glabrata* were recovered only in 40 % of samples analysed. Out of 80 strains of lactic acid bacteria, isolated from forty samples of kodo ko jaanr, 44 strains were coccii-tetrads and 36 strains were non-sporeforming rods. Species of lactic acid bacteria were identified as *Pediococcus pentosaceus* Mees and *Lactobacillus bifermentans* Kandler, Schillinger and Weiss. Both of them were recovered in all samples of kodo ko jaanr. Marcha used as starter supplemented the dominant microorganisms in kodo ko jaanr fermentation.

Due to cooking prior to fermentation, moisture content of the product was slightly higher in fermented product. The pH, titratable acidity and alcohol content of the product was 4.1, 0.27 % and 4.8 %, respectively. Kodo ko jaanr is mild-alcoholic sweet-flavoured beverage. It is rich in crude fibre and is high calorie food beverage. Because of high calorie, ailing persons and post-natal women consume the extract of kodo ko jaanr to regain the strength. Samantray *et al.* (1989) reported that finger millet also called 'ragi' in South India, is a good source of iron, calcium, magnesium and phosphorus. Fermentation of finger millet to kodo ko jaanr enhanced bioenrichment of minerals such as Ca, Mg, Mn, Fe, K, P. Kodo ko jaanr thus contributes to the mineral intake in daily diet of the local people.

Kodo ko jaanr was prepared in the laboratory following the traditional method by using marcha, collected from Aho village, as mentioned in 3.3.6. Changes in microbial population, physico-chemical and enzymatic activities in fermenting finger millet seeds during kodo ko jaanr

fermentation were investigated. Mould population, which was originated from marcha, declined significantly ($P<0.05$) every day during fermentation and finally disappeared after 5 d. Mucoraceae fungi have roles in initial phase of fermentation mostly in saccharification of the substrates. Population of yeasts increased significantly ($P<0.05$) from 10^5 cfu/g to 10^7 cfu/g within 2 d indicating that yeasts played important roles in amylase production. Among the yeasts, population of *Saccharomycopsis fibuligera* was more dominant on second day than that of *Pichia anomala*, *Saccharomyces cerevisiae* and *Candida glabrata*. Subsequently, load of lactic acid bacteria also increased significantly ($P<0.05$) from 10^6 cfu/g to 10^8 cfu/g in first day and decreased significantly ($P<0.05$) to a level of 10^3 cfu/g at the end.

Temperature of fermenting finger millet increased significantly ($P<0.05$) from 26° C to 30° C within 2 days due to exponential growth activities of mixed population of microorganisms. However, after second day of fermentation, temperature of fermenting millet remained constant around the incubation temperature of 28° C. The cause of increase in acidity and consequent drop in pH during fermentation was likely due to utilization of free sugars of the substrate by yeasts and lactic acid bacteria. Since all the strains were able to ferment glucose. Alcohol content increased significantly ($P<0.05$) from 0.1 % to 6.9 % within 6 d. The result showed that reducing sugar content increased significantly ($P<0.05$) till 3 d followed by decrease in total sugar content. This is due to maximum break down of starch of substrates to reducing sugars by amylolytic enzymes produced by moulds and yeasts during fermentation. Maximum activities of

saccharification (glucoamylase) and liquefaction (∝-amylase) of finger millets were observed on second day of fermentation. Saccharifying activities were mostly shown by *Rhizopus* spp. and *Saccharomycopsis fibuligera* whereas liquefying activities were shown by *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*. *Lactobacillus* and *Pediococcus* showed no amylolytic activities. The result indicated that *Saccharomycopsis fibuligera* and *Rhizopus* spp play the important role in saccharification process of *jaanr* fermentation, breaking starch of substrates into glucose for ethanol production. *Mucor* spp., *Pichia anomala* and *Candida glabrata* may supplement the saccharification.

Sterilised finger millet seeds were allowed to ferment with different combinations of strains of moulds, yeasts and lactic acid bacteria, previously isolated from marcha samples to test the ability of isolates to produce kodo ko jaanr under controlled conditions. Jaanr prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 showed significantly ($P<0.05$) high reducing sugar and alcohol contents on 6 d with slightly acidic pH than jaanr samples fermented by other strains. Sensory evaluation result showed that kodo ko jaanr prepared by a cell suspension mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 had significantly ($P<0.05$) highest score in general acceptability and was significantly ($P<0.05$) acceptable to judges. As seen from Table 7, *Saccharomyces cerevisiae* strains posses strong tendency to ferment glucose, galactose, maltose, raffinose, sucrose and starch into ethanol. Kozaki and Uchimura (1990) observed that *Saccharomyces cerevisiae* plays main role in alcohol fermentation in tapuy,

rice wine of the Philippines. *Rhizopus chinensis* MJ:Rh3 had highest glucoamylase activities thus breaking the starch of cooked millet to glucose which was fermented by *Saccharomyces cerevisiae* into alcohol and also retained some sugar to give a sweet taste with desirable alcoholic flavour. Whereas jaanr prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomycopsis fibuligera* MS:YDS2 had sweet-sour taste but due to low-alcohol content, the product had unpleasant odour, which could not be considered as good quality jaanr. Reddy and Basappa (1996) observed that *Saccharomycopsis fibuligera* produces high biomass during fermentation of cassava starch, which leads to lesser ethanol yield.

The consumers' preference trial showed that kodo ko jaanr prepared in the laboratory by cell suspension mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 as starter was more acceptable than the kodo ko jaanr prepared by conventional marcha. Sanchez and Kozaki (1984) stated that sensory evaluation in wine analysis is indispensable because even the accurate chemical analysis is not enough to guarantee the quality of a good wine. Laboratory-made kodo ko jaanr may have more advantages over the jaanr prepared by using marcha due to better quality in product, maintaining consistency and maximum utilization of substrates.

The results of the present studies demonstrate that to make a good quality jaanr, cell suspension mixture of a selected strain of mould *(Rhizopus)* and one amylolytic yeast (*Saccharomyces cerevisiae*) may be used as starter.

5.5. BHAATI JAANR

Bhaati jaanr is a soft, juicy and sweet-sour with mild-alcoholic beverage prepared from steamed glutinous rice and is consumed directly. Sometimes, yellowish-white supernatant liquor called nigaar was collected at the bottom of the earthenware crock, where bhaati jaanr was stored. It is a traditional diet for new mothers in villages who believe that it helps them to regain their strength.

Yeasts population was found higher than that of lactic acid bacteria in all twenty-four samples of bhaati jaanr samples. As in kodo ko jaanr, moulds were not recovered in any bhaati jaanr product analysed. Out of 127 strains of microorganisms isolated from bhaati jaanr samples, 69 isolates were yeasts and 58 isolates were lactic acid bacteria. Only two types of yeasts were recovered from bhaati jaanr samples and were identified as *Pichia anomala* (E.C. Hansen) Kurtzman and *Saccharomyces cerevisiae* Meyen ex Hansen. The association of *Saccharomyces cerevisiae* with bhaati jaanr fermentation can be attributed by its strong ability to ferment sugar as well as being ethanol tolerant and would be able to produce alcohol. Presence of *Pichia anomala* in bhaati jaanr samples may contribute ester odour to product. Cronk *et al.* (1979) reported that *Hansenula anomala* (now *Pichia anomala*) contributed ester odour to tapé which is similar to bhaati jaanr. *Pediococcus pentosaceus* and *Lactobacillus bifermentans* were found in bhaati jaanr samples. Probably the presence of lactic acid bacteria in bhaati jaanr contributes characteristic flavour to the product and making the product slightly acidic in nature.

The moisture content in bhaati jaanr was higher than unfermented steamed rice due to juicy nature of the fermented product. The pH, acidity and alcohol contents of the product were 3.5, 0.24 % and 5.9 %, respectively indicating bhaati jaanr as sour, acidic and mild-alcoholic beverage. Fat, protein and calorie contents remained same as the substrate. Considerable increase in calcium, manganese, iron, zinc, sodium, potassium and phosphorous was observed in bhaati jaanr over the substrate, which revealed that fermentation enhanced the bioenrichment of minerals in the product. Though bhaati jaanr is consumed as beverage, it can also be eaten as dissert or staple food.

Loads of moulds, decreased significantly ($P<0.05$) during fermentation and disappeared after the fifth day of fermentation. Population of yeasts increased significantly ($P<0.05$) from 10^5 cfu/g to 10^8 cfu/g within 2 d, and decreased to a level of 10^5 cfu/g in 10 d. Exponential increase in load of lactic acid bacteria was significant ($P<0.05$) till second day of fermentation, and then declined slowly.

Temperature of fermenting rice increased above the incubation temperature of 28° C due to growth of mixed microorganisms within 3 d and remained constant to that of incubation temperature. During fermentation, pH decreased and acidity increased significantly ($P<0.05$). The cause of increase in acidity and decreased in pH during fermentation was likely due to utilization of free sugars of the substrate by yeasts and lactic acid bacteria. Since all the strains were able to ferment glucose. Thus alcohol content increased significantly ($P<0.05$) during fermentation. The result showed that reducing sugar content increased significantly ($P<0.05$)

till 3 d followed by decrease in total sugar content. This is due to maximum break down of starch of substrates to reducing sugars by amylolytic enzymes produced by moulds and yeasts within 3 d during fermentation. Maximum activities of saccharification and liquefaction of glutinous rice were observed on the third day of fermentation. This result suggested that *Saccharomycopsis fibuligera* and *Rhizopus* spp contribute in saccharification and liquefaction of glutinous rice, breaking starch of substrates into glucose for alcohol production and also in aroma formation in bhaati jaanr preparation. Suprianto *et al.* (1989) reported that *Saccharomycopsis* is important in aroma formation in tapé preparation and *Rhizopus* sp. is important in liquefy and saccharify the glutinous rice.

5.6. MAKAI KO JAANR

Makai ko jaanr is viscous, slightly bitter, mild-alcoholic beverage, made from maize and the fermented mass extract is drunk directly. Africans consume varieties of fermented maize products, which include staples, gruel and beverages, and most of these are naturally fermented involving lactic acid bacteria or yeast or mixture of lactic acid bacteria-yeast (Niche, 1995). Unlike African fermented maize products, in the Sikkim Himalayas, maize is used only as mild-alcoholic beverage prepared by using starter.

Yeasts were found 10 times higher than that of lactic acid bacteria in makai ko jaanr samples. Out of 100 strains of microorganisms isolated from twelve samples of makai ko jaanr, 54 isolates were yeasts and 46 isolates

were lactic acid bacteria. Filamentous moulds were not recovered in the final product. Among the yeasts, 30 strains were identified as *Pichia anomala* (E.C. Hansen) Kurtzman and 24 strains were identified *Saccharomyces cerevisiae* Meyen ex Hansen. Out of 46 lactic acid bacteria strains, 28 were coccoid cells in tetrads (*Pediococcus pentosaceus* Mees) and 18 strains were non-sporeforming rods (*Lactobacillus bifermentans* Kandler, Schillinger and Weiss).

The pH and acidity of makai ko jaanr was 3.3. and 0.38 %, respectively indicating the products is slightly acidic in nature. Alcohol content of the product was 2.5 %, comparatively less than that of other cereal-based jaanr products. Makai ko jaanr is low-alcohol content food beverage. Increase in crude fibre content was observed in makai ko jaanr. Calorie value remained almost same in both unfermented and fermented maize. Remarkable increase in iron, potassium and phosphorous was observed in makai ko jaanr indicating that bioavailability of minerals is enhanced during fermentation.

Filamentous mould count declined significantly ($P<0.05$) during makai ko jaanr fermentation and disappeared after the fourth day, as seen in other jaanr fermentation, indicating that these organisms have roles only in the initial phase of fermentation, mainly in production of amylase. The load of yeasts increased significantly ($P<0.05$) within 2 d and remained constant till eighth day, and decreased significantly ($P<0.05$) till an end of fermentation. This result indicated that maximum activities of saccharification and liquefaction of maize was shown by yeasts, mainly by *Saccharomycopsis fibuligera* within 2 d and, consequently the population

of these yeasts declined gradually towards the end of fermentation. This is the reason why *Saccharomycopsis fibuligera* was not recovered in any final product of makai ko jaanr. Lactic acid bacteria population increased significantly ($P<0.05$) from 0 day to 2 day and declined gradually till the end of fermentation. This may be due to availability of free sugars, after breaking starch of maize by moulds and yeasts during saccharfication, for the growth of lactic acid bacteria, originated from marcha. Ekundayo (1969) and Achi (1990) proposed that extracellular amylolytic enzymes of microorganisms help to break down the starch of maize and sorghum into beverage which are likely to stimulate the growth of lactic acid bacteria.

Temperature of fermenting maize remained relatively constant to incubation temperature throughout the fermentation. During fermentation, pH dropped significantly ($P<0.05$) within 10 d and acidity increased significantly ($P<0.05$) on each day. Reducing sugar increased significantly ($P<0.05$) within 1 d followed by maximum activities of saccharification by moulds and yeasts. However, liquefying activities was maximum till third day of fermentation. Saccharification and liquefaction of fermenting maize seeds were carried by *Saccharomycopsis fibuligera* and *Rhizopus* spp., thus increasing free sugars to produce alcohol. Alcohol content increased significantly ($P<0.05$) during fermentation of makai ko jaanr. *Mucor* spp. and *Pichia anomala* may supplement the saccharification. The decrease in total sugar content of maize could be attributed to the utilization of the sugars by fermenting microorganisms. Adegoke *et al.* (1995) observed that microorganisms are known to depend on reducing sugars for their metabolic processes.

5.7. GAHOON KO JAANR

Gahoon ko jaanr is wheat-based alcoholic beverage and is drunk directly by filtering the fermented grits. Gahoon ko jaanr is mostly used for distillation to get raksi.

Yeasts population was 10 times higher than that of lactic acid bacteria in ten samples of gahoon ko jaanr. Moulds were not recovered in the finished product. Out of 47 strains of microorganisms isolated from ten samples of gahoon ko jaanr, 25 isolates were yeasts {*Pichia anomala* (E.C. Hansen) Kurtzman and *Saccharomyces cerevisiae* Meyen ex Hansen} and 22 strains were lactic acid bacteria (*Pediococcus pentosaceus* Mees and *Lactobacillus bifermentans* Kandler, Schillinger and Weiss). The microbial composition was almost same as that of other jaanr products, since the microorganisms were originated from marcha, used for preparation of this alcoholic product.

The pH, acidity and alcohol content of the product was 3.9, 0.35 % and 3.1 %, respectively indicating that this product is acidic and mild alcoholic beverage. Fermentation resulted in increase in crude fibre content in gahoon ko jaanr. Minerals were enhanced due to fermentation. Gahoon ko jaanr is rich in iron, sodium, potassium and phosphorous.

5.8. RAKSI

Raksi is a clear, distilled wine with characteristic aroma prepared from fermented cereal beverages such as kodo ko jaanr, bhaati jaanr, makai ko jaanr, gahoon ko jaanr. It is drunk directly without addition of water. Unlike other cereal-based wine of Asian countries, where the fermented rice or other cereal is decanted or filtered, in the Sikkim Himalayas fermented cereals or starchy substrates are distilled to get high alcoholic drink, which can stored for long. Raksi preparation is done by alcoholic distillation method in traditional way by the rural women. The crude method is still operating in most of the villages, and some people have improvised the apparatus. The indigenous knowledge of alcohol distillation by mountain women is worth noting. Alcohol content of raksi is higher than the jaanr due to distillation. Raksi is slightly sour in taste. Raksi distilled from bhaati jaanr mixed with few petals of *Rhododendron* showed highest alcohol content (~ 27 %) comparable to raksi prepared from other fermented cereals.

6. SUMMARY

Traditional fermented beverages constitute an integral part of dietary culture and have strong ritual importance among the ethnic people in the Darjeeling hills and Sikkim of the Sikkim Himalayas in India. Common indigenous fermented beverages are kodo ko jaanr, bhaati jaanr, makai ko jaanr and gahoon ko jaanr. Raksi is distilled liquor prepared from fermented starchy materials. About 57.6 % and 76.7 % of rural people prepared fermented beverages for home consumption in the Darjeeling hills and in Sikkim, respectively.

Marcha, a dry, spherical or flattened, solid ball-like starter used for production of indigenous alcoholic beverages. It is traditionally prepared from rice mixed with wild herbs and spices. Sixty-six samples of marcha, collected from eleven different important marcha-making villages of the region, were analysed and microbial load of filamentous moulds, yeasts, lactic acid bacteria and total viable counts were found at the level of 10^6 cfu/g, 10^8 cfu/g, 10^7 cfu/g, and 10^8 cfu/g, respectively. Out of 733 strains of microorganisms, 152 were filamentous moulds, 321 strains were yeasts and 260 were lactic acid bacteria. Filamentous moulds were identified as *Mucor circinelloides* forma *circinelloides*, *Mucor* sp. (close to *M. hiemalis*), *Rhizopus chinensis* and *Rhizopus stolonifer* variety *lyococcus*. Species of *Mucor* were more prevalent than species of *Rhizopus* in marcha samples analysed. Yeasts were identified as *Saccharomycopsis fibuligera*, *Pichia anomala*, *Saccharomyces cerevisiae* and *Candida glabrata*. Among yeasts, *Saccharomycopsis fibuligera* was most dominant in marcha. Lactic acid bacteria *Pediococcus pentosaceus* and *Lactobacillus bifermentans* were isolated from marcha. *Pediococcus pentosaceus* was more predominant

than *Lactobacillus bifermentans* in marcha. Marcha contained 14 % moisture, pH 5.58, 0.01 % acidity and 1.4 % ash.

Saccharomycopsis fibuligera and *Pichia anomala* showed strong phosphatase and peptidase activities in API-zym system applied. All isolates showed relatively weak esterase and lipase activities except by *Pichia anomala* as compared with phosphatase activities. Strains of moulds and yeasts showed the amylolytic activities whereas none of the lactic acid bacteria showed amylolytic activity. *Saccharomycopsis fibuligera* MS:YD4 showed highest α-amylase activity and *Rhizopus chinensis* MJ:Rh3 showed highest glucoamylase activity.

Kodo ko jaanr, prepared from finger millets, is common fermented beverage in the Sikkim Himalayas. Milky white extract of kodo ko jaanr is sipped through a narrow bamboo straw called pipsing in a bamboo-or wood-made vessel called toongbaa, which is unique food culture in the region. Population of yeasts was detected at the level of 10^7 cfu/g whereas that of lactic acid bacteria was comparatively less (~10^5 cfu/g) in forty samples of kodo ko jaanr. Filamentous moulds were not recovered in any finish product. Out of 161 strains of microorganisms, 81 isolates were yeasts *Pichia anomala*, *Saccharomyces cerevisiae* and *Candida glabrata* and 80 isolates were lactic acid bacteria *Pediococcus pentosaceus* and *Lactobacillus bifermentans*. *Pichia anomala* and *Saccharomyces cerevisiae* were present in all samples whereas *Candida glabrata* were present only in 40 % of samples analysed. Lactic acid bacteria were recovered in all samples. The proximate composition of kodo ko jaanr sample was analysed. The pH, moisture, acidity and alcohol content of the product was 4.1, 69.7

%, 0.27 % and 4.8 %, respectively. Kodo ko jaanr is rich in crude fibre. Ash, fat and protein content remained the same as that of substrate. Fermentation of finger millet enhanced bio-enrichment of minerals.

Kodo ko jaanr was prepared in the laboratory following the traditional method by using marcha. Changes in microbial population, physico-chemical and enzymatic activities in fermenting finger millet seeds during kodo ko jaanr fermentation were investigated. Mould population declined significantly ($P<0.05$) every day during fermentation and finally disappeared after 5 d. Population of yeasts increased significantly ($P<0.05$) from 10^5 cfu/g to 10^7 cfu/g within 2 d. Lactic acid bacteria also increased significantly ($P<0.05$) from 10^6 cfu/g to 10^8 cfu/g in first day and decreased significantly ($P<0.05$) to a level of 10^3 cfu/g at the end. Total viable count increased significantly ($P<0.05$) within 1 d and decreased. Temperature of fermenting finger millet remained between 26° C to 30° C during fermentation. The mean pH decreased and acidity increased during fermentation. Alcohol content increased significantly ($P<0.05$) from 0.1 % to 6.9 % within 6 d. Reducing sugar content increased significantly ($P<0.05$) till 3 d and decreased, followed by decrease in total sugar content. Maximum activities of saccharification and liquefaction of finger millets were observed on second day of fermentation.

Different combinations of moulds, yeasts and lactic acid bacteria isolates, previously isolated from marcha, were inoculated aseptically on sterilized finger millets to test the ability of isolates to produce kodo ko jaanr under controlled conditions. Jaanr prepared by a combination of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 showed

significantly (P<0.05) high reducing sugar and alcohol contents than jaanr fermented by other strains. Sensory evaluation result also showed that kodo ko jaanr prepared by this combination had significantly (P<0.05) highest score in general acceptability. The consumers' preference trial was carried out. Kodo ko jaanr prepared in the laboratory by mixture of *Rhizopus chinensis* MJ:Rh3 and *Saccharomyces cerevisiae* MJ:YS2 as starter was more acceptable than product prepared by conventional marcha.

Bhaati jaanr is a soft, juicy and sweet-sour with mild-alcoholic beverage prepared from steamed glutinous rice and is consumed directly. Yeasts population was found higher than that of lactic acid bacteria in all twenty-four samples of bhaati jaanr samples. Moulds were not recovered in any final product analysed. Out of 127 strains of microorganisms isolated, 69 isolates were yeasts *Pichia anomala* and *Saccharomyces cerevisiae*, and 58 isolates were lactic acid bacteria *Pediococcus pentosaceus* and *Lactobacillus bifermentans*. The pH, moisture, acidity and alcohol contents of the product were 3.5, 83.4%, 0.24 % and 5.9 %, respectively. Ash content was slightly higher than the substrate. Fat, protein and calorie contents remained same as the substrate. Mineral contents increased in bhaati jaanr over the substrate.

Moulds decreased significantly (P<0.05) during fermentation and disappeared after the fifth day of fermentation. Loads of yeasts increased significantly (P<0.05) from 10^5 cfu/g to 10^8 cfu/g within 2 d, and decreased to a level of 10^5 cfu/g at the end. Lactic acid bacteria increased significantly (P<0.05) till second day of fermentation, and then declined slowly. Temperature of fermenting rice remained between 28° C to 30° C. During

fermentation, pH decreased, and acidity and alcohol content increased significantly (P<0.05). Reducing sugar content increased significantly (P<0.05) till 3 d, and decreased followed by decrease in total sugar content. Maximum activities of saccharification and liquefaction of glutinous rice were observed on the third day of fermentation.

Makai ko jaanr is a viscous, mild-alcoholic beverage, made from maize and is drunk directly. Yeasts were found 10 times higher than that of lactic acid bacteria. Out of 100 strains of microorganisms isolated from twelve samples of makai ko jaanr, 54 isolates were yeasts and 46 isolates were lactic acid bacteria. Filamentous moulds were not recovered in the final product. Among the yeasts, 30 strains were *Pichia anomala* and 24 strains were *Saccharomyces cerevisiae*. Out of 46 lactic acid bacteria strains, 28 were *Pediococcus pentosaceus* and 18 strains were *Lactobacillus bifermentans*. The pH, moisture and acidity of makai ko jaanr were 3.3, 81.9 % and 0.38 %, respectively. Alcohol content of the product was 2.5 %, comparatively less than that of other cereal-based jaanr products. Ash content was slightly higher in the product. Fat, protein and calorie value remained same. Crude fibre content increased in makai ko jaanr. Mineral contents increased in makai ko jaanr.

Mould count declined significantly (P<0.05) during makai ko jaanr fermentation and disappeared after the fourth day. The load of yeasts increased significantly (P<0.05) within 2 d and remained constant till eighth day, and decreased significantly (P<0.05) till an end. Lactic acid bacteria population increased significantly (P<0.05) from 0 day to 2 day and declined gradually. Temperature of fermenting maize remained relatively

constant to incubation temperature. During fermentation, pH dropped significantly ($P<0.05$) whereas acidity and alcohol increased significantly ($P<0.05$). Reducing sugar increased significantly ($P<0.05$) within 1 d followed by decrease in total sugar. Maximum activities of α-amylase and glucoamylase were observed on third and first day, respectively.

Gahoon ko jaanr is a fermented wheat-based, mild-alcoholic beverage and is drunk directly by filtering the fermented grits. It is mostly used for distillation to get raksi. Yeasts population was 10 times higher than that of lactic acid bacteria in ten samples. Moulds were not recovered in the finished product. Out of 47 strains of microorganisms, 25 isolates were yeasts *Pichia anomala* and *Saccharomyces cerevisiae*, and 22 strains were lactic acid bacteria *Pediococcus pentosaceus* and *Lactobacillus bifermentans*. The pH, moisture, acidity and alcohol content of the product was 3.9, 73.7%, 0.35 % and 3.1 %, respectively. Ash content was slightly higher whereas, fat and protein remained the same. Fermentation resulted in increase in crude fibre content. Minerals were enhanced due to fermentation.

Raksi is a clear, distilled wine with characteristic aroma prepared from fermented cereal beverages and is drunk daily. Raksi is prepared using traditional distillation apparatus, used by the rural women. The pH, acidity and alcohol content of raksi were 3.6, 0.06% and 22.9% respectively.

Outlines of findings are ☞

- ❖ Traditional method of sub-culturing of mixed inocula using rice as base substrates for preparation of starter called marcha was investigated.
- ❖ Indigenous knowledge on beverages making technology practiced by rural women belonging to ethnic communities of the Darjeeling hills and Sikkim was documented.
- ❖ Microorganisms ranging from filamentous moulds (*Mucor circinelloides, Mucor* sp. (close to *Mucor hiemalis*), *Rhizopus chinensis, Rhizopus stolonifer* variety *lyococcus*;) to amylolytic and alcohol-producing yeasts (*Saccharomycopsis fibuligera, Pichia anomala, Saccharomyces cerevisiae* and *Candida glabrata*) and acid producing bacteria (*Pediococcus pentosaceus* and *Lactobacillus bifermentans*) associated with marcha and various types of jaanr products were isolated, characterised, identified, indexed and preserved.
- ❖ Proximate composition of these indigenous fermented beverages was determined to know their food value.
- ❖ Attempt was made to upgrade the traditional processing of kodo ko jaanr using selected strains instead of conventional marcha.

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