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Occurrence and diversity of phenotypically distinct yeast strains isolated from starter cultures used in alcoholic fermentation by two ethnic tribes of Arunachal Pradesh

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Abstract

The present investigation is an attempt to isolate and identify the yeasts used by some local aborigines of Arunachal Pradesh and observe their behaviour under *in-vitro* conditions. Interestingly, all the isolates have shown a preference of growth at neutral pH; a character not usually observed in yeasts who generally prefer an acidic medium for growth. The results obtained in the present findings suggest that all the isolates are different in terms of their physiological or biochemical activities. One important aspect of the studies was the successful isolation of pure cultures from a consortium whose contents were previously unknown (Sarma, 2002). Protein profiling of the isolates have revealed that the isolates are indeed variants at the genetic level as was evident from expression of whole cell proteins. The isolates might therefore be mutagenic variants, which arose during segregation through time immemorial. It can be predicted that careful identification and selection of yeasts from starter cultures employed in traditional brewing in northeast India can yield industrially important and beneficial yeast strains for the exploitation and benefit of mankind.

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Introduction

Yeasts have been regarded as major sources of ethanol and other alcoholic beverage production since time immemorial. The identity of yeasts for wine, both for household consumption and large-scale industrial uses has been appreciated after the discovery of the fermentation process by Louis Pasteur (Black, 1999). The diverse and dynamic activities of yeasts have impact on many aspects of wine making (Fleet, 1998). The northeastern part of India is well known for production of household liquors; which is associated with the regions rich indigenous knowledge system and this knowledge is extricably linked to its social, cultural, environmental and institutional contexts (Sharma and Mazumdar, 1980, Ghosh, 1992). Traditional knowledge of wine making in northeast India has been both explicit and tacit that has been codified into words or transferred from one generation to another through ages; thereby suggesting a sense of common or communal ownership amongst different communities (Ghosh, 1992). The various ethnic tribes of northeast India represent a concoction of various original aborigines, which include the Mongoloid, the Chinese and the Aryan descent (Ghosh, 1992). On the other hand, traditional alcoholic brewing is an important household cum soci *et al,* drinking activity associated to many religious ceremonies among different ethnic tribal groups of this region (Ghosh, 1992 ; Demuyter *et al.,* 2004). The methods for wine and beverage production among the tribes differ as all of them follow their own indigenous protocols employing different starter cultures, although most of them use similar substrates for fermentation (Karotemprel, 1984). These locally produced alcohols and alcoholic beverages have several limitations like bad odour, turbidity, toxic metabolites, texture and inconsistency which not only lower down the quality and yield but also contribute to obstacles on the commercialization of the fermented products (Karotemprel, 1984, Demuyter *et al.*, 2004). Most of the tribes use either rice or millet as sources of starter cultures for perpetuation of yeast strains (Karotemprel, 1984). As is common, the Nyshi and Apatani tribes of Arunachal Pradesh have been employing yeasts for various fermentation processes, since time immemorial. They prepare indigenous alcoholic beverages for domestic consumption by utilizing yeasts as substrate cakes. The exploitation of various yeast isolates and their perpetuation has been in practice since ancient times (Demuyter *et al.*, 2004, Ramirez *et al.*, 2004). The processes of alcoholic fermentation by various tribes have been observed to have changed with the progress of individual tribal communities. The consequential effect of this perpetuation is that with time, the nutritional status of the strains have changed and shifted, which might be attributed to certain spontaneous mutations of the strains under natural conditions.

No study on isolation and yeast strains from starter cultures used in alcoholic fermentation by two ethnic tribes of Arunachal Pradesh is available in the literature. Therefore, the present investigations were studied in an attempt to isolate and identify the yeasts used by some local aborigines of Arunachal Pradesh and observe their behaviour under *in-vitro* conditions.

Materials and methods

Sources of yeast starter cultures

Starter culture cakes used for fermentation and household production of alcoholic beverages by Apatani and Nyshi tribal population of Arunachal Pradesh were collected from remote villages (Barnett *et al.,* 2000).

Yeast isolation and identification

The fermentative cakes thus collected were carried to the laboratory in polypropylene packs and were allowed to dry in the shade for 2 days. 1.0 gram of the crude samples were weighed, dissolved into 10ml of double distilled water and diluted 1000 fold for successful isolation of yeast pure cultures by repetitive streaking in solid YPD-agar {containing 1% Yeast extract (Sigma, USA), 2% Bactopeptone (Difco, Detroit, USA), 2% Dextrose (Merck, India) and 2% Agar (Qualigens, USA)} media supplemented with

antibiotics {Chloramphenicol 100 mg l^{-1} (Sigma C-0378) and 50 mg l^{-1} Chlortetracycline (Sigma C-4881)} following methodology mentioned earlier by Even, 1996. The Petri plates were incubated at 30 °C and after 5 successive streaking of the original consortium for a number of batches; pure cultures of single colony isolates were obtained which were identified in an inverted microscope (Leica MPS-30, Germany) at a magnification of 200X. Altogether 4 different yeast strains from the collected starter culture materials were obtained (Table 1) which were inoculated into fresh liquid YPD medium and incubated at 30 °C to obtain mass culture of the strains.

Purification and maintenance of yeasts

Cells were initially grown under anaerobic conditions for 36 hours to an optical density at 595nm, yielding 260 grams of cells (Mills *et al.*, 2002). After 72 hours of incubation, the samples were diluted 1000 folds followed by recording of colony forming unit (cfu) for each isolate (Table 1). From a suitable dilution (2 X 10^6 cells/ml) of each sample, the 8 representative isolates were picked up and recultivated in YPD liquid media for 48 hours to ensure the purity of the cultures obtained. Aliquots of the pure yeast isolates obtained from batch cultures as stated above were designated with codes (Table 1); maintained in glycerol stock concentration of 15% (v/v) and on YPD agar slants and stored at −80 °C for further investigations. For routine studies of the yeast isolates, scrapings from the frozen cultures were transferred into fresh YPD medium every 30 days as was described by Even, 1996.

Phenotypic characterization of the isolated yeasts

Micromorphological description of the isolates obtained was performed as was described by Yarrow, 1998. Growth of the isolates in solid media was determined by visual examination of 4 days old cultures incubated at 30°C. The gross morphology of cells was determined by microscopic examination of 24-h old cultures grown in YPD broth incubated at 30°C (Table 1).

Physiological characterization of the isolated yeasts

The variation in growth pattern of the yeast isolates under study was obtained by analyzing extracellular protein content secreted by the isolates in 100ml YPD liquid media (pH 7.0) incubated in a BOD incubator (ICT, India) at 30°C for 10 days following the methodology of Yarrow (1998) with some modifications (Yarrow,1998). The observations for growth behaviour of the isolates were measured on the basis of extracellular protein content calculated from their extinction coefficient taken at 660nm in a UV-VIS spectrophotometer (U-2001 Hitachi, Japan). Axenic cultures of yeasts obtained after successive sub-culturing were subjected to different temperature regimes to observe their thermotolerance. 1µl of all the isolates grown homogenously at a cell concentration of 2 X 10^6 cells/ml in liquid YPD media were transferred to 100 ml liquid YPD media (pH 7.0) in 250ml Erlenmeyer flasks which were incubated at three different temperatures viz. 28°C, 35°C and 40°C respectively in a BOD incubator for a period of one week. 1ml of the incubated cultures after 3 days were diluted 10 times and the growth potential of the isolates were calculated from protein content which was determined by observing the extinction coefficient at 660nm in a UV-VIS spectrophotometer. Sensitivity of the isolates to growth conditions of variable pH (5.0, 5.5, 6.0, 6.5 & 7.0) was studied from protein concentrations of 72 hours incubated YPD liquid cultures obtained by measuring the extinction coefficients at 600nm in a UV-VIS spectrophotometer following the methodology followed by Gao and Fleet (1998). The pH of the prepared media before inoculation was optimized with the addition of 0.5M Na_2HPO_4 to attain the desired pH.

Isolation and determination of whole cell proteins

For whole cell protein isolation, 130 grams of frozen cells were grounded in a pestle and mortar (HiMedia, India) in presence of liquid nitrogen. Cells were thawed and resuspended in 90 ml of KPF1 lysis buffer {50mM HEPES-KOH (pH 7.6), 10% glycerol, 0.3% Nonidet P-40, 2mM $MgSO_4$, 2mM $CaCl_2$, 150mM potassium acetate (KOAc), 2.5mM β-mercaptoethanol and 1mM phenylmethylsulfonyl fluoride} following

the methodology followed by Lowry *et al.*,1951. To the cell suspension, an equal volume of 425-600µm glass beads (HiMedia) was added and the cells were further lysed in a Bead Beater (Bio-spec, USA) using 15 cycles of 20 s on and 100 s off. The suspension was centrifuged at 5000 Xg for 10 minutes in a refrigerated centrifuge, supernatant was decanted in 2.0ml eppendorf tubes while the cell pellet containing cell debris and glass beads was discarded. The total whole cell protein content was determined following the methodology as was described by Lowry *et al.*,1951, using Folin-Ciocalteau reagent. The protein equivalent was calculated from a standard protein assay curve of Bovine serum albumin (BSA, Merck, Germany).

Protein profiling of yeast isolates using one-dimensional SDS-PAGE

The resolution of total cellular protein profiling was conduced employing one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the procedure of Laemmli, 1970, with slight modifications as was mentioned by Sambrook *et al.* (1989). Electrophoresis was carried out in a vertical slab gel electrophoretic apparatus (Vertical Dual Mini Gel System, Bangalore Genei, India) with 12.5% resolving gel of 0.75mm thickness and a 4.5% stacking gel. The gels made were 12cms in height and 14cms in width. For preparing working samples, the protein extracts prepared and stored earlier were thawed to room temperature and vortexed gently for 1 minute to make a homogenous solution. 5X commercial gel loading buffer (Genetix, USA) was added to a concentration of $1/5^{th}$ the volume of sample extracts while 20X reducing agent (DTT) was added to a concentration of $1/20^{th}$ the volume of sample extracts. The amount of sample extract was calculated from protein concentrations in µg/ml and was made upto 140µg/ml for a total volume of 25µl. The sample mixtures were heated at 100°C for 3 minutes, vortexed for a few minutes and 25µl of the sample preparations were finally loaded directly to SDS-polyacrylamide gels. For comparing the molecular weight (MW) of the polypeptide bands, 10µl of a standard 14.3-97.4 kDa protein ladder (Bangalore Genei, India) was used and electrophoresed alongwith the yeast protein sample preparations as per manufacturer's recommendations. The gels were stained with Commassie Brilliant Blue (CBB R-250, Sigma B-2025) destained several times for obtaining greater contrast of the bands.

Results and discussion

Isolation of yeasts and their growth behaviour under in-vitro conditions

The isolated yeasts were observed to be different in morphology and growth pattern. During the first plating from the starter culture, a total of eight isolates were obtained based on colony morphology (Fig.1). However, subsequent tests confirmed their status as four distinct isolates which were designated with codes viz. A, B, C and D respectively so as to facilitate their smooth manipulation under laboratory conditions. Of the four isolates obtained, isolate B and D were found to turn yellowish in YPD solid media (Fig.1); while the other two isolates A and C were grayish white and opaque in colour (Fig.1). The growth behaviour of the isolates suggested a characteristic profile with isolate C depicting the slowest growth while isolate B the fastest in YPD media incubated at 30°C (Fig. 2).

Table 1. Morphological features of isolated yeast strains**.**

Strain type	Designated code	Gross Morphology	Pigmentation	Cfu/ml*
1	FY-A	Grey white colony, lawn type growth	None	3.2 X 10^4 (0.28)
2	FY-B	Light yellowish colony, lawn type growth	Light yellowish	3.0 X 10^4 (0.21)
3	FY-C	White colony, spiky growth on surface	None	2.6 X 10^4 (0.23)
4	FY-D	Filamentous, sticky & amorphous colony	Yellowish	2.6 X 10^4 (0.21)

Cell counts were determined after incubation at 30°C for 72 hours. Cell counts were done with a haemocytometer**.**
*Average values of three determinants are provided. Standard deviations are shown in brackets.

Thermotolerance of the isolates

Out of the four pure yeast isolates A, B, C and D, isolate D showed the highest growth at 35°C (116 µg ml^{-1}) and varying sensitivity to different temperature ranges (Fig. 2). Interestingly, isolate D did not show appreciable growth either at 28°C or 40°C which suggest that the isolate has an ambient temperature range for expressing suitable fermentation activity. The isolate has also been found to be the most thermotolerant in comparison to the other isolates in question (Fig. 2). All the other three isolates viz. A, B, and C showed almost similar growth at all the three temperature ranges viz. 28°C, 35°C and 40°C. Isolates A and B showed better growth potential at 35°C while isolate C depicted poor growth at all the temperatures suggesting it to be a slow grower compared to the others (Fig. 2).

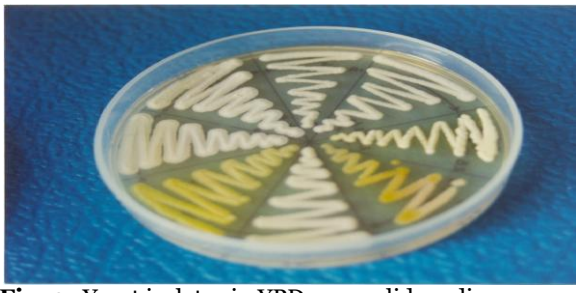


Fig. 1. Yeast isolates in YPD agar solid medium.

pH sensitivity studies

All the four isolates were observed to grow well at a neutral to near neutral pH, a characteristic quite unusual in yeasts. pH sensitivity results indicated that the four pure cultured isolates had remarkably fine sensitiveness to different pH ranges of growth. In the first 24 hours of incubation at the different pH ranges, all the isolates showed marked tolerability to different pH levels (Fig. 2). At an acidic pH 5 and 5.5, isolates A, C and D depicted very poor growth behaviour in the first 24 hours of incubation. However, it depicted an increase in growth during the next 48 hours of incubation (Fig. 2). The same was the case in other pH ranges of 6.0 and 6.5 were observed where an increase in growth patterns for all the isolates could be recorded with an increase in alkalinity of the medium (Fig. 2). As proposed, isolate B showed the maximum growth behaviour at a neutral pH 7.0 when compared to the other three isolates in question.

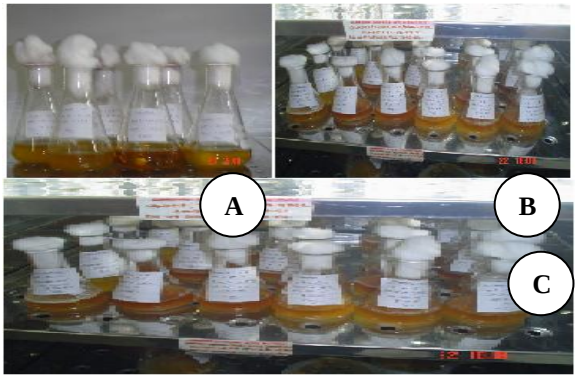
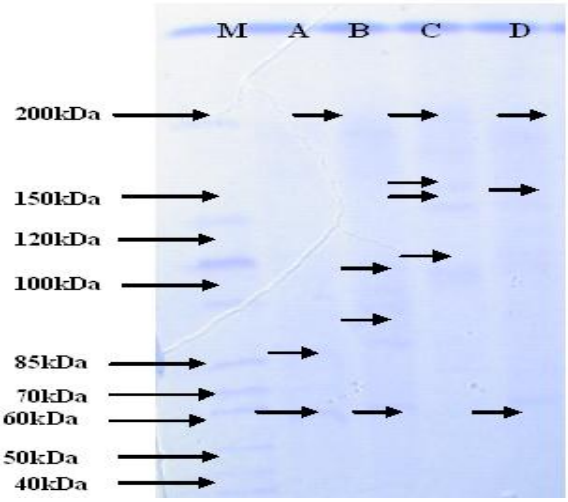


Fig. 2. A= Growth behaviour of yeast isolates in liquid YPD media at 30°C; B= Thermotolerance of the Isolates in liquid YPD media; C= pH sensitivity of the isolates in liquid YPD media at 30°C.



Lane 1: 200kDa Molecular marker (Fermentas Inc., USA)
Lane 2: Isolate A
Lane 3: Isolate B
Lane 4: Isolate C
Lane 5: Isolate D

Fig. 3. SDS-PAGE protein profile of the four yeast isolates.

Total whole cell protein analysis

Total whole cellular protein content in all the isolates varied considerably. It was observed that the isolate B showed the maximum amount of whole cell protein content followed by isolates A and D. Isolate C depicted a marked decrease in whole cell protein content which directly corresponds to growth of the isolates in YPD media (Table 2).

Table 2. Total cellular protein content of the yeast isolates.

Sl No.	Isolate type	Protein content in μg ml^{-1}
1	A	95.4
2	B	116.4
3	C	55.9
4	D	73.2

Protein profiling

Protein profiling of all the four yeast isolates could be studied employing SDS-PAGE of the whole cell lysates (Fig. 3). The gel analysis revealed that all the four isolates are similar at intra-species level but dissimilar at interspecies level (Towbin *et al.*, 1979). A band of 199.6 kDa was present in three of the isolates viz. B, C, and D but absent in A. One particular band of 173.2 kDa was observed in isolate C and not in others. Another band of 168 kDa was observed in isolates C & D and not in A & B. Isolate C depicted the characteristic presence of another band of 116.7 kDa. Two lower molecular weight bands of 111.4 and 88.2 kDa were observed in isolate B but was absent in others (Fig. 3). A very low molecular weight band of 59.5 kDa was present in all three isolates except isolate C. The band pattern obtained provides a clear indication that all the isolates are different to some extent. Isolate C might represent a different clade of origin provided the genetic origin is evaluated using advanced molecular techniques like ARDRA, RAPD and RFLP's (Puig *et al.*, 2000, Barre *et al.*, 1993).

Conclusion

Alcoholic fermentation successively involves different microorganisms, although yeasts are the most prominent species observed (Sarma, 2002). In the present investigations, it was not surprising to notice the role of yeasts in starter culture for indigenous alcoholic fermentation although their true identity is yet to be ascertained (Tsuyoshi *et al.*, 2004). It is also to be noted that *Candida* sp. (a group of diploid sexual fungus) had been reported earlier by researchers (Tsuyoshi *et al.*, 2004, Fleet, and Heard, 1993, Puig *et al.*, 2000), and its presence in the present findings cannot be ruled out.

Traditional alcoholic brewing is an important household cum soci*etal* drinking activity among different ethnic groups of Arunachal Pradesh. The methods for wine and beverage production among the tribes have different indigenous protocols and therefore the perpetuation of yeast strains also differ. The results obtained in the present findings suggest that all the isolates are different in terms of their physiological or biochemical activities. One important aspect of the studies was the successful isolation of pure cultures from a consortium whose contents were previously unknown (Sarma, 2002). A cursory look into possible clues have suggested that originally there was a common starter culture used by all the tribes of north-east India which eventually underwent a variation and selection in parallel with their segregation pattern as different groups followed different protocols for fermentation thus yielding variants that got selected either due to cultural selection or extensive selection pressure (Demuyter *et al*, 2004, Puig *et al.*, 2000, Ramirez *et al.*, 2004). However the studies nullify the presence of adenine deficient yeast isolates as was reported earlier on *Candida* strains isolated from cakes used by certain other tribes of Assam. Protein profiling of the isolates have revealed that the isolates are indeed variants at the genetic level as was evident from expression of whole cell proteins (Ramirez *et al.*, 2004). The isolates might therefore be mutagenic variants, which arose during segregation through time immemorial. A clear and vivid picture can be obtained by subjecting the isolates to various molecular analyses, which could not be performed in the present studies (Yarrow, 1998, Dijken, 2000). Nevertheless, the studies have provided an appreciable amount of data to compare the isolates with earlier reports (Tsuyoshi *et al.*, 2004). Interestingly, all the isolates have shown a preference of growth at neutral pH; a character not usually observed in yeasts who generally prefer an acidic medium for growth. Growth of one of the isolates (B) was found to be higher compared to others under optimum conditions while another

isolate (D) showed an appreciable thermotolerance and maximum growth at 35°C which was clearly astonishing. Notwithstanding the experiments performed and results obtained, it can be predicted that careful identification and selection of yeasts from starter cultures employed in traditional brewing in northeast India can yield industrially important and beneficial yeast strains for the exploitation and benefit of mankind.

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