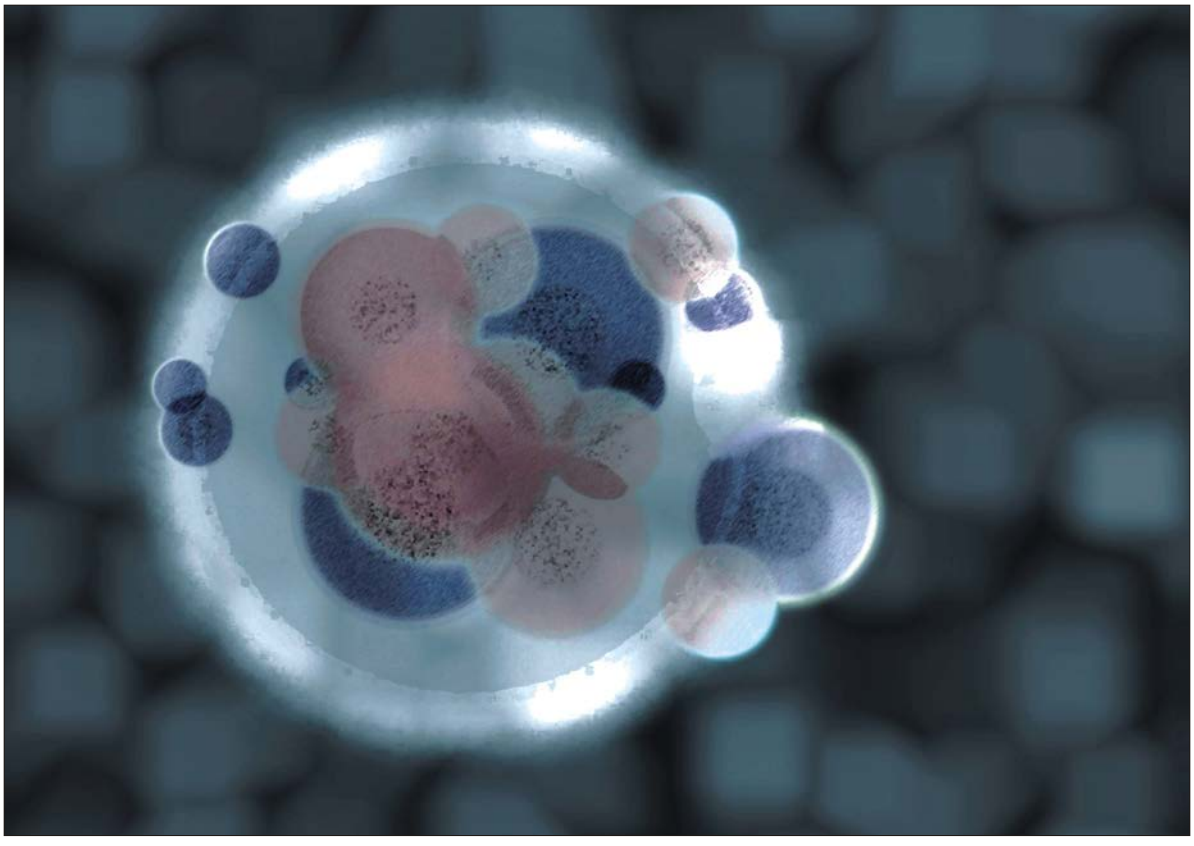

Journal of Biological Sciences

ISSN 1727-3048

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2017.91.105

Research Article
Exploring the Genealogy and Phenomic Divergences of Indigenous Domesticated Yeasts Cultivated by Six Ethnic Communities of Assam, India

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Abstract

Background and Objective: Wild and spontaneous fermentation are popular practice among home brewers representing indigenous communities of the world. Assam, a biodiversity rich region is a treasure house of traditional knowledge and multiple ethnicities which has not been properly documented. Household fermentation is highly rampant amongs't different races who follow their own inherited system, extricably linked to social, cultural, environmental and institutional contexts. In this study, 'Starter materials' from six ethnic communities were collected to enumerate and comprehend the assortment of yeasts that are fundamental to ethnic fermentation. **Methodology:** Starter materials from six ethnic communities were collected and yeasts were isolated in yeast extract-peptone-dextrose (YPD). These were identified on the basis of morphology, comparative physiology, biochemical responses and ultra miscoscopy. Establishment of molecular identity were done through 18S ribosomal DNA analysis using inter transcribed sequence (ITS1 and ITS4) primers for amplifying D1/D2 regions of yeast DNA. Phylogenetic relationships were established through maximum parsimony and Unweighted Pair Group Method with Arithmetic mean (UPGMA) methods. Interrelationships among yeasts representing interspecies and intercommunity variations were sorted out using five point summary plot analysis. **Results:** A total of 64 isolates were identified on the basis of morphological, physiological and biochemical differences that represented 31 variants. The 31 variants could be categorized further into 7 different genera representing 9 different species viz., *Candida tropicalis*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida glabrata*, *Pichia burtonii*, *Saccharomycopsis fibuligera*, *Meyerozyma caribbica* and *Rhodotorula taiwanensis* based on molecular analysis. *Wickerhamomyces anomalus* dominated the entire spectrum of starter cultures except Ahom starters, where *Candida* isolates appeared predominant. **Conclusion:** Study indicated that starters used by the communities originate from one place which eventually underwent a parallel segregation and perpetuated in different regions exposed to varied stress and diverse maintenance processes practiced by individual communities. This domestication and human selection through continuous breeding led to the development of cultivated variants that thrive in man shaped environments and behave sub-optimally in nature which corroborate further to consider the fact that the originality of a common starter culture cannot be denied. It is apparent that subtle differences in yeasts fundamentally originated from different lineages in the wild.

Key words: Yeasts, ethnic communities, phylogeny, geographical distribution

Received: October 24, 2016 Accepted: December 21, 2016 Published: January 15, 2017

Citation: Deep Prakash Parasar, Hridip Kumar Sarma and Jibon Kotoky, 2017. Exploring the genealogy and phenomic divergences of indigenous domesticated yeasts cultivated by six ethnic communities of Assam, India. J. Biol. Sci., 17: 91-105.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ethnic fermentation starters are believed to have originated in China which with time has been transferred to most of the countries in Asia[1]. Almost all nations and regions all over the world have the tradition of preparing indigenous fermentation products including alcoholic beverages utilizing indigenous agricultural produce and exploiting micro-organisms that had been tamed and cultivated for hundreds of years. Wild and spontaneously fermented food products are a popular practice among home brewers representing indigenous communities and are mostly prepared in solid state materials like rice, barley, sorghum, maize, wheat, rye or millets[2]. This age old practice was initiated via spontaneous fermentation using captured microbes from the local environment and was perpetuated through generations inculcating indigenous knowledge system inherent in the culture, tradition and institutionalized customs of ethnic communities. In the present perspective, a study was conducted on yeasts from traditional fermentation practiced by a few native communities of Assam, a region rich in biodiversity and multiple human ethnicity contributed by races like Caucasian, Mongoloid, Mandarin, Aryan Tibetian and the Burmese descent. Household fermentation is highly rampant whereby each race follow their own inherited knowledge system, extricably linked to social, cultural, environmental and institutional contexts. Such indigenous household produce are thought to have potential for commercialization, provided limitations such as aesthetically disagreeable odour, turbidity, undesirable metabolites, texture and inconsistency are eliminated[2]. The communities use similar substrates like rice or millet as sources of starters for perpetuation of yeasts but the yeasts involved in the fermentation appear different and unique for each community, an attribute that profoundly varies from community to community[3,4]. In this current study, it was examined 'Starter materials' from six native communities to enumerate and comprehend the assortment of yeasts that are fundamental to ethnic fermentation. This baseline study shall contemplate the probability of selecting specific yeasts for a variety of purpose including preparation of suitable consortia with better fermentation efficiency, screening out yeasts with probable industrial uses or even studying their intra and inter social behavior in terms of association and relationships critical to their sustenance and beneficial from the point of metabolite production useful to humans.

MATERIALS AND METHODS

Sampling and collection of starter culture materials: For the present study, samples were collected from six districts of Assam viz., Sibsagar, Dhemaji, Karbi-Anglong, Goalpara, Kokrajhar and Kamrup (Fig. 1). From each of the districts; locations were randomly selected based on population densities of ethnic communities in specific areas. The sampled areas ranged from 25.97-27.45° North latitudes and 90.16-94.82° East longitudes (Table 1). Samples included fermentation cakes used by the Ahoms (inhabitants of Sibsagar district), the Mishings (inhabitants of Dhemaji district), the Karbis (inhabitants of Karbi Anglong district), the Rabhas (inhabitants of Goalpara district), the Bodos (inhabitants of Kokrajhar district) and the Kacharis (inhabitants of Kamrup district) of the mentioned locations within the districts (Table 1).

Isolating yeasts from starter cultures: The fermentative starter cakes collected were carried to the laboratory in polypropylene bags and were allowed to dry in shade for 2 days. About 1.0 g of crude samples were weighed, dissolved in 10 mL distilled water, diluted 10^{-3} times and were inoculated onto petriplates containing YPD agar media (1% yeast extract, 2% bactopeptone, 2% dextrose and 2% agar) supplemented with 100 mg L^{-1} chloramphenicol (Sigma C-3175) and 50 mg L^{-1} chlorotetracycline (Sigma 26430) following the methodology of Even[5]. The plates were incubated at 30°C. Yeast isolates from initial mother cultures were sub-cultured five times, screened and identified in a bright field microscope (Olympus BX-50) at 800X magnification[6]. Growth characteristics of the pure cultures were compared in YPD broth incubated at 30°C for 24 h while mass cultures were obtained in broth incubated for 48 h.

Purification and maintenance of yeasts: For asserting purity of the yeasts, cells were initially grown for 24 h to a maximum optical density at 600 nm, yielding 260 g of cells[6]. Packed Cell Volume (PCV) for each isolate was recorded after incubating cells for 48 h. Specific growth rate and generation time were measured as per Kratz and Myers[7]. From a suitable dilution (2×10^6 cells mL^{-1}) for each sample, representative isolates were picked up and recultivated in YPD liquid media. Aliquots of the purified yeasts from batch cultures were designated with codes (Table 2), maintained in glycerol (15% v/v) stocks and YPD agar slants and were stored at -80°C for further studies. Scrapings from the frozen cultures were transferred onto fresh YPD medium[5].

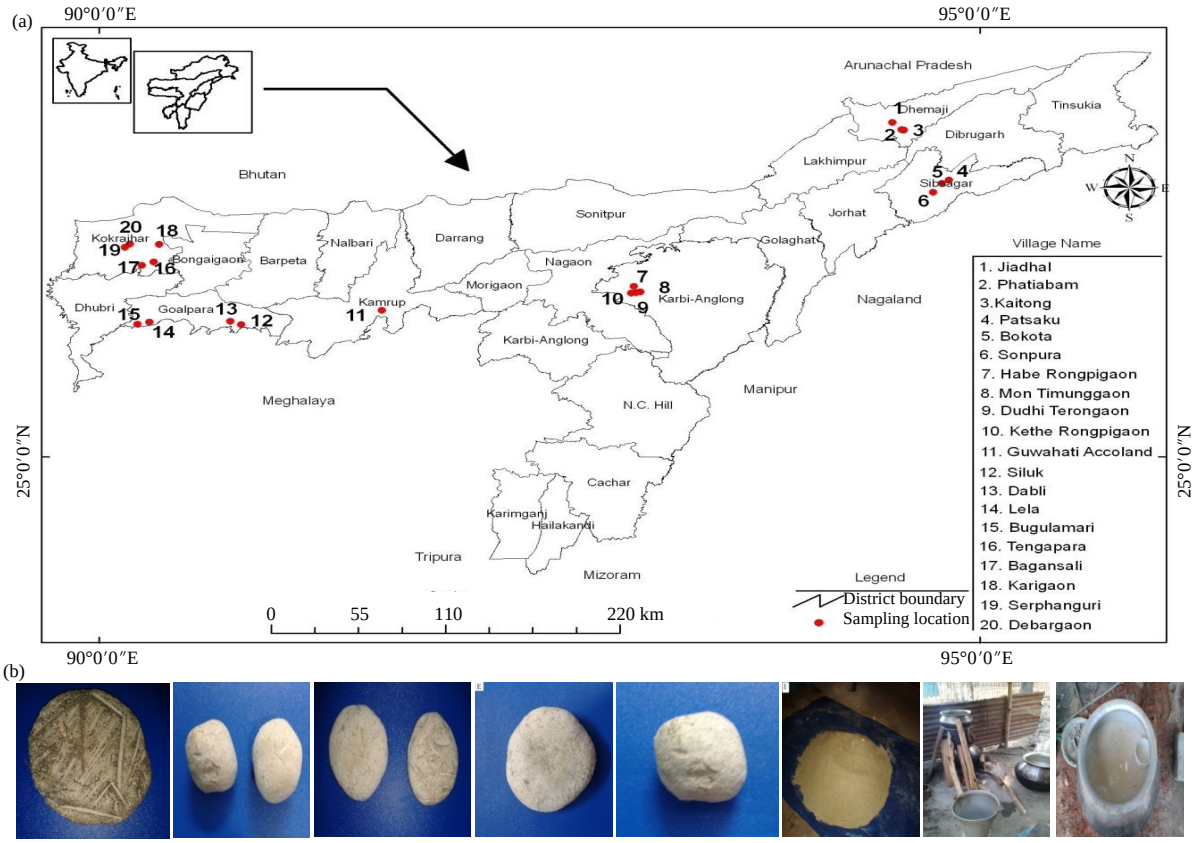

Fig. 1(a-b): (a) Map of Assam showing the locations of sampling and (b) Snapshots of fermentative cakes from (left to right) Mishing, Rabha, Bodo, Kachari and Karbi community; rice stock for starter culture; process of crude rice beer preparation and fermented rice beer

Table 1: Geographical attributes of the collection sites of sampled areas within six districts of Assam

Districts	Sampled areas	Latitude and longitude	Location
Goalpara	1	25.99°N, 90.74°E	Dabli
	2	25.96°N, 90.80°E	Siluk
	3	25.98°N, 90.28°E	Lela
	4	25.97°N, 90.21°E	Bugulamari
Kokrajhar	5	26.40°N, 90.27°E	Bagansali
	6	26.40°N, 90.27°E	Tengapara
	7	26.55°N, 90.17°E	Debargaon
	8	26.54°N, 90.16°E	Serphanguri
	9	26.55°N, 90.34°E	Karigaon
Dhemaji	10	27.39°N, 94.57°E	Kaitong
	11	27.39°N, 94.55°E	Phatiabam
	12	27.45°N, 94.50°E	Jiadhal
Sibsagar	13	26.93°N, 94.73°E	Sonpura
	14	27.00°N, 94.78°E	Bokota
	15	27.02°N, 94.82°E	Patsaku
Kamrup	16	26.07°N, 91.60°E	Rani
Karbi Anglong	17	26.19°N, 93.02°E	Kethe Rongpi Gaon
	18	26.20°N, 93.03°E	Habe Rongpi Gaon
	19	26.20°N, 93.04°E	Dudhi Teron Gaon
	20	26.20°N, 93.04°E	Mon Timung Gaon

Comparative physiology of yeasts in selective media: Micro-morphological description of the yeasts were performed as was described by Yarrow[8]. Comparative physiology was studied in six different media formulations

Table 2: Physiological characteristics of the yeast isolates in YPD media incubated at 30°C for 24 h

Community	Laboratory code designation	Specific growth rate (μm cm^{-3} min^{-1})	Generation time (h^{-1})	PCV/mL (×10^6) After 24 h	Colony texture in YPD media	Colony colour in YPD media
Bodo	BOA1X	0.123± 0.027	8.13±0.635	4.9±0.71	Smooth, flat, semi opaque	White
Bodo	BOA1Y	0.122±0.021	8.19±0.641	4.5±0.65	Smooth, flat, semi opaque	White
Bodo	BOA2Y	0.122±0.021	8.19±0.641	4.6±0.61	Smooth, flat, semi opaque	Yellowish white
Bodo	BOB1X	0.122±0.021	8.19±0.641	4.7±0.69	Smooth, flat, semi transparent	Creamy white
Bodo	BOB2X	0.063±0.007	15.87±0.998	3.9±0.49	smooth, flat ,Semi opaque,	Yellowish white
Bodo	BOB3X	0.123±0.027	8.13±0.635	4.9±0.71	Rough, elevated, semi transparent	Yellowish white
Bodo	BOB1Y	0.120±0.022	8.33±0.655	4.4±0.63	smooth, opaque, elevated	Yellowish white
Bodo	BOB2Y	0.124±0.023	8.06±0.601	3.4±0.44	Smooth, flat, semi opaque	Creamy white
Bodo	BOB2.2Y	0.150±0.044	6.660±0.512	5.6±0.77	Rough, opaque, flat	Creamy white
Bodo	BOB3Y	0.124±0.023	8.06±0.678	4.7±0.69	Smooth, flat, semi transparent	Creamy white
Bodo	BOC1X	0.067±0.006	14.92±0.763	3.8±0.47	Smooth, opaque, elevated	Creamy white
Bodo	BOC2X	0.122±0.021	8.19±0.597	4.8±0.70	Smooth, flat, opaque	Yellowish white
Bodo	BOC1Y	0.124±0.023	8.06±0.570	4.4±0.63	Smooth, opaque, elevated	Creamy white
Bodo	BO1.7	0.121±0.018	8.26±0.613	5.1±0.70	Smooth, semi opaque, elevated	Yellowish white
Bodo	BO2.3	0.149±0.043	6.71±0.397	5.8±0.79	Rough, elevated, opaque	Yellowish white
Bodo	BO3.8	0.122±0.021	8.19±0.597	4.6±0.61	Smooth, elevated, opaque	Yellowish white
Bodo	BO4.1	0.121±0.018	8.26±0.613	4.1±0.59	Smooth, elevated, opaque	Creamy white
Bodo	BO4.2	0.148±0.042	6.75±0.438	6.3±0.87	Smooth, flat, transparent	Yellowish white
Rabha	RAA1X	0.122±0.021	8.19±0.597	4.6±0.61	Smooth, flat, semi opaque	Yellowish white
Rabha	RAA1Y	0.120±0.022	8.33±0.641	4.5±0.65	Smooth, opaque, elevated	Yellowish white
Rabha	RAB1X	0.121±0.018	8.26±0.613	4.6±0.61	Rough,Opaque, flat	Creamy white
Rabha	RAB2X	0.072±0.019	13.88±0.711	5.9±0.80	Smooth, semi opaque, elevated	Yellowish white
Rabha	RAB3X	0.122±0.021	9.19±0.597	5.1±0.70	Smooth, opaque, elevated	White
Rabha	RAB4X	0.124±0.023	8.06±0.601	4.6±0.61	Rough, opaque, flat	Creamy white
Rabha	RAB1Y	0.123±0.027	8.13±0.635	4.8±0.70	Smooth, opaque, flat	White
Rabha	RAB1.1Y	0.122±0.021	8.19±0.597	4.7±0.69	Smooth, flat, semi opaque	Creamy white
Karbi	KR1X	0.066±0.007	15.15±0.929	3.8±0.47	Smooth, flat, semi opaque	White
Karbi	KR2X	0.123±0.027	8.13±0.635	4.9±0.71	Smooth, elevated, opaque	White
Karbi	KR1Y	0.066±0.007	15.15±0.929	4.2±0.63	Smooth, elevated, opaque	Creamy white
Karbi	KR2Y	0.065±0.006	15.38±0.933	4.0±0.57	Smooth, elevated, opaque	Creamy white
Karbi	KR1.4	0.066±0.007	16.15±0.929	4.9±0.57	Smooth, flat, opaque	White
Karbi	KR1.5	0.067±0.008	14.92±0.763	3.6±0.45	Smooth, flat, opaque	White
Karbi	KR2.11	0.066±0.007	15.15±0.929	4.0±0.57	Smooth, flat, opaque	Yellowish white
Karbi	KR2.12	0.064±0.005	15.62±0.978	3.9±0.46	Smooth, transparent, flat	Yellowish white
Karbi	KR4.9	0.066±0.007	15.15±0.929	4.0±0.58	Smooth, elevated, opaque	White
Karbi	KR4.10	0.069±0.010	14.49±0.763	4.1±0.59	Smooth, flat, opaque	White
Karbi	KR5.6	0.070±0.011	14.28±0.738	4.1±0.59	Rough, opaque, elevated	White
Kachari	KC2X	0.124±0.023	8.06±0.603	4.6±0.61	Smooth, opaque, elevated	White
Kachari	KC3X	0.116±0.018	8.62±0.882	5.2±0.73	Smooth, opaque, elevated	White
Kachari	KC4X	0.059±0.053	16.94±0.993	3.9±0.46	Smooth, opaque, elevated	White
Kachari	KC5X	0.063±0.008	15.87±0.947	3.5±0.45	Smooth, opaque, elevated	White
Kachari	KC6X	0.121±0.018	8.26±0.612	4.7±0.69	Smooth, flat, semi transparent	Yellowish white
Kachari	KC8X	0.143±0.035	6.99±0.634	3.6±0.45	Rough, opaque, flat	White
Kachari	KC1Y	0.125±0.024	8.00±0.603	4.8±0.70	Smooth, opaque, elevated	White
Ahom	1A	0.150±0.044	6.66±0.404	6.2±0.86	Smooth, flat, semi opaque	White
Ahom	2A	0.066±0.007	15.15±0.929	4.1±0.59	Smooth, opaque, flat	White
Ahom	2B	0.068±0.009	14.70±0.793	4.2±0.63	Smooth, elevated, opaque	White
Ahom	2D	0.153±0.047	6.53±0.392	6.4±0.88	Smooth, flat, semi opaque	Yellowish White
Ahom	3D	0.061±0.006	16.39±0.946	3.9±0.46	Smooth, elevated flat	Yellowish White
Ahom	C1	0.066±0.007	15.15±0.929	4.1±0.59	Smooth, flat, opaque	Yellowish White
Ahom	C7	0.139±0.033	7.19±0.475	3.5±0.45	Smooth, elevated, opaque	Yellowish White
Ahom	e7	0.067±0.008	14.92±0.798	4.2±0.63	Smooth, elevated, opaque	Yellowish White
Ahom	AH3SA	0.149±0.043	6.71±0.415	6.2±0.86	Smooth, flat, semi opaque	Yellowish white
Ahom	AH3SB	0.145±0.039	6.89±0.427	5.7±0.77	Smooth, transparent, flat	Yellowish White
Ahom	AH2SA	0.12±0.022	8.33±0.655	5.1±0.70	Smooth, elevated, opaque	White
Ahom	AH4SA	0.147±0.041	6.80±0.421	5.5±0.76	Smooth, elevated, opaque	White

Table 2: Continue

Community	Laboratory code designation	Specific growth rate (μm cm^{-3} min^{-1})	Generation time (h^{-1})	PCV/mL ($\times 10^6$) After 24 h	Colony texture in YPD media	Colony colour in YPD media
Ahom	Ade8	0.140±0.034	7.14±0.468	5.8±0.79	Smooth, elevated, opaque	Yellowish White
Mishing	MIE1X	0.127±0.026	7.87±0.503	3.2±0.41	Smooth, elevated, opaque	Creamy white
Mishing	MIB4X	0.124±0.023	8.06±0.598	4.6±0.61	Smooth, elevated, opaque	Creamy white
Mishing	MID3X	0.143±0.037	6.99±0.455	3.3±0.43	Smooth, elevated, opaque	Creamy white
Mishing	MID3.1X	0.149±0.043	6.71±0.431	3.2±0.41	Smooth, flat, transparent	Creamy white
Mishing	MID 4Y	0.138±0.032	7.24±0.479	5.4±0.75	Smooth, elevated, opaque	Creamy White
Mishing	MID4.2X	0.144±0.038	6.94±0.450	3.8±0.49	Smoothe, elevated, transparent	Creamy white
Mishing	MIC5X	0.145±0.039	6.89±0.441	3.9±0.51	Smooth, elevated, opaque	Brick red

±: SE (n = 3), Packed cell volume (PCV) was measured with a heamocytometer

viz., Yeast extract-peptone-dextrose (YPD) agar, czapek dox agar, Drop out Base (DOB) agar, HiCrome OGYE agar, HiCrome *Candida* differential agar and Yeast Morphology Agar (YMA) to determine colony characteristics and select out representatives indicating similar genus and/or species including *Candida* isolates. The YPD was used to observe the growth of heterotrophic yeasts while Czapek Dox was used as a semisynthetic medium to screen filamentous yeasts in presence of sodium nitrate as sole nitrogen source. The DOB agar was used to estimate the number of auxotrophic mutants in absence of lysine, histidine, adenine and uracil. HiCrome OGYE agar containing oxytetracycline was used as a differential medium to screen yeasts from other microbes at low pH and to inhibit the growth of Lactic Acid Bacteria (LAB). HiCrome *Candida* differential agar was used for differentiating *Candida* isolates at the species level. Yeast morphology agar was employed to study the cellular morphology, formation of mycelia, pseudomycelia, vegetative cells and other cultural characteristics.

Biochemical response of yeasts to varied carbon and nitrogen sources

Carbon sources assimilation test: Determination of carbon sources assimilation of the yeast isolates were tested in Yeast Nitrogen Base (YNB) liquid media supplemented fructose, maltose, cellobiose, raffinose, melibiose, arabinose, trehalose, galactose, inositol, mannitol, mannose and rhamnose following the methodology of Das and Pandey[9] with modifications. About 10 μL of precultured inoculum (2.6×10^5 cells at OD_{600}) of mid log phase cells were inoculated on to 100 mL YNB media containing each of the carbon sources, followed by incubation at 30°C for 24 h in an orbital shaker incubator (Genie, Merck) set at 100 rpm. Growth pattern of yeasts were recorded based on turbidity of the media[9].

Nitrogen sources assimilation test: Comparative growth of the selected yeasts in four nitrogen sources was studied independently. These included viz., ammonium sulphate (7.5 g L^{-1}) and amino acids {glycine (84.2 mg L^{-1}), leucine (71.4 mg L^{-1}), methionine (20.3 mg L^{-1}) and tryptophan (482.6 mg L^{-1})} following the methodology of Albers *et al.*[10]. Each of the nitrogen sources were added to 100 mL of Synthetic Medium (SM) containing the mentioned nitrogenous sources and cells were cultured under similar conditions.

Organic acid utilization test: Five different organic acids viz., tartaric, malic, citric, pyruvic and propionic acids were used as substrates to determine their assimilation in yeasts following the methodology of Fonseca[11]. About 0.5 g of each of the organic acid sources was added to 100 mL of Mineral Medium (MM), pH 4.5[12] and cells were cultured under similar conditions.

Establishing inter-relationships and segregating yeasts on the basis of molecular identity: From the above mentioned experiments, 64 yeast isolates were obtained and were segregated into groups based on their similarities and differences. Notably, 31 isolates appeared distinctly unique with distinguishing characters encompassing two or more isolates with the same catenation. For establishing their genetic identity, genomic DNA was extracted following the methodology of Hanna and Xiao[13] with modifications. Details of the procedure, primers used and methodology followed for molecular identification will be published elsewhere. Briefly, isolated genomic DNA was purified using a Himedia DNA purification kit (MB552) was subjected to PCR amplification using universal primers ITS1 and ITS4 for amplifying D1/D2 regions[14] and the amplicons were sequenced in a 96 capillary sequencer (Shimadzu). Sequences obtained were analyzed through a BLAST search against the non-redundant nucleotide (nt) database and were submitted to NCBI Genbank and DDBJ databases (Accession numbers in Table 3). Multiple sequence alignment was performed in Clustal-X programme Version 2.0[15].

Table 3: Molecular identity and accession number of the yeast isolates

Isolates	Identification	No. of accession	Approx. base pair (bp)
BOC1X	*Wickerhamomyces anomalus*	KM603603	482
BOB2X	*Pichia anomala*	KM603606	251
BOB3Y	*Wickerhamomyces anomalus*	KM603608	359
BOB2.2Y	*Pichia burtonii*	KM603610	429
BOA1X	*Wickerhamomyces anomalus*	KM603612	378
BOA2Y	*Wickerhamomyces anomalus*	KM603614	222
BOB1X	*Wickerhamomyces anomalus*	KM603616	457
BOC2X	*Wickerhamomyces anomalus*	KM603617	432
BO1.7	*Wickerhamomyces anomalus*	KM603624	571
RAB3X	*Wickerhamomyces anomalous*	KM603601	593
RAB1X	*Wickerhamomyces anomalus*	KM603604	277
RAB1Y	*Wickerhamomyces anomalus*	KM603609	601
RAB2X	*Meyrozyma caribbica*	KM603611	520
RAA1X	*Wickerhamomyces anomalus*	KM603613	288
RAB1.1Y	*Wickerhamomyces anomalus*	KM603618	464
KR4.10Y	*Saccharomyces cerevisiae*	KM603619	250
KR5.6	*Saccharomyces cerevisiae*	KM603620	582
KR1.4	*Saccharomyces cerevisiae*	KM603621	345
KR2.11	*Saccharomyces cerevisiae*	KM603622	703
KR4.10X	*Saccharomyces cerevisiae*	KM603623	272
KC4X	*Saccharomyces cerevisiae*	KM603607	287
AH3SA	*Candida tropicalis*	KM603605	319
2D	*Candida tropicalis*	KM603615	315
AH2SA	*Candida glabrata*	KM603625	224
MIB4X	*Wickerhamomyces anomalus*	KM603602	440
BOB3X	*Wickerhamomyces anomalus*	LC011409	180
KR2X	*Wickerhamomyces anomalus*	LC011408	143
KC8X	*Saccharomycopsis fibuligera*	LC011407	133
KC6X	*Wickerhamomyces anomalus*	LC011410	178
KC3X	*Candida glabrata*	LC011411	112
MIC5X	*Rhodotorula taiwanensis*	LC011412	141

Phylogenetic analysis was performed through maximum parsimony and UPGMA methods[16]. The UPGMA dendogram are provided in Fig. 2.

Screening out yeast representatives at the intercommunity and interspecies level: The identity of 31 yeasts on the basis of molecular characterization is provided in Table 4. To further circumscribe the isolates on the basis of same identity of a particular community, thermo-stability (from 29-37°C with an increase in 2°C for each set of experiment) and pH tolerance (from 5-9 with increase in 0.5 in each set of experiment) of the isolates were analyzed. Finally, 16 of the 31 yeasts were separated out from the rest as representatives of the communities for further study.

Reference yeast strains: For comparing the growth of the selected 16 yeasts, three reference strains viz., MTCC3979 (*Wickerhamomyces anomalus*), MTCC3984 (*Candida glabrata*) and MTCC3092 (*Saccharomyces cerevisiae*) were procured from IMTECH Chandigarh, India[3].

Growth behaviour of selected yeasts in YPD: The variation in the pattern of growth of the segregated 16 isolates were compared in YPD for 48 h until late log phase was achieved for assessing resemblances representing inter-community and inter-species diversity. The protein content of the samples were measured at 600 nm using folin-ciocalteau reagent[17] using Bovine Serum Albumin (BSA) as standard. Data obtained in Fig. 3(a).

For statistical discernment of the entire range of data, the procedure of cluster analysis was adopted to group the homogenous isolates from the non-homogenous ones differing in the utilization/assimilation of substrates tested. Corresponding dendograms were constructed for each of the substrate types (viz., carbon, nitrogen, organic acids) generating clusters that are pictorially represented. Initial data generated enormous, messy and disorganized dendrograms that was divided in two parts i.e., Rabha, Bodo and Karbi in one dendrogram and Ahom, Mishing and Kachari in another dendrogram. To comprehend the data into more compact form, a second slot

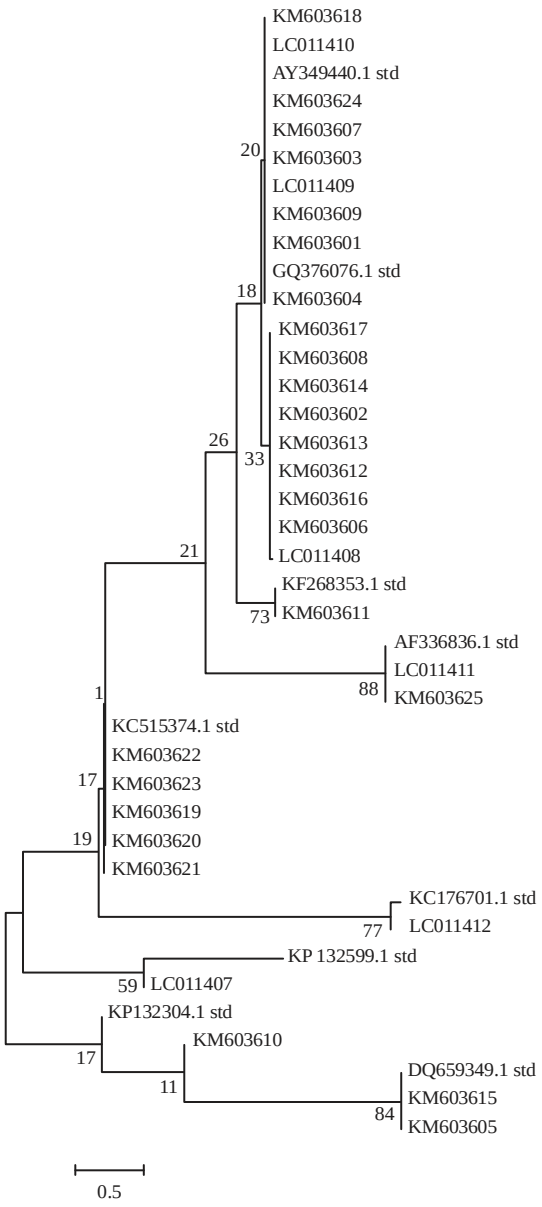

Fig. 2: Bootstrap analysis of UPGMA and Neighbor-joining tree showing the phylogeny of isolated yeasts

Scale bar specifies 0.5 changes per nucleotide position

of dendrogram was constructed wherein isolates that showed homogeneity to all the characters representing a single clade were segregated again by selecting one representative from each homogenous class. Exploratory data analysis five point summary plots meant for elucidating basic resistant statistical summaries of the 16 isolates was performed using R-software version 3.0.2[18].

Scanning electron micrographs and phase contrast imagery of selective isolates: Scanning electron and phase contrast microscopy were carried out in nine isolates representing 7 genera and 9 species from all six communities. For phase contrast microscopy, 1 µL of 24 h incubated YPD liquid cultures containing yeast cells were smeared over clean glass slides and heat fixed for 5 min before observing in a Leica DM 750 phase contrast microscope[19]. Scanning Electron Microscopy (SEM) was performed as per Oliveira *et al.*[20]. Briefly, the isolates were fixed with 2.5% glutaraldehyde for 24 h at 4°C and washed thrice in 0.1 M phosphate buffer (pH 7.2). Post fixation was carried out for 2 h in 2% osmium tetroxide followed by dehydration in 30, 50, 70, 95 and 100% ethanol gradients. Dried forms of the cells were retrieved in liquid CO_2

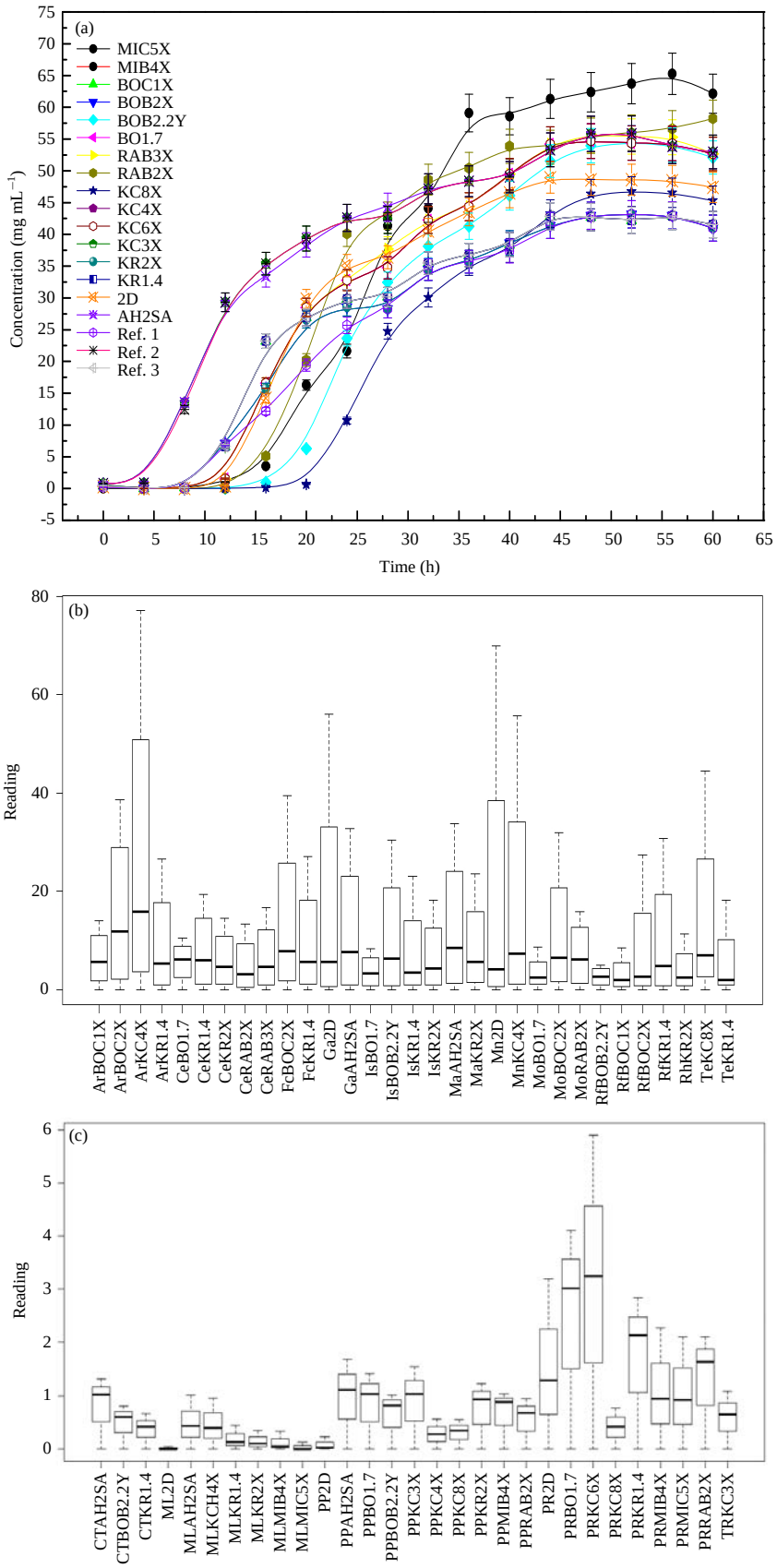

Fig. 3: Continue

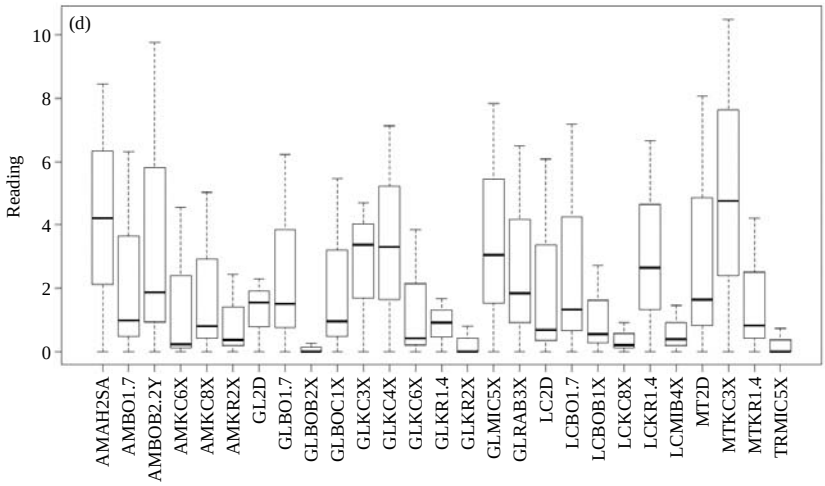

Fig. 3(a-d): (a) Growth behaviour of the selected yeast isolates in YPD medium {MTCC3979 (*Wickerhamomyces anomalus*), MTCC3984 (*Candida glabrata*) and MTCC3092 (*Saccharomyces cerevisiae*) are reference sample}. Double bars indicate ±SE of triplicate records. Five point summary box plot grouped by cluster dendogram of homogenous yeast utilizing, (b) Twelve carbon sources, (c) Five organic sources and (d) Five nitrogen sources

after acetone wash, coated with gold particles in a vacuum evaporator and examined under SEM (JSM-6360, JEOL). Details of cell shape, size and budding types observed are shown in Table 4.

RESULTS

Obtaining pure cultures of yeasts: A total of 64 yeasts were obtained from the entire repertoire of representative starter culture cakes of six ethnic communities that differed in morphology, growth performance and colony characteristics (Table 2). The isolates were designated laboratory code against each serial number (Table 2).

Comparative morphology of the isolated yeasts: Colony morphology of the isolates varied in YPD media wherein 7 of the 64 isolates showed rough morphology, whilst rest 57 exhibited smooth appearance. Except for three Bodo isolates, growth of rest of the 61 isolates appeared fast in YPD. In DOB media, not much difference could be observed although the surface morphology varied significantly. About 26 isolates showed rough surface appearance contradicting observations in YPD in case of Bodo isolates. Karbi isolates did not show any colouration and appeared transparent. None of the isolates showed fast growth rate compared to YPD and fascinatingly 25 isolates grew very slow taking almost 48 h for detectable growth. In HiCrome OGYE agar, it could be ascertained that most of the isolates were pure and not mixed or impure cultures. Notably, 56 isolates showed smooth colony surface morphology in contrast to 8 with rough morphology. The isolates showed fast growth except MIC5X (*Rhodotorula taiwanensis*) that did not grow at all in HiChrome OGYE. In Czepak Dox medium, no rough surface morphology was evident in mishing isolates while no smooth surface colonies were visible in Rabha isolates. Of the 64 isolates, 29 displayed rough surface morphology while 29 displayed smooth surface morphology. The only distinction in colony colour was observed in Ahom isolates that appeared transparent. In HiCrome *Candida* differential agar, 16 isolates appeared rough in surface morphology, while the rest appeared smooth. In case of colony colour, 15 isolates appeared transparent white, 15 appeared light purple (i.e., 6 Bodo, 8 Rabha and 1 Kachari) 10 isolates showed transparent purple colouration (i.e., 7 Karbi and 3 Kachari), 8 Ahom isolates depicted bluish white colonies while all the Mishing isolates (except MIC5X) displayed transparent white colour. In case of yeast morphology agar, the note worthy observation was the appearance of rough colony morphology of Kachari isolate KC8X (*Saccharomycopsis fibuligera*) while others exhibited smooth surface.

Biochemical responses of studied yeasts

Carbon, nitrogen and organic acids utilization test: To compare carbon, nitrogen and organic acids utilization, the samples were categorized on community basis. Growth rate was measured in terms of no growth (-), less growth (+), moderate growth (++) and luxuriant growth (+++). Isolates pertaining to a corresponding pair with exactly similar pattern

Table 4: Distribution of the isolates according to species and community representing sources of collection. (Below): Details of the cellular morphology yeasts as was observed under bright field and scanning electron microscope

	Segregation of yeasts on the basis of molecular identity								
Community	*Candida glabrata*	*Candida tropicalis*	*Pichia anomala*	*Pichia burtonii*	*Saccharomyces cerevisiae*	*Wickerhamomyces anomalus*	*Saccharomycopsis fibuligera*	*Meyerozyma carribica*	*Rhodotorula taiwanensis*
Bodo	--	--	BOB2X	BOB2.2Y	BOC1X	BOA1X, BOA2Y, BOB1X, BOB3X, BOB3Y, BOC2X, BO1.7	--	--	--
Karbi	--	--	--	--	KR2Y, KR1.4, KR2.11, KR4.10, KR5.6	KR2X	--	--	--
Rabha	--	--	--	--	--	RAA1X, RAB1X, RAB3X, RAB1Y RAB1.1Y	--	RAB2X	--
Kachari	KC3X	--	--	--	KC4X	KC6X	KC8X	--	--
Ahom	AH2SA	AH3SA,2D	--	--	--	--	--	--	--
Mishing	--	--	--	--	--	MIB4X	--	--	MIC5X

Representative yeast isolates from different communities

Laboratory code of isolate	Molecular identity of isolates (determined through 18S-28S ITS rDNA sequencing)	Cell size diameter (μm m^{-2})			Cell Shape	Budding
		Replicate-I	Replicate-II	Replicate-III		
RAB3X	*Wickerhamomyces anomalus*	4.78±0.21	4.38±0.11	4.93±0.19	Ogival	Yes
KC8X	*Saccharomycopsis fibuligera*	4.01±0.13	4.20±0.09	4.19±0.27	Apiculate	Yes
BOB2X	*Pichia anomala*	3.09±0.10	3.11±0.11	3.32±0.18	Ellipsoidal	Yes
BOB2.2Y	*Pichia burtonii*	3.68±0.16	3.14±0.10	3.75±0.18	Ellipsoidal	Yes
RAB2X	*Meyerozyma carribica*	1.99±0.12	2.12±0.13	2.03±0.18	Ogival	Yes
2D	*Candida tropicalis*	5.02±0.12	4.72±0.15	4.76±0.20	Globose	Yes
KR1.4	*Saccharomyces cerevisiae*	2.94±0.18	3.43±0.42	2.77±0.27	Spherical	Yes
KC3X	*Candida glabrata*	2.83±0.10	2.95±0.13	3.18±0.13	Apiculate	Yes
MIC5X	*Rhodotorula taiwanensis*	6.91±0.19	7.23±0.13	6.06±0.13	Cylindrical	Yes

±: SE, ±: Three observations

of biochemical responses were selected and was segregated out from their counterparts for further studies. For example, from the Bodo community, the isolates (BOB3X and BO4.2), (BOA1X and BOA1Y), (BOA2Y and BOB1Y), (BOB3Y and BOB2Y), (BOB1X and BO1.4), (BOC2X and BO4.2), (BO1.7 and BO3.8), (BOC1X and BOC1Y) showed exactly similar pattern of biochemical responses and hence one isolate from each corresponding pair was segregated out for further studies. Remaining two isolates viz., BOB2X and BOB2.2Y showed individual characters and hence were chosen independently with the above. Similarly, from the Rabha community RAA1X and RAB1X were selected from their counterparts (RAA1Yand RAB4X) along with isolates RAB2X, RAB3X, RAB1Y and RAB1.1Y. From the Karbi community, six isolates viz., KR2X, KR2Y, KR1.4, KR2.11, KR4.10 and KR5.6 were selected from the eleven initial isolates. From the Kachari community, 2 isolates (viz., KC3X and KC4X) were selected from their counterparts KC2X, KC5X and KC1Y while 2 other isolates viz., KC6X and KC8X with different attributes were selected independently. From the Ahom community, only 3 isolates (viz., AH2SA, AH3SA, 2D) were selected from a repertoire of 13 isolates that fell into three categories based on their biochemical profiles. These included category-I (AH3SA and AH3SB), category-II (2D, 3D, Ade8, C1, C7, e7) and category-III (AH2SA, AH4SA, 1A, 2A, 2B) respectively. From the Mishing community only 2 isolates were selected viz. MIB4X and MIC5X from the rest viz., MIE1X, MIB4X, MID3X, MID3.1X, MID4Y, MID4.2X. Henceforth, a total 31 isolates viz. 10 from Bodo community, 6 from Rabha community, 6 from Karbi community, 4 from Kachari community, 3 from Ahom community and 2 from Mishing community were finally subjected to molecular identification (Table 4). Sequences of the isolates have been submitted to NCBI and DDBJ data base. The accession numbers are provided in Table 2. The phylogenetic tree constructed is provided in Fig. 2.

From the molecular analysis, all the 31 isolates could be categorized into 7 different genera representing 9 different species (Table 4). *Wickerhamomyces anomalus* dominated the entire spectrum of starter cultures from all six communities, except Ahom starters, where no *Wickerhamomyces* was found. *Saccharomyces cerevisiae* was present only in three communities with maximum occurrence in starters of Karbi community. *Candida glabrata* and *Candida tropicalis* were the only fermenting yeasts retrieved from starters of Ahom community, which was interesting and unique in terms of ethnic fermentation.

Growth behaviour of the selected isolates: The growth behavior of isolate AH2SA (*Candida glabrata)* was fastest in YPD liquid medium compared to others. Its log phase started almost 4 h from the time of inoculation and reached the stationary phase at approximately 45th h of incubation. On the contrary, isolate KC8X *(Sacchoromycopsis fibuligera)* from Kachari community depicted the slowest growth rate with its log phase starting from 20 h of inoculation and reaching the stationary phase at 48th h of incubation. In the case of remaining isolates, the log phase remained persistent in-between 8th-12th h of incubation. Stationary phase of the isolates initiated from 40 h, except isolate MIC5X (*Rhodotorula taiwanensis)* where the stationary phase appeared early at almost 35th h of incubation (Fig. 3a).

Statistical analysis: Cluster dendrograms were prepared to select out the representatives of homogenous groups in case of carbon, nitrogen and organic acids utilization. Intriguingly, a total of 12 isolates were found to assimilate all the tested carbon sources differently as analyzed through five point summary plot in Fig. 3(b). Utilization of arabinose was significant amongst *Saccharomyces cerevisiae* isolates while arabinose metabolism was highly pronounced in Kachari isolate KC4X (*Saccharomyces cerevisiae*). Cellibiose and fructose utilization was not very significant except a few Rabha and Karbi isolates. Galactose utilization was more pronounced in *Candida* isolates while inositol metabolism was diverse in Bodo and Karbi isolates representing *Pichia burtonii*, *Wickerhamomyces anomalous* and *Saccharomyces cerevisiae*. Maltose and mannitol assimilation were noteworthy in *Candida glabrata* (AH2SA), *Wickerhamomyces anomalous* (KR2X), *Candida tropicalis* (2D) and *Saccharomyces cerevisiae* (KC4X). Substantial assimilation of trehalose was evident in *Saccharomycopsis fibuligera* (KC8X), which otherwise was insignificant in other isolates.

The utilization of nitrogen sources by the representatives of homogenous groups are depicted in Fig. 3(d). Overall, 15 isolates could differentially utilize all nitrogen sources. *Candida glabrata* (AH2SA) appreciably utilized ammonia compared to other isolates. *Candida tropicalis* (2D) displayed unequal utilization pattern of methionine, glycine and lysine while *Wickerhamomyces anomalous* (BO1.7) utilized ammonia, mehionine and glycine differently. *Pichia burtonii* (BOB2.2Y) and *Pichia anomalous* (BOB2X) showed high assimilation of ammonia compared to other nitrogenous compounds. *Saccharomyces cerevisiae* (BOC1X) appreciably utilized glycine. Assimilatory pattern of glycine was unusual in 11 of the 16 isolates with remarkable utilization by *Rhodotorula taiwanensis* (MIC5X) as was apparent from balanced distribution of quartiles. Utilization of leucine was discrete in 6 isolates compared to others and appeared

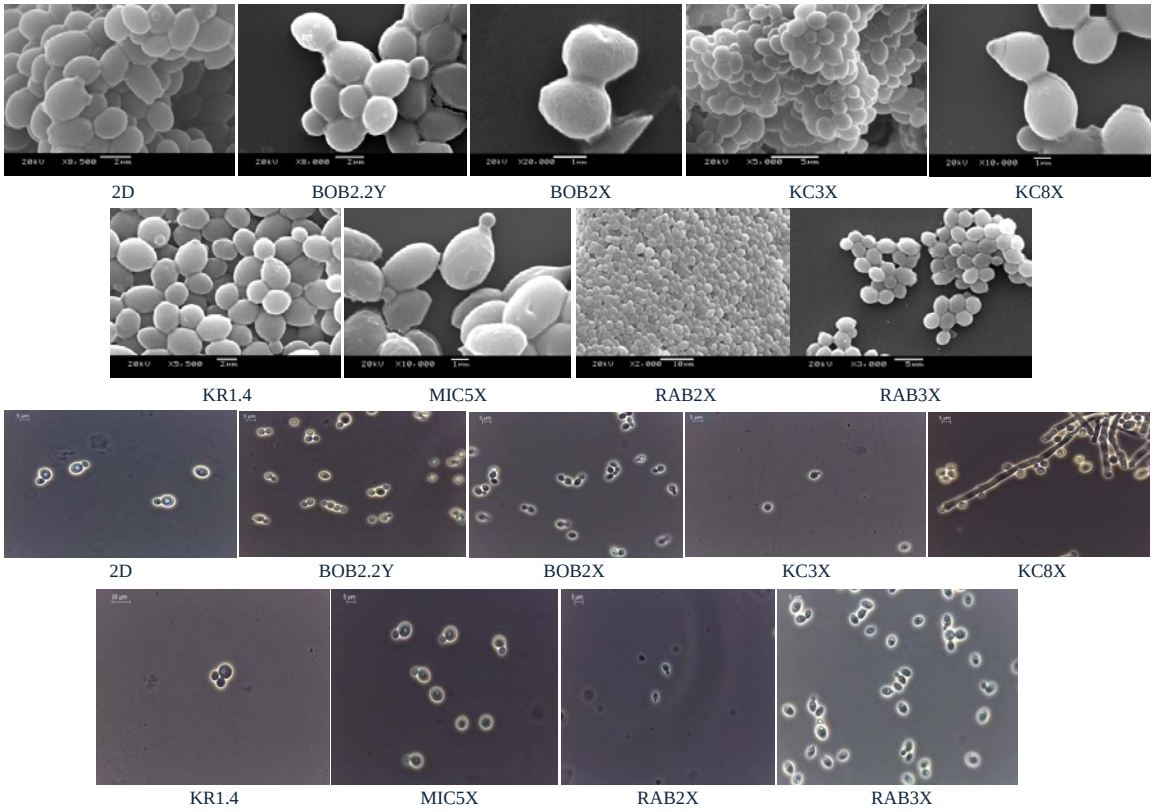

Fig. 4: Scanning electron microscopic images and phase contrast microscopic images of the nine isolates representing seven genera and nine species retrieved from six different communities

2D: *Candida tropicalis*, BOB2.2Y: *Pichia burtonii*, BOB2X: *Pichia anomala*, KC3X: *Candida glabrata*, KC8X: *Saccharomycopsis fibuligera*, KR1.4: *Saccharomyces cerevisiae*, MIC5X: *Rhodotorula taiwanensis*, RAB2X: *Meyerozyma caribbica* and RAB3X: *Wickerhamomyces anomalus*

insignificant in *Saccharomycopsis fibuligera* (KC8X) and *Wickerhamomyces anomalous* (MIB4X). *Saccharomyces cerevisiae* (KR1.4) showed a balanced pattern of leucine utilization. Similarly, methionine utilization was noteworthy in *Candida tropicalis* (2D) and *Candida glabrata* (KC3X) respectively (Fig. 3d).

In case of organic acids, utilization pattern by the isolates are depicted in (Fig. 3c). The five point box plot summary revealed that majority of the isolates responded very poorly to citric and malic acids. Citric acid was differently metabolized by *Candida glabrata* (AH2SA), *Pichia burtonii* (BOB2.2Y) and *Saccharomyces cerevisiae* (KR1.4) with some significant variations. Malic acid was assimilated differentially but insignificantly low by all the isolates with some observable changes in *Candida glabrata* (AH2SA) and *Saccharomyces cerevisiae* (KC4X). Similar pattern of propionic acid utilization was also evident in 10 of the isolates that did not support growth beyond a certain perimeter. Assimilation of pyruvic acid was much significant in *Candida tropicalis* (2D), *Wickerhamomyces anolaous* (BO1.7), *Saccharomyces cerevisiae* (KR1.4), *Wickerhamomyces anomalous* (MIB4X) and *Rhodotorula taiwanensis* (MIB5X), a testimony of similar substrate utilization by genetically variant yeasts. Incidentally, *Saccharomycopsis fibuligera* (KC8X) assimilated pyruvate but did not respond to other organic acids. Tartaric acid did not support the sustenance of any of the isolates except *Candida glabrata* (KC3X) which was henceforth unique and exceptional (Fig. 3c).

Scanning electron micrographs and phase contrast imagery of selective isolates: Both phase contrast images and scanning electron micrographs confirmed the cellular morphology of the isolates as distinctly different from one another (Fig. 4). The cell shape could be identified by consulting yeast monographs and were observed to represent six forms (Table 4). Incidentally budding was observed in all the isolates while isolates KC8X *(Saccharomycopsis fibuligera)* depicted hyphale branching with septation. The cell sizes were maximum in case of *Rhodotorula taiwanensis* (MIC5X), followed by *Candida*

tropicalis (2D) while *Meyerozyma carribica* (RAB2X) appeared smallest in cell diameter (Table 4).

DISCUSSION

This is the first complete report on the genealogical identity and phenomic divergences of yeasts from traditional starter cultures perpetuated by six ethnic communities of Assam, a biodiversity hotspot region of the eastern Himalayas. In the present study, the diversity of yeasts in fermentative starter cakes of six ethnic communities of Assam viz. Ahom, Mishing, Bodo, Rabha, Kachari and Karbi had been quite intriguing and revealed the presence of 9 different isolates characterizing 7 different genera. The identified yeast species falls under the subkingdom *Dikarya* in the taxonomical hierarchy representing two different families Sporiodiobolaceae and Debaromycetaceae with diverse phenomic and genomic interrelationships. The presence of *Wickerhamomyces anomalous* in almost all the fermentative starters, the presence of only *Saccharomyces cerevisiae* in starters of Karbi community while the absence of specific fermenting yeast in starters of Mishing community was interesting. The occurrence of only *Candida* isolates in starters of Ahom community is really intriguing, although not surprising[3]. Notably, the extent of *Candida* species in fermentation starters of southeast Asian region is not so widespread which also support the findings of this study. The presence of *Saccharomycopsis fibuligera*, *Pichia burtonii*, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalous* in starters of Kachari, Bodo, Karbi, Rabha and Mishing communities did provide a clue about the possible link of the origin of starters with reports from Thailand[1], China[21] and Tibet[22]. The isolated species are not unfamiliar to rest of Southeast Asia[23] which corroborate further to consider the fact that the originality of a common starter culture cannot be denied. Across continents, too, yeast diversity varies with distance, location, climate and adaptation. The diversity of wine yeasts has been extensively described from European countries. The *Saccharomyces* spp. could assimilate glucose, maltose, galactose, sucrose but not lactose, trehalose, cellobiose, raffinose, arabinose, inositol and xylose, contradicting our findings. Chandrasena *et al.*[24] had reportedly evaluated 1000 wild indigenous yeasts from natural sugary sources of Sri Lanka intended to benefit industrial fermentations. The study reiterates our findings that indigenous yeasts (including *Pichia* and *Candida*) isolated, can be explored for industrial fermentations as they exhibit enormous diversity in terms of behavior, metabolism and stability. Kadjogbe *et al.*[25] had reported the presence of *Candida glabrata* in fermentation starter that could ferment only glucose and trehalose while two *Rhodotorula* isolates that could metabolize a host of carbon sources including galactose, sucrose, maltose, trehalose, raffinose and cellibiose respectively were identified. In the present findings, it was found that our *Candida* isolates could assimilate all the tested carbon sources and assimilate ammonia, glycine, methionine and leucine as sole nitrogen sources but could not assimilate tryptophan. Contrarily, tryptophan was metabolized very well by *Rhodotorula taiwanensis*. The *Candida* isolates assimilated citrate, malate and propionate poorly but showed exceedingly appreciable metabolism in pyruvate. *Rhodotorula taiwanensis* could ferment most of the tested sugars including trehalose, glycine, tartrate, citrate and pyruvate quite well. *Candida* isolates grew fastest in YPD while *Saccharomycopsis fibuligera* grew slowest in YPD. A *Saccharomyces cerevisiae* isolate could exceptionally utilize arabinose while trehalose assimilation was balanced in *Saccharomycopsis fibuligera*. Likewise, it was observed that all the isolates were unique in terms of their physiological and biochemical parameters and hence assures excellent possibility for both household and industrial applications.

Traditional fermentation and alcoholic brewing is an important household cum social activity of the ethnic communities who follow different protocols for generating fermented produce[26]. It had been a herculean task for the researchers of this region to excavate the possible linkages of the origin of communities and their fermentative habits, since a majority are primary descendents from Thailand, Myanmar, Tibet, China and rest of Southeast Asia which further necessitates the relevance of such study. In Southeast Asia, the diversity of indigenous fermenting yeasts had been reportedly enormous and diverse[27]. Limtong *et al.*[1] had reported the predominance of *Saccharomycopsis fibuligera* (3.9×10^4-2.9×10^7 CFU g^{-1}) in loog pang; a traditional fermentative drink of Thailand while the abundance of *Pichia anomala* in bubod (Philippines), murcha (Sikkim, India), *Candida* in ragi (Indonesia), *Saccharomyces cerevisiae* and *Pichia burtonii* in nuruk (Korea), *Zygosaccharomyes rouxii* in miso and *Saccharomyces sake* in sake (Japan), *Saccharomycopsis*, *Saccharomyces*, *Candida*, *Pichia*, *Sporobolomyces* in Hong Qu and chu (China) and other *Saccharomyces* sp. in banh-men (Vietnam), bubod, loog-pang and ragi has also been reported[23]. Incidentally, in all the reports, the predominance of *Candida*, *Saccharomyces*, *Saccharomycopsis* and *Pichia* in traditional fermentation had been well documented which appear similar to our findings. The diversity of indigenous yeasts in traditional fermentations from northeast India had also been reported earlier[27,28]

although much explorations are still to be covered over a wide range of geographical area encompassing a colossal population of ethnic inhabitants representing different tribes and communities. In a seminal study, Jeyaram *et al.*[29] had reported the molecular identification of 163 yeast species from 'Hamei', a solid state culture material of glutinous rice of the state of Manipur in India and it appears that although Assam and Manipur fall in the same bio-geographic region, subtle differences in yeast types is perhaps due to geographical distances of the residing communities who settled in minute pockets across the entire sub-Himalayan region, remained detached from one another through natural barriers, evolved their own traditions and tamed their own yeasts that fundamentally originated from different lineages in the wild.

CONCLUSION

This study can be concluded that except for the starters of the Ahom and the Mishing communities, the rest of the starters used by the other four communities seem to originate from one place which eventually underwent a parallel segregation in due course of time and perpetuated in different regions of Assam exposed to varied stress and diverse maintenance processes practiced by individual communities. The present finding also recommends that careful selection of yeasts can facilitate in developing consortia that harbor important traits of both economic and oenological value and thus can be sorted for large scale production of metabolites through industrial fermentation.

ACKNOWLEDGMENTS

Authors are thankful to the indigenous communities for providing raw materials and NEHU-SAIF, Shillong for electron microscopy. Also, thanks to Prof. A.K. Mishra of Banaras Hindu University, Mr. Vivek Verma of Gauhati University and Mr. Bhakar Jyoti Nath of Gauhati University for molecular analysis, statistical inputs and experimental help. The DPP is a recipient of BSR-UGC fellowship and is a research student under HKS. This study was supported by a 'DBT Twining' project (BT/303/NE/TBP/2012 dt. 04/01/2013) sanctioned to HKS by the Department of Biotechnology, Govt. of India. JK is a Senior Professor working with the IASST and a collaborator of the presented study.

STATEMENT OF SIGNIFICANCE

- The study has ascertained the persistence of diversified yeasts in starter cultures of ethnic communities from Assam that have tremendous fermentative potential
- The fermentative goods and beverages are thought to have potential for commercialization, provided limitations such as aesthetically disagreeable odour, turbidity, undesirable metabolites, texture and inconsistency are eliminated
- This baseline study shall contemplate the probability of selecting yeasts for applications like preparation of suitable consortia with better fermentation efficiency, screening out yeasts of industrial use, identifying yeasts that are pathogenic to human health or even studying their intra and inter social behavior critical to their sustenance and beneficial from the point of metabolite production useful to humans

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