BIOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF RICE BEER PRODUCED IN ASSAM AND THERAPEUTIC APPLICATION OF A NOVEL ESTER SYNTHESIZED FROM ITS COMPONENTS

ABSTRACT

The thesis is divided into six chapters, which are briefly discussed below:

Chapter 1: Introduction

This chapter describes in brief about the some population of people with different ethnic background in North East India. Most of the people of this region are tribal and bear their own methods of fermenting food materials for the purpose of preservation and taste enhancement and they have been carrying these from time immemorial. All the fermented products are region specific and have their own unique substrates and preparation methods. The fermented alcoholic beverages prepared in this region are discussed, which are unique from the rest of the world in several aspects and bears deep attachment with the socio-cultural lives of the people. The starter cultures used and the utilization of indigenous microbes have reflected the expertise of these people in customary microbiology.

Chapter 2: Biochemical and microbiological characterization of rice beer and starter cultures produced in different regions of Assam

Some of the rural areas where rice beer is predominantly prepared were visited and the process of preparation was observed and documented. The methodologies followed by the Karbi, Deori, Ahom, Mising, Bodo and Dimasa communities of Assam were studied. Also the methodologies practised by the Angami tribe of Nagaland, Khasi tribe of Meghalaya and Adi-Galo tribe of Arunachal Pradesh, which are neighbouring states, were studied for comparison. The plant species used for starter cake preparation were collected from the places visited and their taxonomical identification was carried out. The starter cakes and rice beer prepared by the various tribes were also collected.

The rice beer samples were studied for the content of organic acids, carbohydrates and amino acids by high performance liquid chromatography. The aromatic compounds were detected by GC-MS method. The analysis evinced a wide variation in content of the major

organic acids. Lactic acid was found in high concentration in all the samples, while the other organic acids were present in variable amounts. Among the carbohydrates glucose was predominant and some other monosaccharides were also detected. Most of the essential amino acids were found to be present and among them aspartic acid was the most abundant. All the samples contained the volatile or semi volatile aromatic compounds with phenylethyl alcohol being the most abundant compound. The overall study revealed that this form of drink has important nutritional values and dietary requirements.

A comparative study of rice beer prepared using the various starter cakes was done based on their physicochemical, biochemical and microbiological properties. Significant variations in the density, hardness and color of the starter cakes was found. The moisture was low in all the cakes and carbohydrate was the major component. The pH, titrable acidity, alcohol content, sugars and starch did not vary much among the prepared rice beer. Polyphenols were present in the final product in various concentrations and the rice beers also evinced considerably high antioxidant activity. Yeasts and lactic acid bacteria were dominant in all the samples and spoilage microbes were absent. The study revealed significant variations among the starter cakes and the final product.

Chapter 3: Evaluation of the antioxidative and antimicrobial properties of plants used in rice beer starter culture preparation

Phytochemicals content and antimicrobial activity of *Artocarpus heterophyllus*, *Cyclosorus extensa*, *Oldendia corymbosa* and *Alpinia malaccensis* were investigated. Maximum alkaloids and terpenoids were found in *A. heterophyllus*; tannins and saponins in *C. extensa*; flavonoids, polyphenols and phytosterols in *O. corymbosa* and anthraquinone, glycosides and anthocyaninin *A. malaccensis*. Aqueous, methanolic, ethyl acetate and hexane extracts were prepared from all the leaves. Attenuated total reflectance fourier transform infrared spectrophotometric (ATR-FTIR) analysis revealed that alkanes and alkyl halides were prevalent in all the extracts and the ethyl acetate extracts contained comparatively higher number of functional groups, which were also more effective against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. The minimum inhibitory concentration values of *A. malaccensis* against the tested pathogens were found to be lesser than the other species.

The purified phenolic extracts of *A. heterophyllus*, *O. corymbosa*, *C. extensa* and *A. malaccensis* were then tested for the presence of various polyphenols and antioxidant activities. The polyphenols were extracted with ethanol and purified by stepwise dialysis and column elution. HPLC was performed to detect and quantitate the polyphenols and ten different *in vitro*

methodologies were used to measure antioxidant properties. Quercetin, ferulic acid and salicylic acid were present in all the species. Chlorogenic and *p*-coumaric acid in *A. heterophyllus*; *p*-coumaric acid, caffeic acid, naringin, catechol and resorcinol in *C. extensa*; chlorogenic, caffeic and quinic acid in *O. corymbosa* and naringin, quinic acid and catechol in *A. malaccensis* were detected with varied concentrations. The DPPH and ABTS assays exhibited high radical scavenging activities and they were also able to combat •OH radicals and H_2O_2. Superoxide anions and nitric oxide radicals were scavenged, and the extracts exhibited ferric ions chelation and ferrous ions reduction capacity. A reduction in the level of lipid peroxidation was also observed. All the four species were found to be potent sources of polyphenols having antioxidative property.

In the next step, the bioflavonoid fractions from the leaves of *A. heterophyllus* and *C. extensa* were purified by Amberlite XAD–2 and analysed by HPLC. Myricetin and epigallocatechin gallate were the major flavonoids in *A. heterophyllus*, whereas kaepferol, luteoline and quercetin were in high amount in *C. extensa*. Rice beer prepared in the laboratory was supplemented with these extracts and subjected to storage at 32 ºC for eight weeks. The beers studied were RBAH – fortified with flavonoids from *A. heterophyllus*, RBCE - fortified with flavonoids from *C. extensa*; RBBHT - fortified with butylatedhydroxytoluene; RBF – filtered and RBC – control. Significant differences ($p \leq 0.01$) in all the parameters were observed among all the beers. The aerobic plate counts were less in RBAH and RBCE, as compared to RBC, but fewer counts were observed in RBF. The pH, total phenols and protein increased in all the beers, with less increment in pH and higher content of phenolics in RBAH and RBCE. Antioxidant activity was observed in RBAH and RBCE, which was even higher than RBBHT, in which activity was shown only till the 1st week. The RSA however went on decreasing in all the beers with time. The change in colour was also sequestered in the supplements beers, while the highest change was observed in RBC. The peroxide value and thiobarbituric acid reactive substances were not detected in RBAH and RBCE till the 6th week and 5th week, respectively, whereas in both RBC and RBF the peroxidation was observed from 1st week. The overall study indicated a higher efficiency of the bioflavonoids in improving the shelf life stability of rice beer over BHT and filtration process.

Finally, the optimal conditions of time and temperature of fermentation for extraction of bioactive compounds from the dried leaves of *C. extensa* were obtained by using response surface methodology (RSM). The central composite rotatable design (CCRD) was employed and thirteen experimental runs based on two factors (extraction time and temperature) five levels design were generated and carried out for each of the solvents. The analysis of variance

of the test data was carried out and the sequential sum of squares, *F*-value, R^2 and adjusted R^2 were deduced. The predicted models for all the response variables were adequately fitted to the observed experimental data ($p \leq 0.001$). The maximum extraction of bioactive compounds under the optimum conditions of extraction temperature and time for hexane, ethyl acetate, methanol and distilled water were found to be 25 ºC for 29.43 h, 28.28 ºC for 41.27 h, 43.95 ºC for 29.61 h and 55.00 ºC for 48.00 h respectively. It was also observed that the solubility of the polyphenols was higher in methanol, followed by ethyl acetate and the highest antibacterial activity against *E. coli* was shown by the ethyl acetate extracts.

Chapter 4: Identification of fungal and lactic acid bacteria isolates from rice beer and starter cultures and evaluation of their functional properties

In the first step, amylolytic properties of the fungi isolated from starter cakes were studied. Two types of starter cakes viz., *amou* and *perok-kushi* used in the production of rice beer in Assam, India by the Bodo and Deori communities, respectively were used for the isolation of amylolytic fungi. Based on the sequencing of their internal transcribed spacer (ITS) regions the fungi were identified as *Amylomyces rouxii* and *Rhizopus oryzae* and given the strain names TU460 and TU465, respectively. Both the strains showed the ability to degrade and saccharify starch. The glucoamylase activity was considerably high in *A. rouxii* TU460 (14.92 µmol/min) as compared to *R. oryzae* TU465, whereas α-amylase activity was found to be closely related i.e. 7.02 and 6.09 unit mL^{-1}, respectively. SDS PAGE for the determination of molecular size of the glucoamylase enzymes revealed production of two distinct units of 59 kDa and 31 kDa by *A. rouxii* TU460 and one unit of 72 kDa by *R. oryzae* TU465. LC MS/MS analysis revealed that no mycotoxins were produced by both the strains. This study indicated good amylolytic property of both the strains and potential to be used in the starch processing industries.

Physiological properties of yeast strains isolated from rice beer starter cakes produced in Assam, India were studied. The isolates were identified as *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus* and *Pichia membranifaciens* based on sequencing of their ITS regions. Test for their biochemical characteristics, growth at different temperatures and osmotic stress exhibited varying results. Their ability to use sugars anaerobically and as sole source of carbon for aerobic growth was assessed and they were found to varyingly ferment a wide array of carbohydrates. Alcohol dehydrogenase activity showed that two strains of *S. cerevisiae* possessed the highest activities of 61.29 and 54.27 unit ADH mg $^{-1}$ protein. Three strains of *S. cerevisiae* were also found to be the highest producers of alcohol in whose culture media the alcohol content reached up to 6.21% on the 5th day. GC-MS analysis for the analysis of volatile

organic compounds revealed that ethanol and phenylethyl alcohol were invariably produced by all the strains. In addition, wide arrays of fusel alcohols were also produced by all the strains. All the strains of yeasts studies were found to have good potential to be used in the brewing and wine industries.

Genetic identification of the LAB isolates was carried out based on 16s rRNA sequencing. A total 29 number of LAB strains were identified from different rice beer and starter cultures prepared by six different tribes of Assam. Eight of the isolates were identified to be *L. casei*, sixteen as *P. pentosaceus*, two to be *L. paracasei*, two as *L. plantarum* and one as *L. pentosus* strains. Most of these isolates could maintain a count of 7 log CFU/ml upto 14% bile concentration. Highest survivability was shown by the isolate *L. pentosus* TEZU174, which had a count 7.8 log CFU/ml at 14% bile concentration. In the acid tolerance test, at pH of 2.5, up to 8 hours, highest resistance was shown by the isolates *L. plantarum* TEZU272 and *L. casei* TEZU374. Moreover, *L. pentosus* TEZU174 showed survivability even up to 48 h. At the lower pH of 1.5, four of the strains survived till 3 h, while *L. pentosus* TEZU174 and *P. pentosaceus* TEZU199 survived till 4 h. In the antibiotic susceptibility test, most of the strains were found to be susceptible, whereby erythromycin, chloramphenicol and linezolid were found to be the most effective. In the autoaggregation assay, *L. casei* TEZU262 and *L. casei* TEZU309 attained 100% aggregation within a period of 5 h. The highest adhesion to xylene (35.66%) and chloroform (51.66%) was showed by *L. casei* TEZU309, whereas *P .pentosaceus* TEZU427 was most adherent to ethyl acetate (96.90%). *L. pentosus* TEZU174, *L. casei* TEZU262, and *P. pentosaceus* TEZU451 showed the highest activity against *E. coli* with zones of inhibition above 40 mm. In case of *S. aureus L. pentosus* TEZU174, *P. pentosaceus* TEZU213, *L. plantarum* TEZU272, *P. pentosaceus* TEZU410 and *P. pentosaceus* TEZU427 showed zones of inhibition above 40 mm. In the test for antioxidant activity, *L. casei* TEZU374 and *P. pentosaceus* TEZU482 were found to be the most effective with DPPH scavenging activity above 70%. The cellular autoaggregation and adhesion to solvents *L. casei* TEZU309 and *L. casei* TEZU374 were assessed, with and without their surface proteins. The LiCl treated *L. casei* TEZU309 and *L. casei* TEZU374 could attain only 14.56% and 18.59% autoaggregation respectively till the 10^{th} hour, while their adhesion to organic solvents was also reduced. Their adherence to CaCo-2 intestinal epithelial cells was also reduced when the surface proteins were removes. Transmission electron microscopy revealed the presence of a distinct layer above the cell surface, relating to the S-layer proteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed the molecular weights of the major surface associated proteins to be of 52 Kda and 51 Kda in *L. casei* TEZU309 and *L. casei* TEZU374

respectively. Finally, utilization of D-glucose, maltose and lactose for production of organic acids by the LAB strains was evaluated. Lactic acid and acetic acid were the major organic acids produced by utilization of glucose by all the strains. Lactic acid, acetic acid and succinic acid were the major organic acids produced by all the strains by utilizing maltose except *L. pentosaceus* TEZU174 and *L. casei* TEZU468 which did not produce acetic acid. Except *L. pentosus* TEZU174, tartaric acid, lactic acid, acetic acid, citric acid and oxalic acid were produced by all the strains by utilizing lactose. These results evinced that the LABs found in rice beer of Assam has probiotic properties and can be used utilized as beneficial organisms with functional properties.

Chapter 5: Laboratory scale optimization of rice beer making process and sensory evaluation of the products

Response surface methodology was employed to obtain the optimal conditions of time and temperature of fermentation for rice beer and the same conditions were applied to prepare beer from cassava (*Manihot esculanta*) and plantain (*Musa* ABB). *Saccharomyces cerevisiae*, *Aspergillus oryzae* and *Lactobacillus plantarum* were used to carry out the fermentation process. Thirteen experimental runs based on two factors five levels design were carried out according to a central composite rotatable design. The independent variables were varied as, fermentation time (24 to 216 h) and temperature (25 to 40 °C).The responses studied were protein content, alcohol content, *L. plantarum* count, total polyphenols content, reducing sugars content and titratable acidity. Numerical optimization predicted that a fermentation period of 143.12 h at a temperature of 33.28 °C would result in the most desired rice beer, with response values of protein content 0.77 %, alcohol content 6.99 %, *L. plantarum* count 7.08 log $CFUml^{-1}$, polyphenols content 34.46 mg/100g, reducing sugars 2.39% and titratable acidity 0.34%. Cassava and banana beers were prepared using the optimized parameters of rice beer and the result exhibited distinctive properties of beer, thereby indicating the applicability of the conditions obtained in preparing beer from wider range of substrates. The response of various substrates on the sensory characteristics of beer was then tested by using fuzzy logic. The panel consisted of 25 judges wherein pre-screening and training were conducted prior to testing. Fuzzy analysis of the responses obtained was carried out using the MATLAB software. Biochemical analysis of the developed products was also carried out. All the beers were found to be above the “not satisfactory” level, with the beer prepared from rice evinced the “very good” level. The incorporation of rice was also found to have positive influence on the other types of beer. This procedure for sensory evaluation will help in precise documentation of the

sensory properties of different types of beers and the effects of different substrates on the sensory perception of the products. The brewing industry is always in search of effective and quicker sensory evaluation techniques for its products. The fuzziness which is associated with the judges' perception in the characterization of sensory profiles of beers could be well represented using fuzzy logic.

Chapter 6: Studies on anti-inflammatory role of compounds derived from rice beer in *in silico, in vitro* and *in vivo* models

$Zn(OTf)_2$-catalyzed selective esterification of salicylic acid and phenylethyl alcohol for the synthesis of salicylic acid phenylethyl ester (SAPE) was confirmed by NMR (13C and 1H) and FTIR studies. ADME-Tox studies revealed the physical and Lipinski-type properties, solubility and volume of distribution. The maximum passive absorption was 100% from trancellular route and permeability for human jejunum was $7.51x10^{-4}$ cm/s with absorption rate of 0.051 min^{-1}. The rate and extent of brain penetration and brain/plasma equilibration rates were -1.8, -0.2 and -3.1 respectively. The probability of oral bioavailability at %F>30% was 0.811 and at %F>70% it was 0.358 and the probability of effects on organs were below 0.6. There was 85% probability that LD50=<5000mg/kg and 95% probability that LD50>300mg/kg. Molecular docking of SAPE and positive controls (indometacin and ibrupofen) was carried out against COX-2 and the MolDock score, rerank score, interaction, internal and H bond of SAPE were found to be favourable. SAPE bonded with Leu353(O) and Tyr3556(OH) with interaction energies of -1.7 and -2.5 and interaction distances of 2.51 Å and 3.02 Å respectively. These values were close to that shown by ibuprofen and indometacin. In the 20 ns molecular dynamics simulation of the SAPE-COX-2 docked complex, the variations of the COX-2 and protein-ligand binding complexes, the average RMSD (~0.13nm) for the complex and the stable dynamic equilibrium condition of the complex (average RMSD of ~0.23nm) was revealed. Thus, SAPE was found to be a good inhibitor of COX-2 and can serve as a novel anti-inflammatory compound.

In the next step, SAPE was tested for its role as a non-steroidal anti-inflammatory drug. *In vitro* cytotoxicity of SAPE in animal cell line models was done. The membrane stability was tested on human erythrocyte suspension and the cytotoxic effect was evaluated by MTT assay upon human primary peripheral blood mononuclear cells and two other cell lines (CaCo-2 and HepG-2). Alamar Blue® assay was also performed to test any cytotoxic effect of SAPE in CaCo2 cells. Membrane stability was observed with the addition of SAPE and the percentage of cell viability was at per with the control in case of all the cell lines up to a concentration of

200 µg/ml of SAPE. The anti-inflammatory activity of SAPE was then tested in rats of Wistar variety in a pre-treatment model of drug administration. Pre-test of the drug was performed by a paw oedema model and indometacin was used as a positive control. The drugs were administered orally for 16 days after which the rats were challenged with intraperitoneal injection of lipopolysaccharides. Measurement of body weight, evaluation of disease activity index and scanning electron microscope study of intestinal morphological changes was done and test for antioxidative and anti-inflammatory biomarkers were performed. Reduction in the extent of tissue disruption was observed in the excised tissues of the treated groups. A significant ($p \leq 0.05$) reduction in the extent of lipid peroxidation was observed in the treated groups. The antioxidative marker enzymes catalase (CAT), γ-glutamyltransferase (GGT) and glutathione S-transferase (GST) were significantly ($p \leq 0.05$) higher in the treated groups as compared to the control group. The pro-inflammatory biomarkers interleukin 6 (IL-6) and Tumor necrosis factor alpha (TNF-α) were also significantly ($p \leq 0.05$) lower in content in the treated groups. These results indicated that SAPE is a safe compound without any cytotoxic effects and has potential as a novel antioxidative and antiinflammatory agent.