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An antioxidant-rich fermented substrate produced by a newly isolated bacterium showing antimicrobial property against human pathogen, may be a potent nutraceutical in the near future.

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Abstract –A specific strain able to produce ethanol (in single step from boiled rice) has been isolated, characterized and its fermented product is analyzed. Ethanol (4.2 %v/v) is produced within 72 hours. Besides, fermented product is rich in antioxidant and showed antimicrobial activity against few gram positive and gram negative human pathogens. The pH, reducing sugar, total sugar, protein and calorific value of the fermented slurry are 3.45±0.05, 6.57±0.22 mg/ml, 53.27±2.02 mg/ml, 0.62±1.13 mg/ml and 54.21±0.81 kcal/100 ml, respectively. The comparative sensory analysis shows this product is comparable to beer. The 16S rDNA analysis exhibits maximum sequence identity with *Bacillus megaterium*, strain IAM 13418 (99%). Although some of the biochemical properties does not match with neither of them.

Keywords – Single step bacterial fermentation; 16S rDNA analysis; Antioxidant rich drink.

1. Introduction

Bioconversion of starch material to ethanol generally involves three steps (i) liquefaction of starch by an endoamylase such as α-amylase, (ii) enzymatic saccharification of the low molecular weight liquefaction products to produce glucose, and (iii) finally fermentation of glucose (Abouzied and Reddy, 1986; Laluce and Mattoon, 1984).

Use of large amounts of amylolytic enzymes to produce fermentable sugar to *Saccharomyces cerevisiae* and high boiling temperature (140°C to 180°C) of to obtain a high ethanol yield requires improvement in the starch to ethanol production process (Shigechi *et al.*, 2004). Several attempts were done to reduce the cost of production via reduction of energy cost for cooking of starchy materials (Matsumoto *et al.*, 1985), using recombinant glucoamylase-expressing yeasts with the ability to ferment starch to ethanol directly (Murai *et al.*, 1997; Nakamura *et al*., 1997), and using co-culture (Shin et al., 2006; Kim *et al*., 1988; Verma *et al*., 2000).

Therefore, the objective of this study is to isolate a strain from available edible fermented rice to develop a process by which bioconversion of steamed rice (starch) to ethanol takes without enzymatic liquefaction and saccharification. Besides, applying the strain obtained from edible fermented rice, rice based fermented drink with natural antioxidative and antibiotic activity was proposed to develop. This work supplies important basis for further research.

2. Materials and Methods

2.1. Isolation, media and culture condition

Fermented rice was collected from Jhargram (22° 27' 0" North, 86° 59' 0" East) in the district of Paschim Medinipur located in West Bengal, India where common people consume this regularly. One ml of sample collected was suspended in 9 ml of sterile distilled water. Then 100 µl of this was amplified on sterilized LB agar plates. The individual colonies were collected and grown in liquid medium overnight in a shaker incubator at 37°C with shaking (150 rpm). The particular strain was selected depending upon its physiological and biochemical property. Isolated strain was stored at-80°C in culture medium containing 50% (v/v) glycerol until use.

2.2. Physiological and biochemical characteristics

Optimum growth phase was determined by inoculating 0.1% from overnight grown parent culture into sterile 50 mL Luria Bertani (LB) broth and optical Density (OD) at 660 nm was measured in spectrophotometer at 30 min interval until the culture reaches stationary phase. To determine of cell dry weight, thirty mL of overnight grown cultured bacteria was filtered through 0.45 µm membrane (millipore) under vacuum. The filter retained mass was washed with 25 mL of sterile water and were dried in a microwave oven and weighed (Cetina *et al*., 2010). Optimum pH and temperature was determined by culturing the bacteria overnight at different pH ranges (3-12) at 37°C and growing at different temperature range (10 to 50°C) with shaking and measuring the OD at 660 nm in a spectrophotometer. Different carbon and nitrogen sources like glucose, lactose, maltose, sucrose, starch cellulose, glycerol, fructose, sorbitol, urea, peptone, ammonium nitrate etc were used for determination of substrate utilization by bacteria. Mineral medium with individual carbon and nitrogen source was used at a time to determine the suitable substrate utilization. Phenol red was used as indicator of bacterial growth. Antibiotic sensitivity of the bacteria was studied by using six commercially available antibiotic disks from HiMedia (India). The sensitivity was determined based on the diameter of the zone of inhibition and evaluation was done according to Manufacturers instruction provided in the kits by Himedia. The extracellular amylolytic activity in culture broth was determined by measuring reducing sugar released from starch as described by Lemmel *et al*. 1980.

2.3.16S rDNA

DNA extraction and purification of the bacterium were done according to the protocols described (Zhou *et al*., 1996). 16S rDNA genes were amplified by polymerase chain reaction (PCR) in 50 µl mixtures according to previously studies (Liu *et al*., 2007). In the reaction, the primers sequences of bacteria-specific 27F, and the universal 1492R were 5´-AGAGTTTGATCCT GGCTCAG-3´, and 5´-CGGCTACCTTGTTACGACTT-3´, respectively. A gene amplifier was used to incubate reactions through an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 55°C for 30 s and 72°C for 1 min and finally at 72°C for 10 min. The PCR product with expected size of about 1.5 kb was purified using PCR purification columns according to the manufacturer's instructions. The 16S rDNA were sequenced by 3730 automatic sequencing equipment (ABI, US). Phylogenetic affiliation of the 16S rDNA sequence derived from the sequencing was estimated by using the program nBLAST-a tool of the National Center for Biotechnology Information (Altschul *et al*., 1997). Depending upon the phylogenetic results, appropriate subsets of 16S rDNA sequences were selected and performed final phylogenetic analysis with Mega 5, phylogenetic development trees were built ((Liu *et al.*, 2007)).

2.4. Nucleotide sequence accession number

The 16S rDNA sequence has been submitted to GenBank with accession number: BankIt1545135 seq1 JX182976.

2.5. Fermentation procedures Analyses of fermented product

Sterilized aspirator bottles containing about 500 grams steamed rice was mixed with 9 ml of inoculum (OD 0.327) and was incubated at 27 ± 2 °C for 3 days. The bottles were topped with tubes to allow gas to escape. Fermented was centrifuged at 4°C for 20 min at 5,000 x g to separate cells from the broth. The broth was used for further characterization.

2.6. Acidity, reducing sugar, proteins, methanol, total soluble solid, higher alcohol and ash content analysis

Centrifuged sample was analyzed by standard methods. Ethanol content, ash content, methanol and higher alcohol was measured using standard Association of Official Analytical Chemists (A.O.A.C), 1990 methods. Total soluble solid content was estimated using a hand refractometer based on the principle of total reflection. Temperature correction was done. Acidity, reducing sugars and total sugar were quantified as described by Teramoto *et al*., 2002. The colour and brightness of the samples were measured with the help of Spectrophotometer (Onkarayya, 1985).

2.7. Screening for bioactive components

Biochemical assays were carried out on the fermented broth for screening the presence of bioactive components according to the methods mentioned in Sofowora et al.(1993) and Harborne et al. (1988)

2.8. Quantitative estimation of bioactive components

Protein in both the samples was estimated by the method of Lowry *et al. (*1951), using serum albumin as standard protein and saponin was estimated according Obadoni and Ochuko et al. (2001). Total phenolic compound was estimated by the method Sadashivam and Manickam (2004). The total phenolic content was measured as gallic acid equivalent using the following linear equation established using gallic as standard: [$A = 0.210C + 0.292$; $R^2 = 0.997$] where A is the absorbance at 765 nm and C the concentration as gallic acid equivalent (µg/ml).

2.9. Estimation of antioxidant and free radical scavenging ability

Total antioxidant activity of the fermented broth was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al*. (1999). Ferric reducing antioxidant power (FRAP) assay of Benzie and Strain et al. (1999) was also followed to measure the total antioxidant activity of the fermented sample. Reducing power of fermented sample checked *by* the method Oyaizu et al. (1986). The free radical scavenging activity of the sample was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method. DPPH solution (0.1 mmol/l) in ethanol was prepared. 2ml of this solution was added to equal volume of water solution containing

different amount of sample. After 30 min absorbance was measured at 517 nm. The % inhibition of the antioxidant was measured as mentioned in referred paper. Ascorbic acid was used as standard. The chelating activity of the fermented sample for Fe2+ is measured according to the methods described by Dinis *et al*. (1994). Scavenging effect of the fermented substrate was studied using ABTS (2,2'-azino-bis- 3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colorization assay according to the method Re *et al*., 1999 .

2.10. Screening of antimicrobial activity

Human pathogenic strains of *Klebsiella pneuminiae (MTCC-109), Staphylococcus aureus (MTCC-96), Bacillus cereus (MTCC-1305), Enterobacter aerogenes (MTCC-111), Pseudomonas aeruginosa (MTCC-741) and Escherichia coli (MTCC-1678) (as positive control)* were collected from MTCC-Chandigarh and cultured according to their specifications. Antimicrobial activity was performed by well diffusion assay placing centrifuged cell free fermented sample (50 µl) on the cups in the solid agar plate where already pathogenic inoculums (10^6 cfu / ml) were spread . Plates were incubated for 24 hours at 37°C and inhibition diameter (mm) were noted.

2.11. Organoleptic Evaluation Assay

Fermented rice is a cultural food product consumed by the local people of Jhargram almost ages. The bacterial strain has been isolated from this product. Therefore, the toxicity study of the product on rat or mice does not make any sense. Based on the traditional uses, sensory evaluation of the alcoholic beverage prepared from isolated bacteria was carried out initially choosing a panel of 40 judges. The judges were selected on the basis of their ability to describe the perceived sensations and to distinguish flavors of beer both qualitatively and quantitatively. The average age of the judges was 25-38. Initially the judges were given some basic taste solution such as sucrose (16g/l), sodium chloride (5g/l), caffeine (0.5 g/l), citric acid (1 g/l). They were asked to familiarize these solutions. Then they were presented with secondary samples and were asked to match. As per ISO 8586-1, International standard guidelines, the judges who achieved more than 80% marks were kept for the final judgment. A rating test with a 16 point was used and the final score was the average of individual scores (Amerine and Ough, 1980). Alcoholic beverage beer was taken as reference.

3. Results and Discussion

3.1. Physiological-biochemical characteristics of the isolated bacteria

A single strain of bacteria was isolated initially depending upon the physiological and biochemical characteristics of the strain mentioned below. The strain is a gram positive, aerobic, spore forming bacteria. The optimum growth time of this bacterium is 48 hours where it reaches the mid-log phase (Fig. 1a). Although, the strain can grow in a wide range of temperature (20 - 45°C), the optimum temperature for growth is 35-37°C. It has also been noted that the bacteria can grow in a vast range of pH, but the optimum pH is 6.5 - 7.0. It has been found that the bacterial strain can produce amylase (Fig. 1b). The amylase activity is optimum between 30-45 hours of growth at pH 7.0 (Fig 1c, d). In addition, the bacteria could use maltose, D-glucose, D-fructose, D-sorbitol, inositol, lactose, pectin, cellulose, and starch as only carbon source in the media for growth (Fig 1e). Furthermore, the bacteria can use peptone, urea, ammonium sulfate, tryptone, and ammonium nitrate as only nitrogen source in the medium (Fig 1f). The cell dry weight calculated was 0.187 gram/30 ml of culture broth.

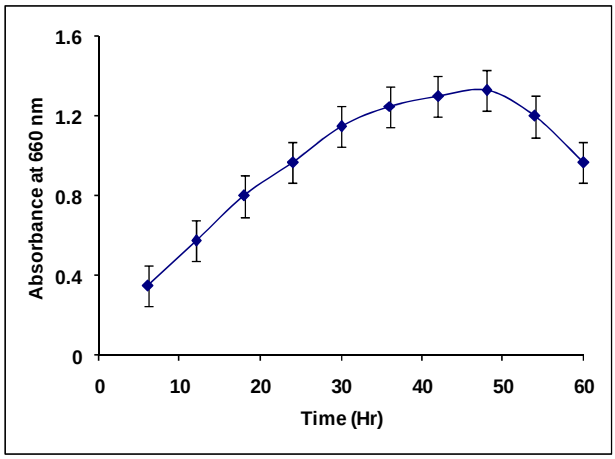

Fig 1a: Absorbance time curves describing bacterial growth in broth at different time interval

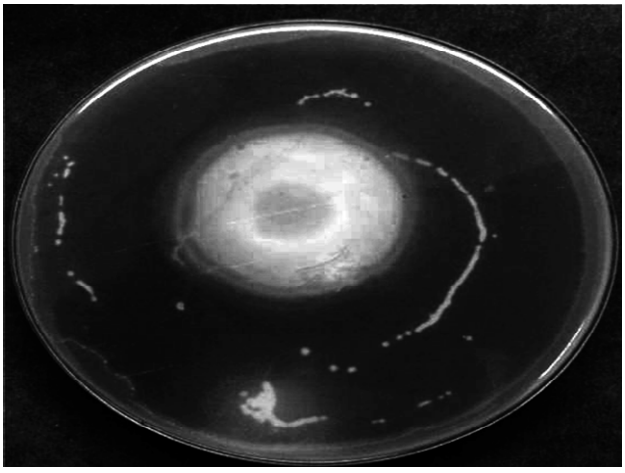

Fig 1b: Zone of clearance of Strain grown starch plate after flooding with iodine and potassium iodide solution

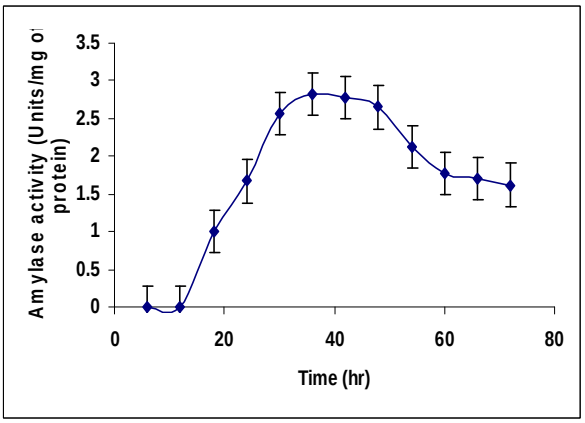

Fig 1c: The effect of time on amylase activity of the bacterial strain.

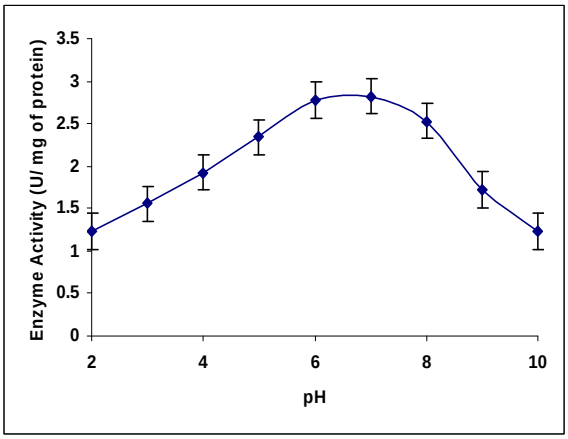

Fig 1d: The effect of Ph on amylase activity of the bacterial strain

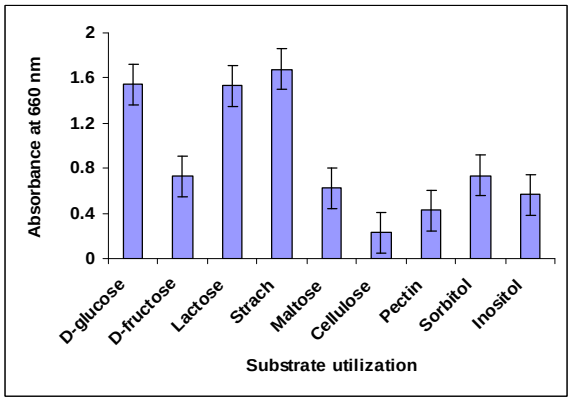

Fig 1e: Utilization of different carbon source By overnight Grown Bacteria

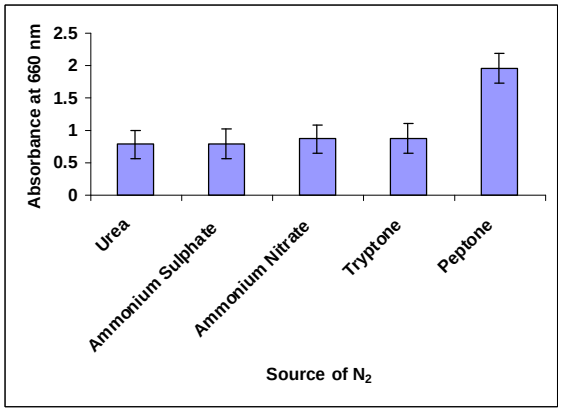

Fig 1f: Utilization of different carbon source By overnight grown Bacteria

3.2. Phylogenetic analysis of 16S rDNA

The 16S rRNA gene sequences which we obtained were blasted using BLAST database (Altschul et al., 1997) of National Center for Biotechnology Information to compare the sequence of the strain with known 16S rDNA sequence database. *Bacillus megaterium* strain IAM 13418, *Bacillus flexus* strain accession number IFO15715, have 99% & 98% sequence identity with the strain BankIt1545135 seq1 JX182976 (will be referred as KAN1 henceforth), respectively. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.17507467 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig 2). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 13 closest nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1363 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

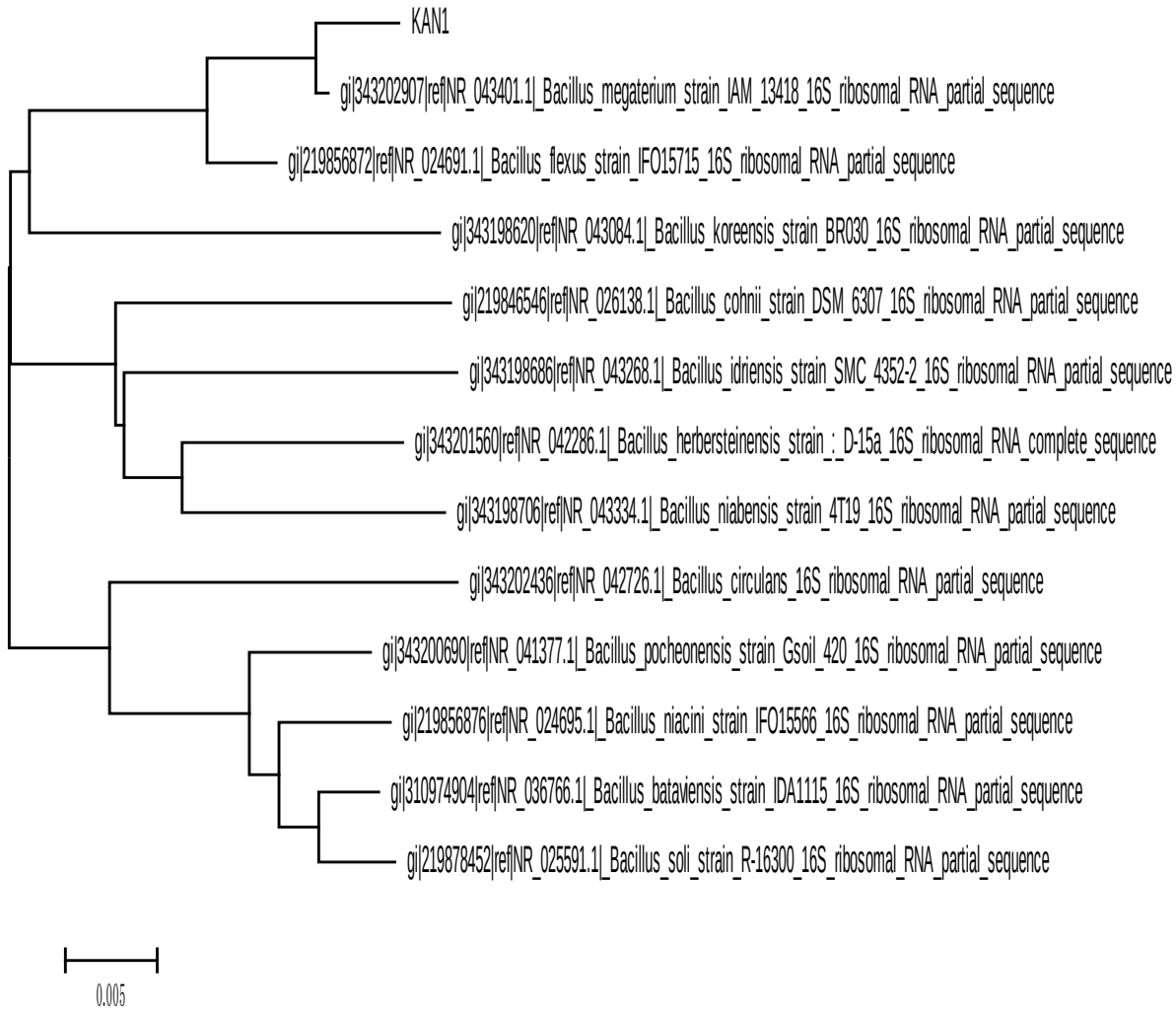

Figure 2. Evolutionary relationships of taxa. Neighborhood joining tree

3.3. Amylolytic activity, Ethanol production by isolated bacteria

Isolated bacteria can produce ethanol within 72 hour by utilizing steamed rice as carbon source. It is assumed that initially it produces amylase that converts starch to simple sugar, and then it utilizes and produces ethanol. The details of the mechanism can only be explored after proper research in the metabolic pathway. The result also signifies that 36 hour is the optimum for amylase production (Fig 1c). The optimum time for the production of alcohol is 72-80 hrs (Fig 3a).

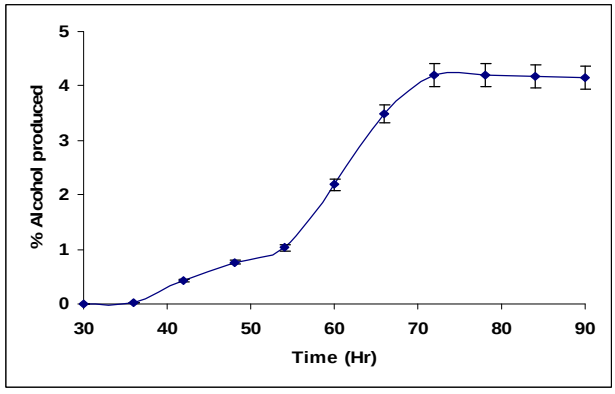

Fig 3a: Alcohol production by the bacteria at different time interval.

3.4. General Analysis of Fermenting supernatant

The fermenting broth was an off-white slurry and without filtration. After centrifugation it becomes clear off white liquid with characteristic aroma. Its alcohol content, pH and acidity were 4.2±0.1 %, 3.45±0.05 and 5.07±0.06 respectively (Table 1 a). It provides 93.2±0.81 kcal /100ml. Total sugar and reducing sugar were observed in an amount of 53.27±2.02, 6.57±0.22 mg/ml of fermented broth. Total soluble solid and ash content was 14.17±0.71

& 0.69±0.015 % respectively. Methanol was absent in the test samples.

Table 1 a. General Analysis of Fermenting supernatant

Characteristics	**Fermented slurry supernatant**
Fermentation Time (hour)	72
Acidity(ml)	5.07±0.06
PH	3.45±0.05
Reducing sugar (mg/ml)	6.57±0.22
Total sugar (mg/ml)	53.27±2.02
Methanol	0
Total soluble solid (%)	14.17±0.71
Ash content (%)	0.69±0.015
Ethanol concentration (% v/v)	4.2±0.1
Calorific Value (kcal /100ml)	54.21±0.81

3.5. Bioactive chemical Screening of the cultured bacteria alone vs fermented broth

Screening of bioactive chemicals results that fermentation yields active and antioxidative compounds (Table 1b). The alkaloids, steroids, sterols, tannins, quinones are absent in the both cases. Phenolics, carbohydrates, amino acids, proteins and the reducing compounds are present in both cases but the prominence of presence was seen for fermenting slurry supernatant rather than supernatant of culture. Sign of saponin production was observed in during fermentation.

Table 1 b. Qualitative analysis bio active chemicals in fermented slurry supernatant

Compound	**Fermenting slurry supernatant**	**Supernatant of culture**
Alkaloids	–	–
Steriods and Sterols	–	–
Flavonoid	–	–
Saponin	+	–
Phenolics	+++	+
Carbohydrates	+++	++
Amino Acids and Proteins	+++	++
Quinones	–	–
Reducing compounds	+++	+
+++: Strongly positive, ++: moderately positive, +: weakly positive, -: negative		

3.6.Quantitative estimation of bioactive chemicals

The product offers a high quantity of protein (0.62±0.13). The yield of total phenolics in the sample is 0.268µg/ml. The supernatant shows the presence of a minute quantity of saponin (0.13±0.01%)

Table 1 c. Quantitative analysis bio active chemicals and total antioxidant capacity fermented slurry supernatant

Characteristics	
Protein Content (mg/ml)	0.62±0.13
Total phenolic compound content (µg/ml, GA equivalent)	0.268±0.0
Saponin (%)	0.13±0.01
Total antioxidant capacity(µg/ml, AA equivalent)	497±6.14
Total antioxidant activity by ferric reducing antioxidant power (FRAP)(µM)	1.25±0.02

3.7. Total antioxidant and free radical scavenging ability

Total antioxidant capacity of the fermented sample was analyzed by phosphomolybdenum method using ascorbic acid as standard. It is expressed as ascorbic acid equivalent using the following linear equation established using ascorbic acid as standard: [$y = 0.0037x + 0.0343$; $R^2 = 0.991$] where y is the absorbance at 695 nm and x the concentration as ascorbic acid equivalent (µg/ml). Total antioxidant capacity of the fermented sample is 497±6.14 µg/ml (ascorbic acid equivalent).

Total antioxidant activity by ferric reducing antioxidant power or the TFRAP value of the sample is 1.25±0.02 µM, where as standard ascorbic acid (1000µM) has a FRAP value 2. Figure 3b confirms the reducing power of the fermented broth using potassium ferricyanide reduction method. Reducing power is associated with the antioxidant activity (Amarowicz *et al*., 2004). Increasing OD value indicates increasing trend of reducing power.

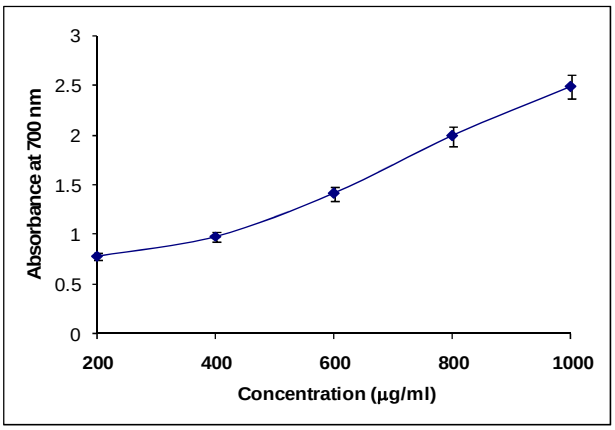

Fig 3b: Reducing power of fermented broth supernatant

Free radical scavenging activity of the fermented substrate was determined by DPPH method and compared with standard Gallic acid (100 µg/ml). Figure 3d is a graphical representation. 50% inhibition or reduction amount (the amount of a particular concentration of antioxidant that reduces the 50% absorbance of DPPH solution) of Gallic acid is 0.3827 ml (concentration 100µg/ml) and 0.7919 ml of fermented broth.

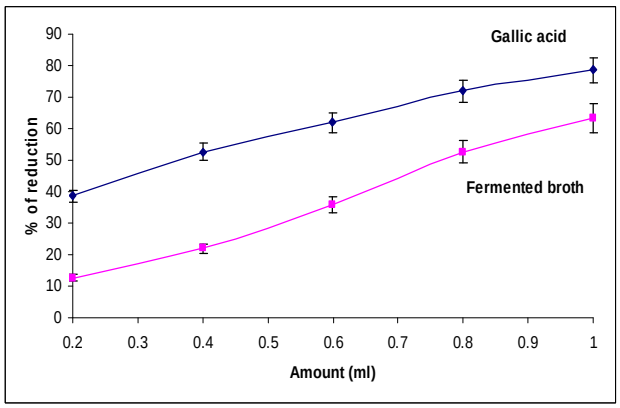

Fig3c: Graphical comparison of antioxidant activity of sample and Gallic acid by 1,1-diphenyl-2- picrylhydrazyl (DPPH) method

The chelating activity of the fermented sample for Fe2+ (ferrozine assay) indicates a minute chelating ability. The standard EDTA (100 µg/ml) of 0.5 ml volume shows a chelating rate of 39.119% where as the fermented substrate of 0.5 ml volume yields only 2.66% chelating rate.

ABTS scavenging effects of fermented substrate of 0.5 ml volume offered 31.71% ABTS radical scavenging ability where as 0 .5 ml ascorbic acid (100µg/ml) offered 87.85 ABTS scavenging ability.

3.8. Antimicrobial Activity

It also shows antimicrobial activity against gram positive and gram negative pathogenic bacteria. The pathogen selected for this purpose were gram positive *Staphylococcus aureus*, *Bacillus cereus* and gram negative, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* showed 14.92 ± 1.06, 7.3 ± 0.3, , 13.08 ± 0.23, 15.92 ± 0.50, and 17.00 ± 0.37 mm zone of inhibition respectively. *E. coli* as positive control resulted 15.75 ± 0.35 mm zone of inhibition.

3.9. Organoleptic Evaluation Assay:

From the organoleptic evaluation, it is found that KAN 1 is comparable with that of commercial beer except differing slightly in color; acidity and flavor which can be further improved (Table 2b). It is clear that the product is not superior to commercial beer. The color of KAN1 product is more probably due to the higher concentration of carbohydrate (Table 2a). pH is very important parameter during fermentation. The most favorable pH for fermentation by using yeast is 4.0-4.5. The yeast strain may tend to demise below this pH. The strain KAN1 may be using this principle where it can grow alone and produce alcohol without any interference from other strains. The calorie value is higher in KAN1 product compared to commercial beer may be due to the occurrence of higher % of carbohydrate. Further tests should also be employed to confirm its use as nutraceutical effect. It should have a great industrial importance as the process offers a low alcoholic drink with rich in antioxidant from steamed rice in single step without addition of external enzyme. Therefore, this process offers a cost effective, high nutritive, natural and easily digestible low alcoholic beverage as a product.

Table 2 a. Chemical parameters of KAN 1 in comparison with commercial beer

No.	Parameters	KAN 1	Commercial beer
1	pH	3.45	4.10
2	Total Phenolics (mg/100 ml)	0.268	0.37
3	Alcohol content (%)	4.2	5.40
4	Reducing sugars (mg/ml)	6.57	2.13
5	Color (optical density)	0.357	0.107
6	Brightness (optical density)	0.497	0.125
7.	Total calorie (kcal/100 ml)	54.21	43.09

Table 2 b. Organoleptic evaluation of KAN 1 in comparison with commercial beer

No.	Characteristics	Total score	Average score given by panel judges based on 16 point scale	
			KAN 1	Commercial beer
1	Appearance	2	1.25	1.75

2	Color	2	1.5	2
3	Aroma	2	1.5	1.75
4	Total acidity	2	1	1.50
5	Body	2	1.5	1.75
6	Flavour	2	1.5	2
7	Astringency	2	1.75	1.50
8	General quality	2	1.5	1.75
9	Overall acceptability	16	11.5	14

7. Discussion

The present study has shown that the isolated microbe is a new strain that has 99% 16S rRNA sequence similarity with *B. megaterium*, strain IAM 13418 and 98% 16S rRNA sequence similarity with *B. flexus*, strain IFO15715. The *B. megaterium* strain KAN1 has the unique property of converting steamed rice to alcohol in a single step process. The isolated microbe exhibits the amylase activity. It is assumed that initially it converts starch to simple sugar by amylolytic activity, and then it utilizes the simple sugar and converts to alcohol. The details of the mechanism can only be explored after proper research in the metabolic pathway. The fermented substrate supernatant contains bioactive molecules and offers high antioxidant and free radical scavenging ability although it didn't offer good metal chelating ability. The supernatant has potential antimicrobial properties against both gram positive and gram negative pathogens. The result of sensory evaluation and calorific value also attributed it to be a potential high nutritive low alcoholic drink as compared to rice wine and beer. Further tests should be employed to confirm its use for human consumption and other nutraceutical effect. It should have a great industrial importance as the process offers a low alcoholic drink with rich in antioxidant from steamed rice in single step without additional of enzymes from foreign sources. It can be summed, this process offers a cost effective, high nutritive, natural and easily digestible low alcoholic beverages.

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