SCREENING FOR BIOGENIC AMINE PRODUCTION BY *LACTOBACILUS* SPECIES AND DEVELOPMENT OF FUNCTIONAL FOOD, TEA CURD.

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INTRODUCTION

- Tea – most popular beverage in the world next to water
- Antioxidants – tea polyphenols – tea catechins
- Reduction of cholesterol, protection against cardio-vascular disease, and cancer
- Curd – Indian Yoghurt, home made, inoculum from previous curd
- Daily food
- Tea curd – functional food – tea polyphenols – biologically active compounds – health benefits

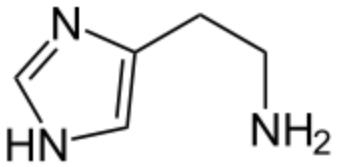
INTRODUCTION

- Biogenic amines - organic, basic nitrogenous compounds with one or more amine groups
- Removal of carboxyl group from amino acids by amino acid decarboxylase enzyme
- Alkaline in nature

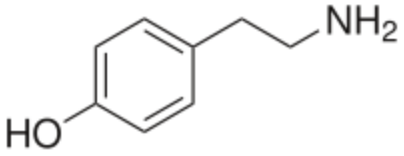
Types of Biogenic amines (BA)

based on amine content

- **Mono amines (MA)**
  - Histamine (HI)
  - Tyramine (TY)
  - Tryptamine (TR)
- **Poly amines (PA)**
  - Putrescine (PUT)
  - Cadavarine (CAD)



(HI)



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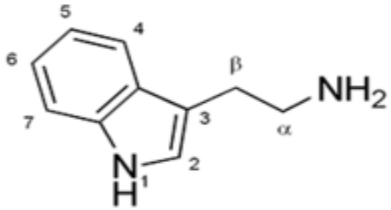


(TR)

(PUT)

(CAD)

INTRODUCTION

- Excessive levels – Hypotension, hypertension, nausea, respiratory distress, heart palpitation
- Histamine – vaso active effects
- Tyramine – rise in blood pressure, mutagen precurson
- Putrescine, cadaverine : can be converted into nitrosamines – potential carcinogens

OBJECTIVES

- To isolate probiotic bacteria from locally available curd (OMFED, Rourkela, Odisha, India and home made)
- To characterize the bacteria for biogenic amine production and tolerance to acidic pH
- To utilize the non biogenic amine producing bacteria for the development of green tea and black tea curd

METHODS

- **ISOLATION OF PROBIOTIC MICROBES**
- OMFED curd, Rourkela, Odisha, India and home made
- Handia – home made rice fermented with inoculum from previously fermented – obtained from local seller
- MRS agar, standardized procedure
- 37°C for 24 hours
- Morphological identification – simple staining
- Grown cultures – repeatedly subcultured for 5 times in MRS broth
- **SCREENIGN FOR BIOGENIC AMINE PRODUCTION**
- Decarboxylase media with different amino acids
- Wells – 0.5 cm in agar plate
- 250 µl cultures in MRS broth – inoculated
- **ACID TOLERANCE TEST**
- MRS broth cultures – centrifuged at 10,000 rpm for 5 minutes – pellet – resuspended in PBS buffer with pH 7.4, 5.5, 4.0, 3.0 and 2.0
- 24 hours incubation at 37°C
- Plated on MRS agar to check viability

Composition of MRS agar

Components	(grams / litre)
Proteose peptone	10.000
Beef extract	10.000
Yeast extract	5.000
Dextrose	20.000
Polysorbate 80	1.000
Ammonium citrate	2.000
Sodium acetate	5.000
Magnesium sulphate	0.100
Manganese sulphate	0.050
Dipotassium phosphate	2.000
Agar	15.000
pH	6.5±0.2

Composition of decaboxylase media

Components	(grams / litre)
Tryptone	5.0
Yeast extract	5.0
Meat extract	5.0
Sodium chloride	2.5
Glucose	0.5
Tween 80	1.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Ferrous sulphate	0.004
Ammonium citrate	2.0
Thiamine	0.001
Di-Potassium phosphate	2.0
Calcium carbonate	0.10
Pryridoxal-5-phosphate	0.05
Amino acid*	1.0
Bromocresol purple	0.06
Agar	20
pH	5.3

***Control media lacks amino acid. Amino acids ornithine, tyrosine, lysine and histidine added separately**

METHODS

- **PREPARATION OF TEA INFUSIONS**
- Green tea and Black tea – *Camellia sinensis* (L) O. *Kuntze* – Parry Agro Industries Ltd., Valparai, Tamil Nadu, India
- 2% (strength of normal cup of tea) – boiled water – 5 minutes – infusion
- Curd manufacture – tea infusions in milk
- HPLC analysis – tea infusions in water

PREPARATION OF TEA CURD (Jaziri, 2009)

Milk – OMFED (toned, 3.0% fat and standardized 8.5% SNF), Rourkela, Odisha, India

⇓

2% green tea and black tea, separately

⇓

5 minutes with stirring

⇓

Filtered through sterile cotton

⇓

Cooled to 45°C

⇓

***Lactobacillus* cultures (grown in MRS broth, 3 X 10^6) – 3.3 mL of each culture (10% v/v inoculation)**

⇓

Sterile, tightly capped tubes (30 mL)

⇓

Inoculation – 6 hours at 42°C

⇓

Stored at 4°C in a refrigerator

SAMPLING

- Periodic sampling
- Each tube – only once to avoid contamination
- After 6 hours – considered as 0 day
- Kept in refrigerator
- Sampling done at the end of 1, 7, 14, and 21 days

MICROBIOLOGICAL ANALYSIS

- Aseptic removal of sample
- serial dilution in 0.1 % peptone water
- Standard spread plate technique using MRS agar
- 37°C incubation for 48 hours under aerobic conditions.

DETERMINATION OF pH

- Electronic pH meter (Orion model 290A)

DETERMINATION OF TITRATABLE ACIDITY

- 10 g sample – titrated against 0.1 N NaOH
- % of lactic acid = ml of alkali× normality of NaOH×9/weight of sample (g)

HPLC ANALYSIS OF TEA POLYPHENOLS

Tea infusion prepared in water and inoculated with microbial cultures

HPLC ANALYSIS OF TEA POLYPHENOLS (Anon, 1999)

5 mL of sample (tea infusion in water) – extracted with 20 mL methanol

↓

Filtered through 0.45 µM membrane filter

↓

10 µl of filtrate

↓

Shimadzu (Kyoto, Japan) – HPLC system with PDA (SPD-M10Avp)

Phenomenex Luna C-18(2) column (4.6 mm ID X 25 cm, 5 uM)

Mobile phase: Mixture of 0.1% orthophosphoric acid (A) and acetonitrile (B)

Gradient elution: 0-12 min, 15% B ; 12-22 min, 25%, 22-30 min, 15% B)

Flow rate – 1.0 mL/min, 35°C

Detection: 280 nm

↓

Resolution peaks recorded – accodring to retention time of compound

↓

Standard curves – quantification of tea polyphenols

RESULTS AND DISCUSSION

ISOLATION OF PROBIOTIC BACTERIA

Table 1: Morphology of bacteria isolated from OMFED curd, home made curd and Handia

Number	Morphology	Bacteria / Yeast
1.OF1	Long rod	Bacteria
2.OF2	Oval shape	Yeast
3.OF3	Long rod	Bacteria
4.OF4	Long rod	Bacteria
5.HM1	Long rod	Bacteria
6.HM2	Long rod	Bacteria
7.HN1	Oval shape	Yeast
8.HN2	Oval shape	Yeast

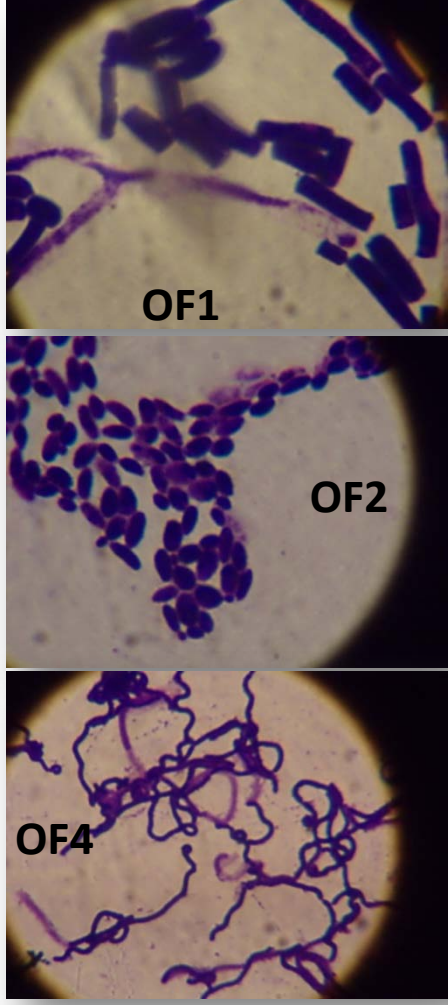
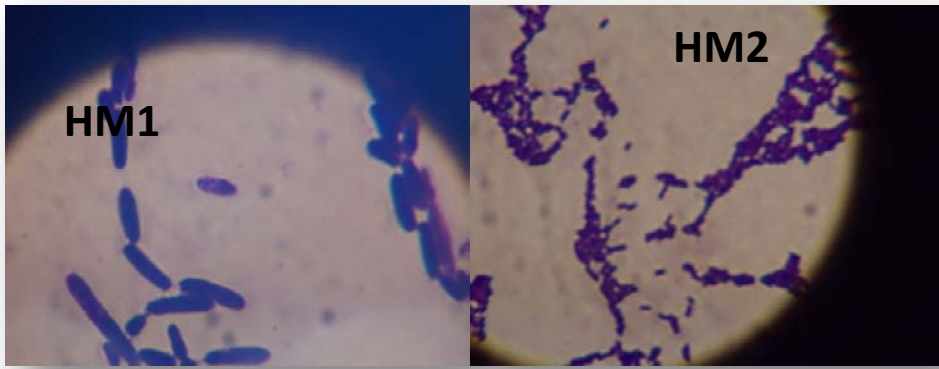
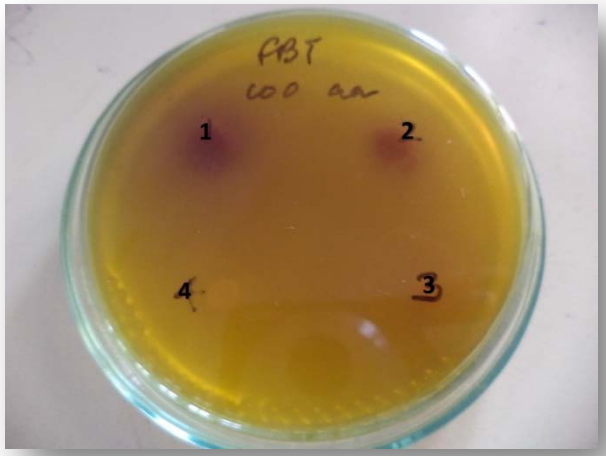


Figure 1: Morphology of bacteria isolated from OMFED curd and home made curd



SCREENING FOR BIOGENIC AMINE PRODUCTION

1.OF1
2.OF2
3.OF3
4.OF4
5.HM1
6.HM2
7.HN1
8.HN2



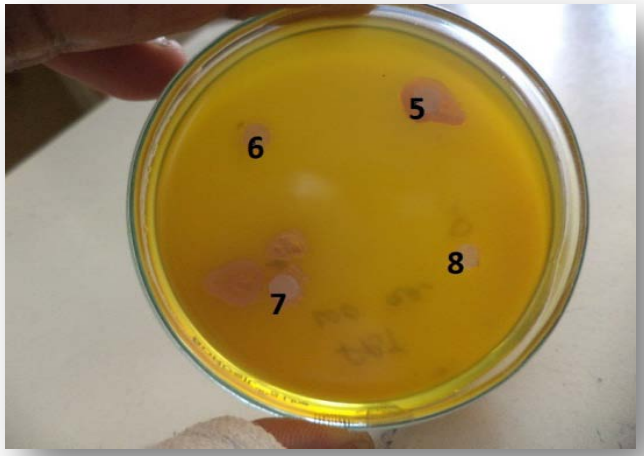


Figure 2: Screening for biogenic amine production in decarboxylase media control lacks amino acid

Control media : color production due to amino acids in proteins

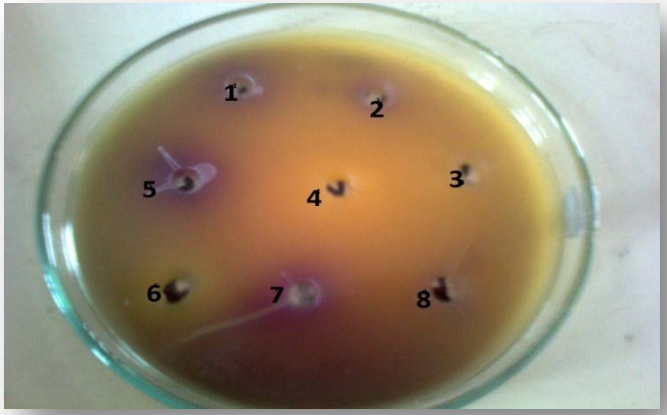
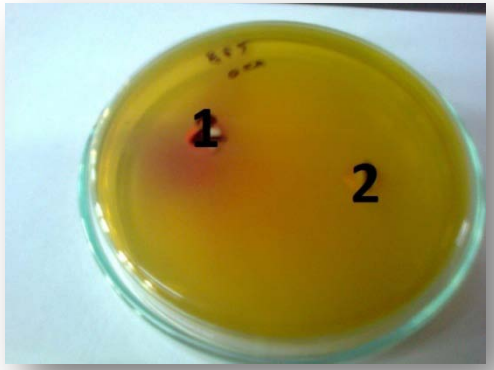


Figure 3: Screening for biogenic amine production in decarboxylase media with tyrosine

Tyramine production
Positive : OF1, OF2, HM1, HN1
Negative: OF3, OF4, HM2, HN2



1.OF1
2.OF2
3.OF3
4.OF4
5.HM1
6.HM2
7.HN1
8.HN2

Figure 4: Screening for biogenic amine production in decarboxylase media with ornithine

Putrescine production
Positive : OF1, HM1
Negative: OF2, OF3, OF4, HM2, HN1, HN2

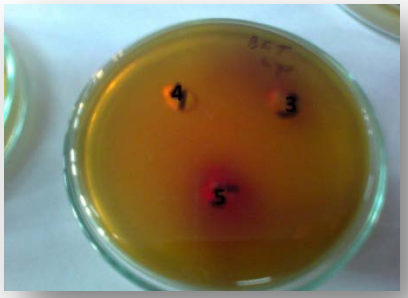
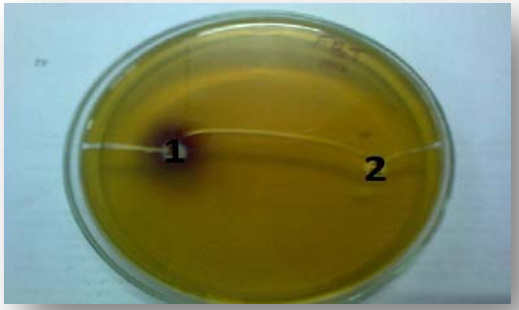
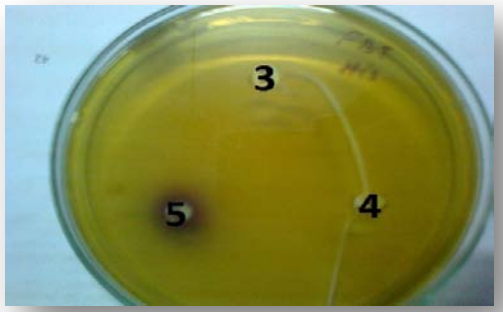


Figure 5: Screening for biogenic amine production in decarboxylase media with lysine

Cadaverine production
Positive : HM1
Negative: OF1, OF2, OF3, OF4, HM2, HN1, HN2





Histamine production

Positive : OF1, HM1

Negative: OF2, OF3, OF4, HM2, HN1, HN2

1.OF1
2.OF2
3.OF3
4.OF4
5.HM1
6.HM2
7.HN1
8.HN2

Figure 6: Screening for biogenic amine production in decarboxylase media with histidine

Table 2: Summary of screening for biogenic amine production

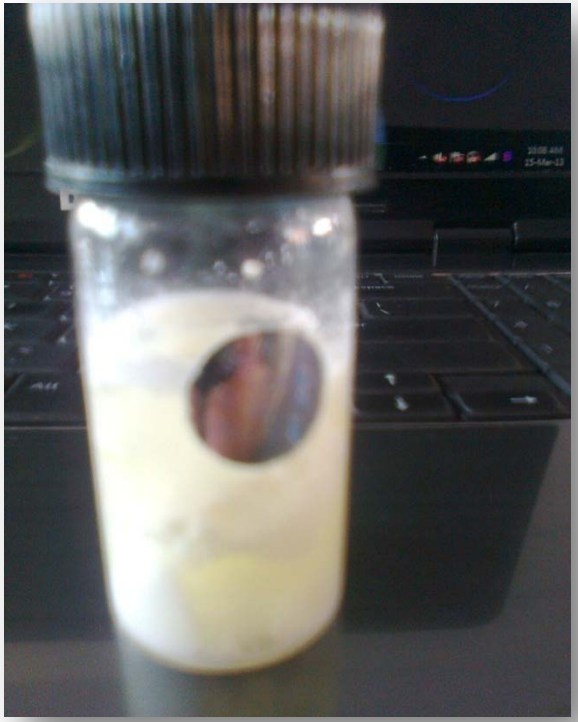
	Tyrosine (Tyramine)	Ornithine (Putrescine)	Lysine (Cadeverine)	Histidine (Histamine)	Selection
OF1	Yes	Yes	No	Yes	No
OF2	Yes	No	No	No	
OF3	**No**	**No**	**No**	**No**	**Yes**
OF4	**No**	**No**	**No**	**No**	**Yes**
HM1	Yes	Yes	Yes	Yes	**No**
HM2	**No**	**No**	**No**	**No**	**Yes**
HN1	Yes	No	No	No	No
HN2	No	No	No	No	

Acid tolerance test

Table 3: Effect of pH on growth of selected non-biogenic amine producing probiotic bacteria

Bacteria	pH 7.4	pH 5.5	pH 4	pH 3	pH 2
OF3	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**
OF4	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**
HM2	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**

Green tea curd



Black tea curd

Figure 7: Green tea curd and black tea curd

Table 4: Effect of time on pH, titratable acidity and number of bacteria of green tea curd

Refrigerated storage (day)	pH	Titratable acidity (gram lactic acid / liter)	Number of bacteria (CFU/mL)
0	4.10	1.26	2.27×10^6
1	4.04	1.35	1.84×10^6
7	4.00	1.71	1.36×10^3
14	3.80	1.17	1.11×10^3
21	4.10	1.62	contamination

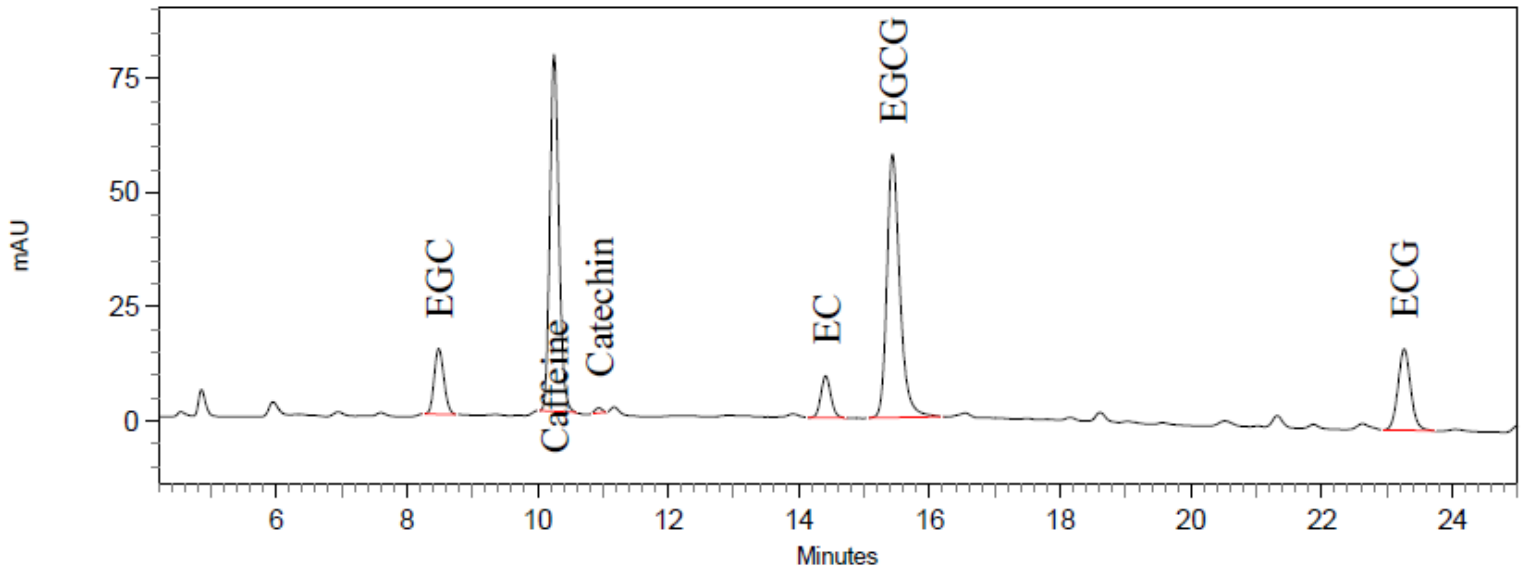
Reduction of bacterial number after 7 days. Study required after 1 day.

Table 5: Effect of time on pH, titratable acidity and number of bacteria of black tea curd

Time Refrigerated storage (day)	pH	Titratable acidity (gram lactic acid / liter)	Number of bacteria (CFU/mL)
0	4.10	1.35	2.30×10^6
1	4.00	1.44	2.00×10^6
7	3.60	1.80	1.61×10^3
14	3.50	1.26	contamination
21	4.05	1.53	contamination

Reduction of bacterial number after 7 days. Study required after 1 day.

Figure 8: Liquid Chromatogram of tea polyphenols



1: 274 nm, 8 nm

Name	Retention Time	Area
Gallica acod	3.904	120713
EGC	8.491	147220
Caffeine	10.251	749813
Catechin	10.944	7921
EC	14.411	96314
EGCG	15.435	794741
ECG	23.264	240513
Totals		2157235

Table 6: Effect of refrigerated storage on content of tea polyphenols in green tea curd

Refrigerated storage (day)	EGCG (mg /L)	EGC (mg /L)	ECG (mg /L)	EC (mg /L)	Catechin (mg /L)	Gallic acid (mg /L)	Caffeine (mg /L)
0	65.2	37.9	11.1	8.5	1.1	5.2	27.1
1	16.7	16.0	2.9	3.7	0.7	2.7	13.9
7	20.9	51.6	2.9	14.8	0.6	7.7	52.1
14	13.3	45.6	0.3	0.1	0.3	6.9	36.5
21	1.5	4.6	0.4	15.7	0.2	2.2	39.5

Varying stability.
Conversion / degradation of complex molecules into simpler molecules
Tea polyphenols are stable at acidic pH.
Hence, microbial enzymes may be the reason for observed varying stability

Table 7: Effect of refrigerated storage on content of tea polyphenols in black tea curd

Refrigerated storage (day)	EGCG (mg /L)	EGC (mg /L)	ECG (mg /L)	EC (mg /L)	Catechin (mg /L)	Gallic acid (mg /L)	Caffeine (mg /L)
0	5.3	1.3	1.7	0.1	0.9	2.9	39.2
1	1.5	1.4	0.4	ND	0.4	0.5	16.9
7	5.1	0.1	0.6	0.3	0.3	5.4	73.9
14	3.4	0.1	0.2	ND	ND	3.7	65.4
21	3.4	0.8	0.6	0.2	0.5	4.6	64.2

ND – Not Detected

Varying stability.
Conversion / degradation of complex molecules into simpler molecules
Tea polyphenols are stable at acidic pH.
Hence, microbial enzymes may be the reason for observed varying stability

CONCLUSION

Among 8 microorganisms isolated, 3 were selected to produce tea curd (non-biogenic amine producing bacteria)

Tolerant up to pH 3.0 and not at pH 2.0

Green tea curd and black tea curd were prepared

Refrigerated storage – reduction of bacterial number after 7 days.

Tea polyphenols – varying stability

Concentration of tea polyphenols after 1 day – can be

FUTURE WORKS

- **Sweet green tea and black tea curd**
- **Frozen green tea and black tea curd (without liquid)**

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Mr. Ajay Dethose,
Ms. Banishree Sahoo

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Thank You