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Extraction of dopamine, serotonin, and gamma-aminobutyric acid from lactobacillus.spp and yeast

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ABSTRACT

Treating the neurological disease is a meticulous process with lots of uncertainties. There is been an increasing demand and improvement of treatments are being under process. Most challenging neurological diseases like Alzheimer`s disease, Parkinson`s diseases are still being under studies for the challenging reasons of their occurrence. Also there is an increasing number of studies are going on with the Micro biome-gut-brain axis related findings to the neurological diseases. With such concept, it is significant to know the potentiality of the microbes which are commonly reside in our body to produce the metabolites i.e, Neurotransmitters related to the diseases. In this study focus is to find such microbes. Lactobacillus spp and the yeast commonly found in gut, shows the potentiality to produce the neurotransmitters with using simple laboratory conditions.


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INTRODUCTION:

“Microbial colonization of mammals is an evolution-driven process that modulates host physiology, many of which are associated with immunity and nutrient intake”—Heijtz et al. (2011). More than a billions of microbes are resides in gut and many of these includes, Bacteria, Yeast and Fungi, and their roles are yet to be find. Both the *Lactobacillus spp* and yeast having the probiotic effect which when administered. These organisms play a vital role in maintaining our micro biome and mycobiome make a balanced healthy environment. Since *Lactobacillus spp* have many health benefits conferring capacity, it’s ideal to use for the treatment too. *Lactobacillus spp* is a facultative anaerobic organisms found in most of the fermented foods. Ingestion of lactobacillus shows the improvement in the cognitive development in the Alzheimer’s patients with supplemented as probiotic [3]. Even the yeast contributes some health conferring effect in maintaining the gut homeostasis as commensally. Neurotransmitters like serotonin are produce in the enterochromaffin cells in the Gastrointestinal tract. And many microbes are also reported for the production of the neurotransmitters. some of the microbes are even helps in the immunological benefits too.

BRAIN-GUT AXIS:

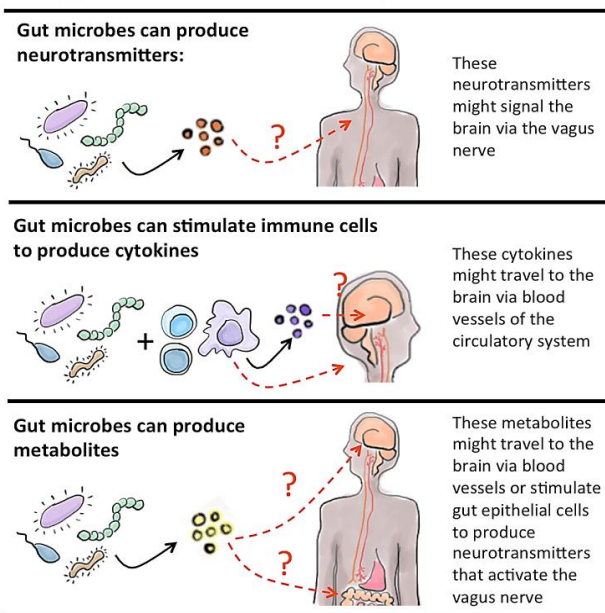
Brain-Gut Axis concept shows an interest in finding the solution for the Neurodisease related problems. Since the longest nerve which is the tenth cranial nerve, the vagus nerve is interconnecting almost all the important organs heart, lungs, and digestive tract to the brain.

Recently it is believed that because of the bidirectional communication between the gut and the brain influencing the neurological function of the brain. Many reviews have also shown that microbes resides in gut have a impact in the brain function by producing the metabolites [3,5, 8]. And even a few experiment were also been conducted to ensure the prediction based on the Brain-Gut axis through the vagal nerve (Holmqvist et al., 2014).

ROLE OF GUT MICROBIOTA IN NEUROCHEMICAL PRODUCTION AND METABOLISM:

The quorum signaling of bacteria helps in acting as a modulator role to maintain the physiological functions properly. Microbes have a direct impact on the neurologic effects and immune responses through the experiment conducted by *Campylobacter jejuni* (Lyte, M, Et. Al 1998). The wide hypothesis around the Gut brain axis is that microbes reside inside the gut may induce the

Hypotheses about the gut-brain axis



immune cells signals through the vagal nerve or by the production of the metabolites there by inducing the nerve signaling. It is proved that the Indigenous Bacteria from the Gut Microbiota Regulate Host Serotonin Biosynthesis (Jessica M et.al 2015). Commonly the lactic acid bacteria is helping in production of the Gamma Amino Butyric acid and considered as the cell factories of Its production (Haixing Li et.al 2010). And the interaction between the serotonin with the Candida(yeast) eliminates the virulence fungi conferring the health benefits (G. Hinterberger et.al 2005). Dopamine as a neurotransmitter also enhances the increase in efficacy of the anti cancer drugs and in the treatments (Chandrani Sarkar et.al 2008). And with the several applications these neurotransmitters study is being increased with various health benefits and through the microbes. (Behav Brain Res et.al. 2015).

MATERIALS AND METHODS:

MATERIALS:

All the materials used for the analysis are from the laboratory and from the Hi Media.

COLLECTION OF SAMPLES:

SAUERKRAUT was prepared by fermenting the Cabbage and uses that as a source for the collection of the *Lactobacillus spp.*, sample. And IDLY BATTER was also used for the yeast isolation. Even the cow's raw milk and Goat's gut were also collected and as a medicinal source symbiotic tablet were collected and brought to the laboratory for further analysis.

CULTURE OF BACTERIA AND YEAST

The collected samples were cultured, and inoculated in the MRS agar medium. The inoculated plates were incubated at anaerobic condition using the anaerobic chamber for 48hrs. After incubation the selected the bacterial colonies and yeast were purified and subcultured for further analysis.

CULTURE MEDIUM AND CONDITIONS:

Lactobacillus spp., MRS Media and Broth was autoclaved at 121°C for 45 mins and plated in petriplates and with 250ml conical flask having precursor amino acids under the anaerobic condition using the anaerobic chamber at room temperature.

S.NO	SAMPLES COLLECTIONS	SOURCES	METHODS ISOLATION	CULTURE MEDIUM
1	CURD	Food samples	Direct inoculation	MRS(De man, Rogosa and Sharpe) agar- incubated at 37°C for 18-24 hours
2	RAW COW MILK			
3	IDLY BATTER			
4	SAUERKRAUT			
5	GOAT GUT	Animal sample		
6	LACTOBACIL	Medical (capsule) sample	Processed to single fold dilution and 10 ⁻⁴ dilution was taken for inoculation.	

IDENTIFICATION OF ISOLATES:

The isolates of the cultured organisms were identified by Gram staining and Biochemical tests such as Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Catalase test,

Oxidase test, Gelatin hydrolysis test and Triple sugar iron agar test.

Yeast got from idly batter and *Lactobacillus spp.* from sauerkraut was used for the further analysis and preliminary screening of the neurotransmitters.

EXPERIMENTAL METHOD:

To know the presence of the neurotransmitters produced by the *Lactobacillus spp.*, and Yeast, as a preliminary test the centrifugation and thin layer chromatographic tests were performed and further continued with the UV spectrophotometric and High performance liquid chromatography.

CENTRIFUGATION:

The broth culture which was grown under 37°C for about a week provided with the amino acid precursors were taken for the centrifugation. Before centrifugation the bacterial cells were undergone cell lysis process with ultrasonication for about 20 mins with glass beads and then were undergone centrifugation for about 25 mins at 8000 rpm. The supernatant was collected with saline water and in case of serotonin analysis the further 10% ZnSo₄ was added followed by collected with 12N HCl for the analysis. The presence of extracted compounds such as Dopamine, Serotonin and GABA(Gamma Amino Butyric Acid) were then been checked for the preliminary test like Thin Layer Chromatography..

THIN LAYER CHROMATOGRAPHY:

Since the Dopamine, Serotonin and GABA are all biogenic amines, visualizing agents were applied for their visualization after the TLC fractionation. Various solvent system were used for the visualizing the compound of interest.

Sample extracts from Yeast and *Lactobacillus spp.* was spotted on the Silica coated TLC plate.

Analysis of Serotonin:

The chromatograms were developed with the mobile phase Acetone: Benzene: 28% Ammonia(20:10:1, v/v). The visualizing effect obtained were by sprayed with Sodium Hypochlorite in 0.1M NaOH. And was observed visually immediately after spraying, under UV irradiation.

Analysis of Dopamine:

The chromatograms were developed with the mobile phase Glacial acetic acid: Butanol: Water (1:4:1 v/v) were sprayed with Bromophenol blue in 5% NaOH which was prepared before use.

The visualizing spots was observed when the silica plate was wet with the reagent.

Analysis of Gamma Amino Butyric Acid:

The chromatograms were developed with the mobile phase Phenol: Chloroform: Isoamyl Alcohol (25:24:1 v/v). The plate was kept in a dark condition for 6-8 hrs, was then sprayed with 0.1% of Ninhydrin in Butanol before use. Colour development were allowed to take place for 24 hrs in a dark room temperature and the visualizing effect were obtained.

SPECTROPHOTOMETRIC METHOD:

Analysis of Serotonin:

Standard Serotonin Solution: A stock solution of Serotonin (1mg/ml) was prepared by dissolving 0.002g serotonin in eppendorf tube with 2ml of saline. The stock solution was used to prepare the working solutions at the different concentration in the range from 0.1 to 1 mg/ml in the test tubes with saline. To the working solutions Ehrlich reagent were added in order to obtain the colour change. Absorbance were measured at 625nm against the reagent blank. Calibration graphs were constructed by plotting absorbance against the final concentration of serotonin.

Analysis of Dopamine:

Standard Dopamine Solution: A stock solution of Dopamine (1mg/ml) was prepared from 10mg/ml dopamine solution by dissolving 1ml dopamine solution in 10 ml of saline. The stock solution was used to prepare the working solutions at the different concentration in the range from 0.1 to 1 mg/ml in the test tubes with saline. To the working solutions 0.5 ml of 0.03M ferric chloride followed by 0.5 ml of 0.0081M of Potassium hexacyanoferrate were added in order to obtain the colour of Prussian Blue complex. Absorbance was measured at 710nm against the reagent blank. Calibration graphs were constructed by plotting absorbance against the final concentration of serotonin.

Analysis of Gamma Amino Butyric Acid:

Glutamate decarboxylase assay: The cells were centrifuged and washed with the 0.5ml of saline

solution and resuspended in 0.5ml of GAD reagent solution containing 0.1g of L-Glutamic acid, 0.03ml of Triton X-100, 9 g of NaCl solution and 0.005g of bromocresol green in 100ml of double distilled water adjusted to pH 4. Development of green colour and blue colour shows the GAD activity. Absorbance was measured at 570nm against the reagent blank. Calibration graphs were constructed by plotting absorbance against the final concentration of GABA.

Analysis under different pH and Temperature:

After sterilization, the MRS Broth and nutrient medium was adjusted with 1 mol/L HCl and 1 mol/L NaOH to the demanded pH values 3, 5, 7, and 9. Then the corresponding *Lactobacillus spp.* and yeast strain was added and cultured at 37°C. After 1 week of the culture, the OD values of Serotonin, Dopamine and GABA produced by the organism were taken out and detected and the same followed for different temperature, - 4°C, 15°C and 45°C measured and tabulated.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC:

The samples were further analysed using HPLC with refractive index detector

RESULTS:

In this study both the organisms *Lactobacillus spp.* and yeast shows a results in finding the neurotransmitters production as by the all above mentioned analysis.

GRAM STAINING:

The cultured bacteria were done gram staining and it was observed as **GRAM POSITIVE** rod shaped bacteria in **SAUERKRAUT** source and **YEAST** were observed in the **IDLY BATTER** source under the microscopic view.

BIOCHEMICAL TEST:

For Biochemical characterization, citrate utilization test, voges proskauer test, Catalase test, Methyl red test, oxidase test, urease test, Gelatin hydrolysis test, Triple sugar iron agar test and Indole test were conducted and found negative.

S.NO	BIOCHEMICAL TESTS	RESULTS
1	VOGES PROSKAUER (VP) TEST	-
2	CITRATE UTILIZATION TEST	-
3	CATALASE TEST	-
4	METHYL RED (MR) TEST	-
5	OXIDASE TEST	-
6	UREASE TEST	-
7	GELATIN HYDROLYSIS TEST	-
8	TRIPLE SUGAR IRON AGAR TEST	-
9	INDOLE TEST	-

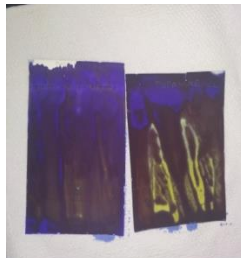
THIN LAYER CHROMATOGRAPHIC ANALYSIS OF SEROTONIN, DOPAMINE AND GABA:

In the analysis of the samples both the *Lactobacillus spp.* and yeast by visually obtained

color spots on the silica plate with the visualizing agents confirmed the presence of the neurotransmitters. The plates were stable with the obtained spots over a period of about nearly a month.



SEROTONIN-TLC



DOPAMINE-TLC



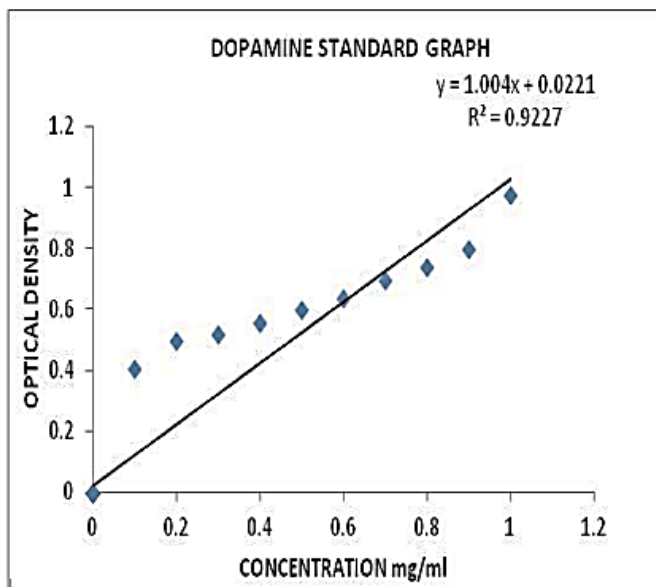
GABA-TLC

UV SPECTROPHOTOMETRIC ANALYSIS OF SEROTONIN, DOPAMINE AND GABA:

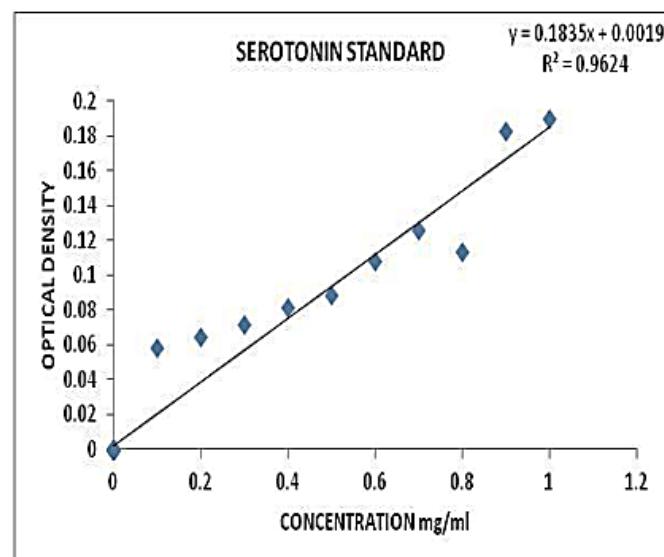
With the identification of the neurotransmitters been confirmed, the quantification were done using the absorbance value obtained through the spectrophotometry and the standard calibration curve were obtained with the linearity in the regression values as follows, Serotonin $r^2 = 0.9624$ from 0.5 Mg/ml to 0.7 Mg/ml and

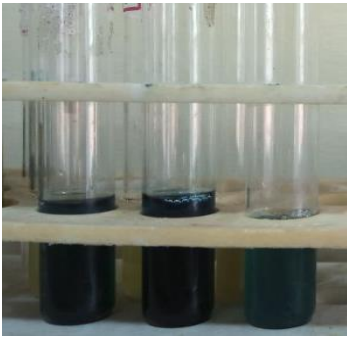
Dopamine $r^2 = 0.9227$ from 0.5 Mg/ml to 1 Mg/ml. And For the GABA with reference to Gokani et.al 1979 standard calibration curve by using the standard calibration curve the concentration of the produced neurotransmitters were calculated using the Microsoft excel software from Microsoft office suite and the value were tabulated.

DOPAMINE STANDARD	
Conc.Mg/ml	OD
0.1	0.41
0.2	0.50
0.3	0.52
0.4	0.56
0.5	0.60
0.6	0.64
0.7	0.70
0.8	0.74
0.9	0.80
1	0.98



SEROTONIN STANDARD	
Conc.Mg/ml	OD
0.1	0.059
0.2	0.065
0.3	0.072
0.4	0.082
0.5	0.089
0.6	0.108
0.7	0.126
0.8	0.114
0.9	0.183
1	0.19

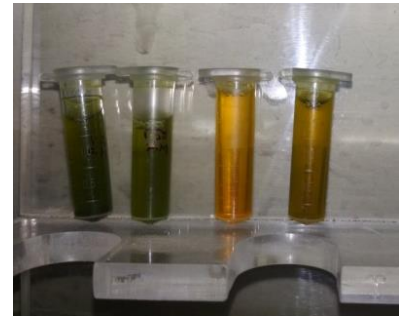




DOPAMINE-PRUSSIAN BLUE COMPLEX



SEROTONIN COLOUR CHANGE



YELLOW SHOWS LESS ACTIVITY
GREEN SHOWS LITTLE HIGH ACTIVITY

RESULTS

DOPAMAINE	
ORAGNISMS	Conc.mg/ml
LACTBACILLUS	0.38
YEAST	0.12

SEROTONIN	
ORAGNISMS	Conc.mg/ml
LACTBACILLUS	0.04
YEAST	0.03

GABA	
ORAGNISMS	Conc.µg/ml
LACTBACILLUS	0.00348
YEAST	0.00136

ANALYSIS UNDER DIFFERENT pH AND TEMPERATURE:

From the anlysis there is not much difference in the production of the Serotonin,Dopamine and

GABA between the yeast and *Lactobacillus spp*. The calculated results were tabulated with using the Microsoft excel software and with the standard graph.

Lactobacillus -Dopamine		
PH	OD	CONC.Mg/MI
3	0.74	0.77
5	0.68	0.74
7	0.46	0.63
9	0.60	0.70

Lactobacillus -Serotonin		
PH	OD	CONC.Mg/MI
3	0.02	0.1
5	0.02	0.1
7	0.02	0.1
9	0.03	0.15

Lactobacillus -GABA		
PH	OD	CONC.µg/MI
3	0.470	11.2
5	0.042	1.2
7	0.077	1.8
9	0.020	1.5

yeast -Dopamine		
PH	OD	CONC.Mg/MI
3	0.46	0.63
5	0.86	0.82
7	0.54	0.67
9	0.96	0.87

yeast-Serotonin		
PH	OD	CONC.Mg/MI
3	0.004	0.03
5	0.003	0.03
7	0.003	0.03
9	0.006	0.03

yeast-GABA		
PH	OD	CONC.µg/MI
3	0.430	10.8
5	0.082	2.1
7	0.027	0.6
9	0.053	1.35

Temperature °C -Dopamine				
ORGANISMS	4	15	45	
LACTBACILLUS				
spp conc.mg/ml	0.77	0.11	0.453	
YEAST	0.72	0.96	0.522	
LACTBACILLUS				
spp conc.mg/ml	0.78	0.47	0.63	
Yeast				
conc.mg/ml	0.76	0.87	0.66	

Temperature °C -Serotonin				
ORGANISMS	4	15	45	
LACTBACILLUS				
spp conc.mg/ml	0.055	0.054	0.407	
YEAST	0.023	0.029	0.267	
LACTBACILLUS				
spp conc.mg/ml	0.04	0.04	0.09	
Yeast				
conc.mg/ml	0.03	0.03	0.07	

Temperature °C-GABA				
ORGANISMS	4	15	45	
LACTBACILLUS				
spp conc.mg/ml	0.514	0.520	0.460	
YEAST	0.540	0.610	0.600	
LACTBACILLUS				
spp conc.µg/ml	0.021	0.021	0.018	
Yeast				
conc.µg/ml	0.022	0.024	0.024	

HPLC analysis of serotonin, dopamine and GABA:

The results confirm the presence of the serotonin, dopamine and GABA by means of the high performance liquid chromatography with the purity of >90% except the Dopamine and GABA of *Lactobacillus spp.* with the recovery of >70% with the obtained peaks.

DISCUSSION:

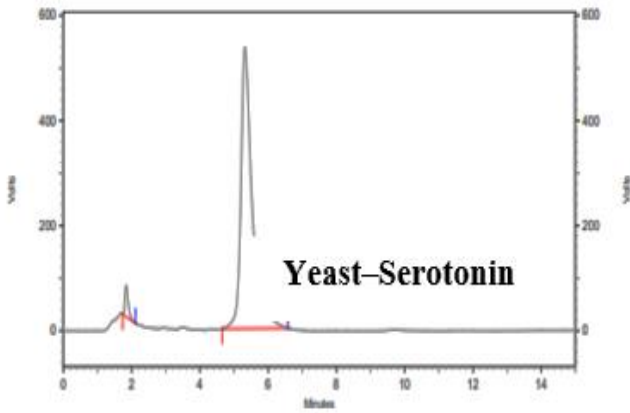
Overall with the study of identification of the potential organisms which is *Lactobacillus spp.* and yeast shows the presence of the production of the serotonin, dopamine and GABA. By Gram staining Got short Rod Gram positive organisms were observed in microscopic as like Short Rod, Single and In pair of arrangements as like *lactobacillus spp.* (Tasneem chowdhury et al., 2016) and Biochemical test confirmed with observed negative results for

the isolates which confirming the *lactobacillus spp.* identification (Kamrun Nahar, et al. 2016 and Soumya Khare Et al., 2016).

TLC plates treated with sodium hypochlorite, potassium hexacyanoferrate as an oxidizing reagent. The reaction product emitted strong, vivid blue fluorescence at ultraviolet irradiation indicates the presence of the serotonin [8]. The tryptophan and metabolites are originally fluorescent under UV at 285 nm they cannot be observed by the naked eye because of their emission in the UV range. Bromophenol were used as an acid-base indicator were used as a visualizing agent of dopamine under the solvent system glacial acetic acid – n-butanol – water (1+4+1 w/v) and under the blue background the dopamine product was visible as yellow spot [15]. As an acid-base indicator, its useful range lies between yellow at pH 3.0- to

Area % Report

Sample ID: Serotonin- Yeast
 Data File: E:\CHROMQUEST\DATA\project\Yaazh Xenomics\16.03.2018\ Serotonin- Yeast.dat
 Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Project.met

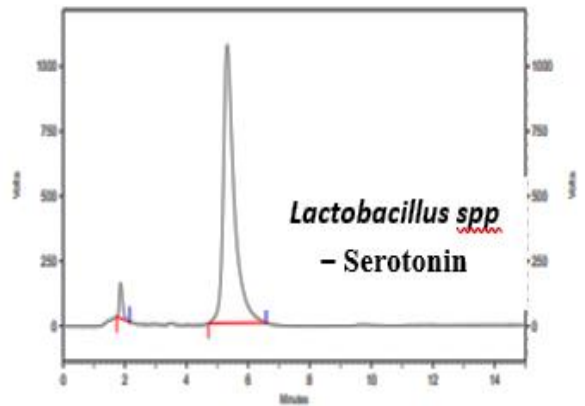


Name	Retention Time	Area	Area %
	1.842	405856	3.16
	5.323	12430319	96.84

Totals		12836175	100.00
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Area % Report

Sample ID: Serotonin- LB
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 Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Project.met

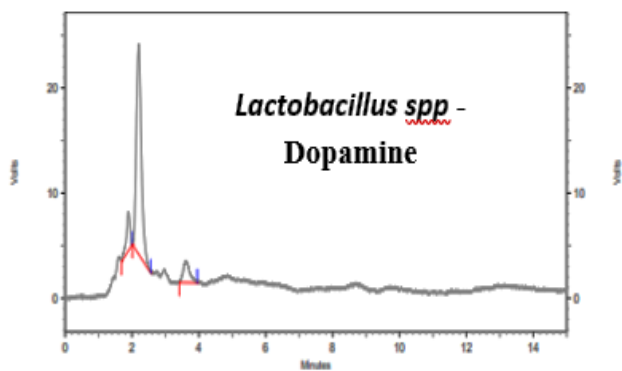


Name	Retention Time	Area	Area %
	1.855	990145	3.61
	5.323	26420171	96.39

Totals		27410316	100.00
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Area % Report

Sample ID: Dopamine- LB
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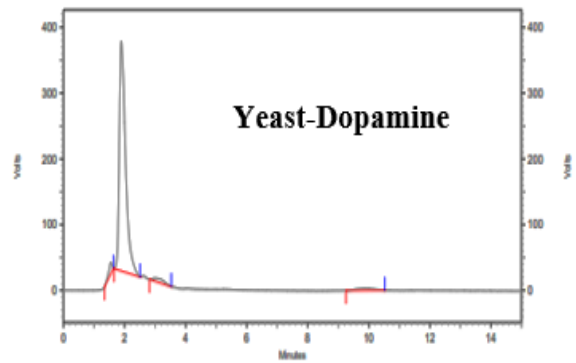


Name	Retention Time	Area	Area %
	1.888	28353	10.72
	2.200	208339	78.81
	3.593	27672	10.47

Totals		264364	100.00
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Area % Report

Sample ID: Dopamine- Yeast
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 Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Project.met

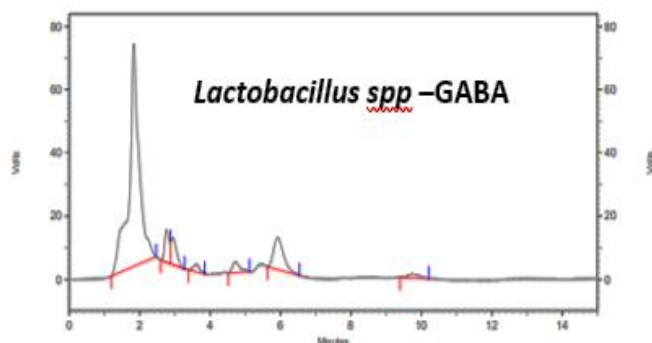


Name	Retention Time	Area	Area %
	1.553	136434	2.54
	1.890	4970603	92.55
	3.015	141786	2.64
	9.893	121988	2.27

Totals		5370811	100.00
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Area % Report

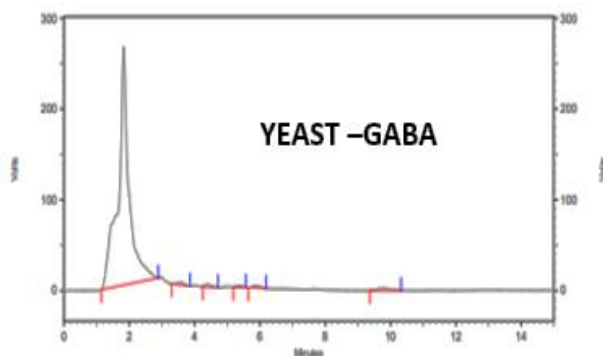
Sample ID: Gaba- LB
 Data File: E:\CHROMQUEST\DATA\project\Yazh Xenomics\16.03.2018\Gaba- LB.dat
 Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Project.met



Name	Retention Time	Area	Area %
	1.825	1393970	73.42
	2.767	92244	4.86
	2.927	98259	5.18
	3.613	30954	1.63
	4.712	51353	2.70
	5.918	199818	10.52
	9.752	32033	1.69
Totals		1898631	100.00

Area % Report

Sample ID: Gaba- Yeast
 Data File: E:\CHROMQUEST\DATA\project\Yazh Xenomics\16.03.2018\Gaba- Yeast.dat
 Method: C:\ChromQuest\Enterprise\Projects\Default\Method\Project.met



Name	Retention Time	Area	Area %
	1.822	6043988	96.73
	3.582	41075	0.66
	4.387	34332	0.55
	5.343	23023	0.37
	5.872	38682	0.62
	9.720	67039	1.07
Totals		6248139	100.00

blue at pH 4.6. TLC plate of GABA was sprayed with the 1% ninhydrin reagent in butanol, which kept in 24 hrs at dark room temperature which developed the colour completely differ from other amino acids which will develop the colour only at higher temperature confirmed the presence[14]. The UV spectrophotometric method based upon the color reaction between serotonin derivatives and *p*-dimethylaminobenzaldehyde (Ehrlich's Reagent) which follows the electrophilic substitution reaction mechanism at the indole ring[10]. on a reaction between dopamine hydrochloride with the mixture of Fe³⁺ and hexacyanoferrate ions to form an intense Prussian blue color complex that has the absorption at 710nm shows the presence of dopamine[1]. By knowing the presence of the Gamma Amino Decarboxylase enzyme activity which plays a significant role in converting the Glutamic acid to GABA ensures the presence of GABA[7].

Analysis under different pH and Temperature shows the optimum condition of the production activity of the microbes which serotonin has no interference over the pH and temperature. GABA shows the highest concentration range in acidic condition and at different temperature conditions its shows the constant concentration range. In case of dopamine the *Lactobacillus spp* shows the optimum range in acidic condition, yeast shows in alkaline condition.

Herein HPLC analysis with obtained peak area %, the purity and presence of the serotonin, dopamine and GABA were confirmed.

CONCLUSION:

In this study with availability of micrograms level of precursor amino acids the organisms shows the results in production of the neurotransmitters, this shows the role of amino acids in influencing the microbial response towards the availability of the Non-essential amino acid Glutamic acid which is been a precursor for the GABA, Tyrosine for the

Dopamine and Essential amino acid tryptophan for the Serotonin production. Also the produced levels of neurotransmitters results are enough for the active transport of nerve impulse to respond the brain. Since the production under different pH and Temperature shows the optimum conditions of the microbial production of neurotransmitters and its responses. These may help in understanding the response of the microbes resides in the host influencing the physiology condition towards the neurological signaling. To the best of our knowledge the quantification of the neurotransmitters produced by the microbes and also the analysis through the HPLC with the Refractive index detector is performed first in this study. However many *Lactobacillus spp.*, was been reported for the production of the neurotransmitters, the yeast response towards the amino acids availability and produced the neurotransmitters may change the focus of study. Hypothetically the organisms which were isolated from the source are mainly from the food based so the regular intake of such foods may confer some health benefits towards the neurological disease.

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