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STUDIES ON PRODUCTION, PURIFICATION AND CHARACTERIZATION OF SEROTONIN

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ABSTRACT

The role of serotonin in the gastrointestinal tract, monoamine serotonin (5-hydroxytryptamine [5-HT}) acts as an essential neurotransmitter and hormone with various biological functions, including regulation of intestinal secretion and motility, leading to well-being and pleasure, mixing of vascular constriction (direct via sympathetic innervation) and dilation (endothelium dependent), Platelet proliferation, stimulation of peripheral nociceptive nerve ends stimulation / inhibition of CNS neurons, Postulated physiological and pathophysiological functions. Pectinase was isolated from bacterial origins and the bacterium was bacillus sp. Production media (composed of (g/l) – Yeast Extract 10gm, NaCl 3gm, KNo3 2gm, KH2Po4 1gm, Dextrose 8 gm, tryptophan 8gm, MgSo4 0.1gm) has been used for enzyme production. Serotonin was detected after 7 days of fermentation; the highest finding was pH 7.5 and enzyme activity.

Key words: Serotonin, Neurotransmitter, Sympathetic innervation, Pathophysiological, Dilation

INTRODUCTION

Enterochromaffin (EC) cells in the intestine contain a large amount of 5-HT (90%) in the human body. In EC cells, 5-HT is synthesized by tryptophan hydroxylase (Tph / TPH) 1, which is a rate-limiting enzyme, and deposited in secreted granules prior to release. The second isoform, TPH2, is also formed in the myenteric plexus; again, this constitutes a much smaller fraction of the overall 5-HT. Tryptophan is an essential amino acid that must be provides in the diet. It is usually a part of protein, but in children, for example, breast milk often includes a more readily available non-protein portion that may be essential for postnatal development.

When drained from the intestine and made usable for circulation, where free and albumin-bound fractions occur, the blood-brain-barrier (BBB) can be crossed by a large amino acid transporter to engage in serotonin synthesis in the CNS. However, the vast majority of serotonin is found in the intestine where it is synthesized from tryptophan in Enterochromaffin (EC) cells of the gastrointestinal tract and is also present in enteric nerves. Tryptophan is first converted to 5-hydroxytryptophan (5-HTP) by a rate-limiting enzyme, tryptophan hydroxylase (TPH), which is not saturated with normal tryptophan concentrations.

As a result, increased concentrations of tryptophan can at least potentially, result in increased metabolic efficiency. Onward metabolism of the short-lived 5-HTP intermediate product to 5-HT is by aromatic amino acid decarboxylase (AAAD).

Nonetheless, the dominant physiopath for tryptophan is currently in the kynurenine is derived from tryptophan by the action of the mostly hepatic enzyme tryptophan by the mostly hepatic enzyme tryptophan-2,3-dioxygenase (TDO) or ubiquitous indoleamine-2,3-dioxydenase (IDO).

Moreover, kynurenic acid, which can be neuro-protective against quinolinic acid induced excitotoxicity, can also induce cognitive impairment when abnormally elevated.

One outcome of the recent intense focus on this 'virtual organ' using metagenomic approaches is the realization that microorganisms in our gastro-intestinal tract out numbers the human cells in our bodies by a factor of 10 and contains 150 times as many genes as our genome (see below for developmental features and health implications)[**4].**

The complex role of the gut microbiota within the brain-gut axis is just beginning to be charted, in contrast to a well-developed understanding of the reciprocal communication between the ENS and the CNS. A number of strategies are available to researchers in this area to help mark out the impact of the gut microbiota on brain and behavior, including the use of microbiota deficient germ-free animals, probiotic supplementation, antibiotic administration, faecal transplantation studies and deliberate infections [5].The gut microbiota can also directly utilize tryptophan, thereby potentially limiting its availability to the host. In addition to the growth requirements for bacteria [6], certain bacterial strains harbor a tryptophanase enzyme that produces indole from tryptophan and serotonin (5-HT) is intermediate product of this pathway. The direct physiological significance of indole 3-acetic acid (IAA) production from tryptophan for the host is not well understood but it is relevant to bacterial physiology and plant-microbe interactions where its effects can be both beneficial and detrimental [7] Unlike eukaryotes, bacteria can also synthesize tryptophan via enzymes such as tryptophan synthase. Intriguingly, specific bacterial strains can also produce serotonin (5-HT) from tryptophan, at least *in vitro* [8, 9].The balance between bacterial tryptophan utilization and metabolism, tryptophan synthesis, serotonin production and indeed the bacterial response to exogenous elevations in serotonin likely plays an important role in determining local gastrointestinal and circulating tryptophan availability for the host in addition to the dietary supply of this essential amino acid.

MATERIALS AND METHODS

Isolation and enumeration of bacterial colonies:

After the collection of rhizospheric soil samples. All samples were serially diluted up to 10^{-6} dilution. 1% of sample from each dilution was transferred to freshly prepared agar plates and incubated at 37° C, for 24 hours. After incubation morphologically distinct colonies were selected and stored at 4°C for further studies.

Inoculum and media preparation: Inoculum was prepared by taking single colony from the stored nutrient agar plates and inoculated into the nutrient broth. In order to produce the serotonin through IAA producing microbe's production medium (YMD medium) was prepared which contained Yeast 10 g/l, Mannitol 8 g/l, NaCl 2 g/l, $MgSO_4$ 0.1 g/l, KH_2PO_4 3 g/l along with pumpkin extract used as tryptophan source.

Optimization of process parameters: The study was carried out in 250 ml Erlenmeyer flasks, pH value of the medium was adjusted at 7.0 with the help of NaOH, autoclaved at 121 ^{0}C for 20 mints, 1 % (v/v) inoculum of bacterial broth inoculated in to the medium and kept it at 30 ^{0}C and 120 rpm in an orbital along with a flask as a control, containing same composition of media and condition but without inoculum. Different set of experiments were studied and medium was optimized through one factor at a time approach on the basis of enzyme activity with different incubation periods, different Carbon sources and their different concentrations, nitrogen sources and their different concentrations and different pH ranges.

Enzyme assay with Salkowski reagent: To determine whether serotonin is producing or not, a colorimetric technique was performed with Van Urk Salkowski reagent using the Salkowski's method (Ehmann, 1977). The isolates were grown in yeast malt dextrose broth (YMD broth) (Himedia, India) and incubated at 28 °C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) and kept in the dark. The optical density (OD) was recorded at 540 and 380 nm after 120 min and 24 hrs.

Calorimetric assay with pumpkin extract:

Another colorimetric technique was performed to determine production of serotonin. YMD broth was centrifuged at 1000 rpm for 10 mints, 2 ml of supernatant was taken and mixed with 2 ml of pumpkin extract and kept at room temperature for 45 mints. The optical density (OD) was recorded at 540 and 380 nm.

Downstream processing to extract the enzyme: Enzyme monooxyginase which is responsible for the production of serotonin was extracted and purified from the YMD broth**.**

Ammonium sulfate precipitation: Ammonium sulfate precipitation is one of the most commonly used methods for protein purification from a solution. In solution, proteins form hydrogen bonds with water molecules through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulfate are added, these groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation. Supernatant of YMD broth was transferred in to a beaker containing a stir bar and placed on magnetic stirrer. While sample was stirring, slowly added saturated ammonium sulfate to bring final concentration up to the 50% saturation. Volume of ammonium sulfate needed is equal to volume of sample. Adding the ammonium sulfate very slowly ensures that local concentration around the site of addition does not exceed the desired salt concentration. Once total volume of ammonium sulfate is added, beaker was moved to 4°C for 6 hours or overnight. Sample was taken and centrifuged the precipitate at 3000g for 30 minutes.

Dialysis: Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semi permeable membrane. This method is used to remove the excess salt concentration from the samples. After ammonium sulfate precipitation Sample was loaded in to dialysis bags, and then this dialysis bag containing samples was placed in another chamber of dialysis buffer (100 mM tris buffer solution) with gentle stirring of buffer. It was allowed to dialyze for 2 hrs. Buffer was changed and again allowed to dialyze for overnight.

RESULTS AND DISCUSSION

Isolation and Identification of Rhizospheric isolates:

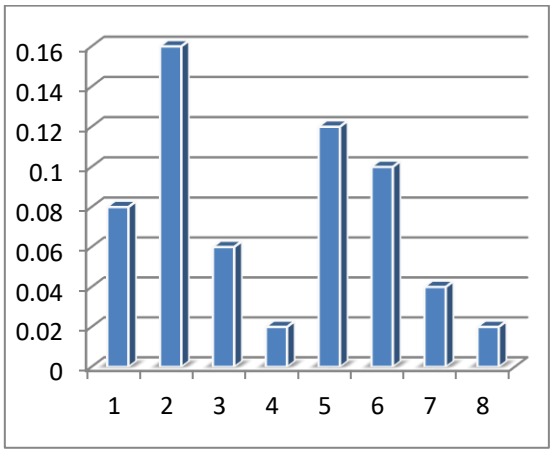
Microbes which were used for the fro production of serotonin were isolated from soil samples and serially diluted and streaked on petri plates to obtained the colonies. 10 bacterial isolates were successfully isolated as IAA producer from rhizosphere soil among which 3 were selected based on IAA production ability.

The isolates were identified based on morphological observation and biochemical characterization.

Fig-1 Rhizosperic Bacterial isolates

Production of serotonin

In this study to produce the serotonin we tried to produce Indole acetic acid because if IAA will produce, serotonin will also produce as an intermediate product compound. So here we used the medium which can utilized by microorganism to produce the IAA. To optimize the production of Serotonin four factors were considered to achieve high production and it was regulated in experimental series, changing one parameter at a time while keeping other variables constant at a particular set of conditions. For this YMD medium was used and further it was optimized in terms of different parameters. Along with YMD medium source of tryptophan (pumpkin extract) was also used because it was found that Tryptophan is a main precursor molecule for biosynthesis of IAA in bacteria Starovic et al (12). Media was optimized on the basis of enzyme activity by changing the different source and concentration of carbons, different source and concentration of nitrogen, metal ions, incubation periods and different PH ranges. In the first set of experiment microbes were grown into nutrient broth medium then different strains were inoculated in to the MAM medium to produce the serotonin. Then calorimetric assay were performed for the strain selection, on the basis of enzyme activity strain S2 was choose to continue the rest of the experiments.



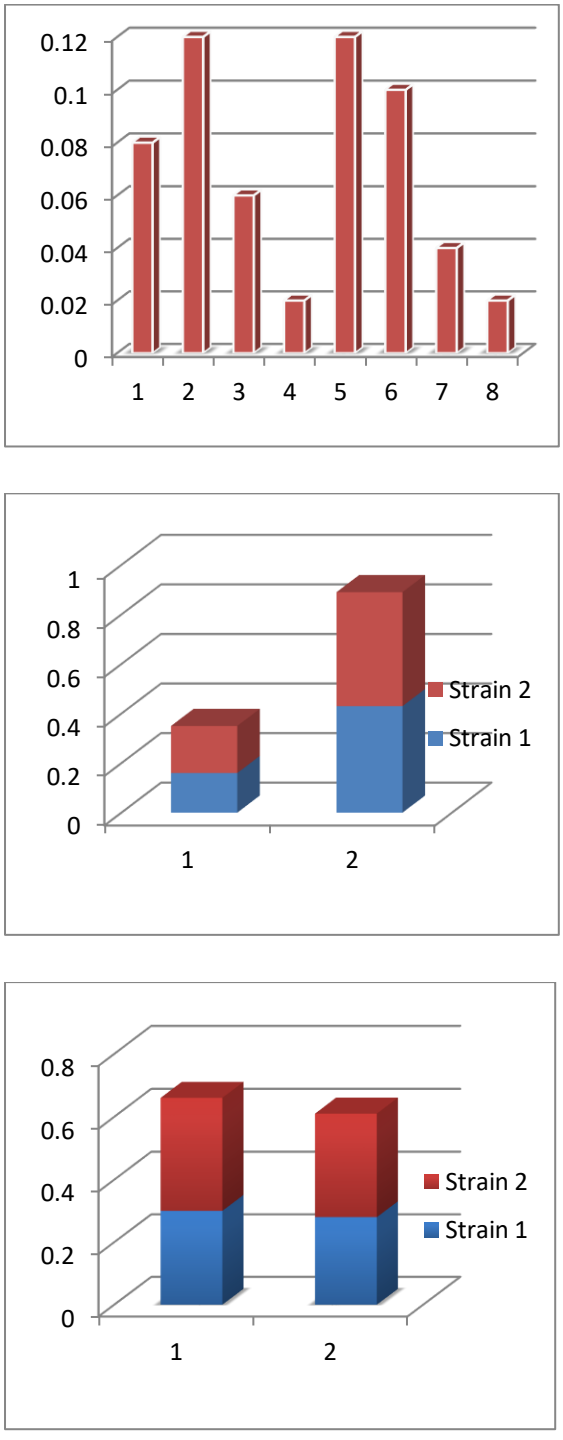
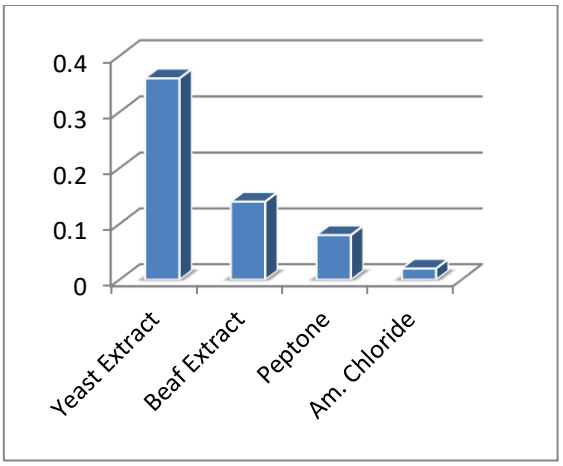


Fig-2 Microbes selection On the basis of absorption (optical density at 380 and 540 nm)

Media optimization with Nitrogen sources:

To enhance the production of serotonin media was optimized by taking the different sources of Nitrogen along with YMD media like yeast extract, beef extract, peptone, ammonium chloride. Colorymetry enzyme assay and enzyme activity with salkowaski reagent were performed and found that yeast extract was more effective among the different nitrogen sources and peptone showed moderate activity.



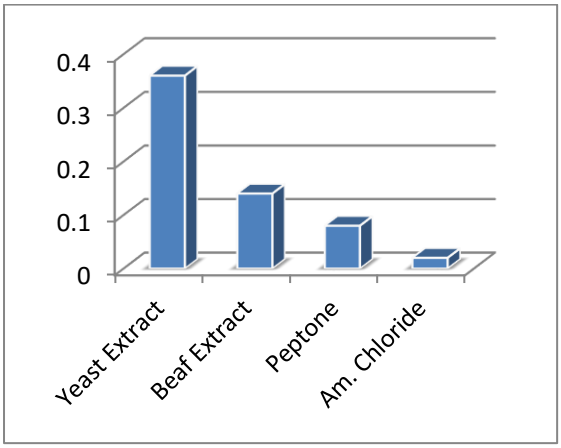


Fig-3 Effect of nitrogen sources on the basis of absorption (optical density at 380 and 540 nm)

Media optimization with different combination and concentration of N-Sources:

After choosing the best nitrogen sources medium was supplemented with different combination and different concentrations of nitrogen sources like Yeast extract + Peptone (5g/l : 5 g/l), Yeast extract + beef extract (5g/l : 5 g/l), yeast extract + peptone (7g/l : 3 g/l and Yeast extract+ beef extract (7g/l :3g/l). and after the enzyme assay observation it was found that combination of Yeast extract and peptone (7:3) showed best activity.

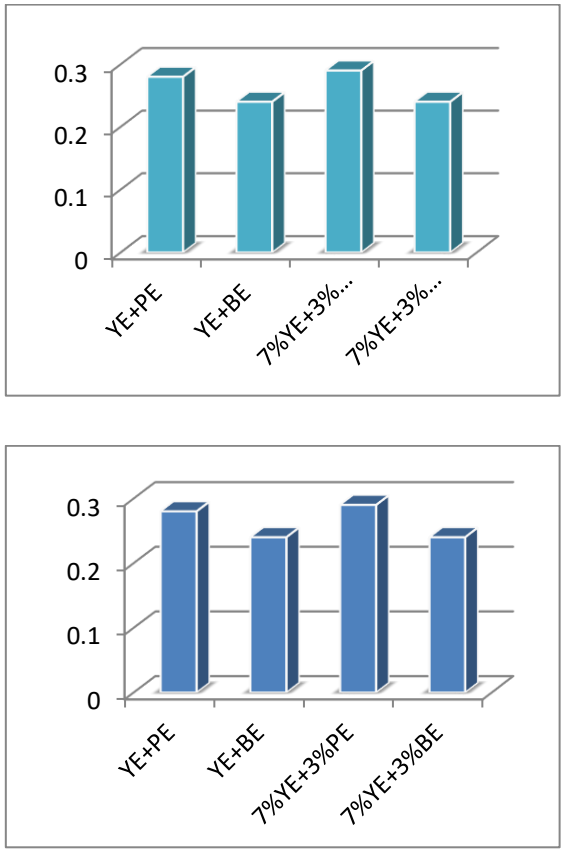


Fig-4 Effect of nitrogen sources on the basis of absorption (optical density at 380 and 540 nm)

Optimization with C-sources:

Carbon sources as a component of production medium play an important role in the growth of microorganisms and in the production of bioactive compounds. Different carbon sources like Dextrose, sucrose, lactose and maltose were used to enhance the production of serotonin.

After enzyme assay dextrose was found as a best carbon supplement. Maltose and sucrose showed moderate effect.

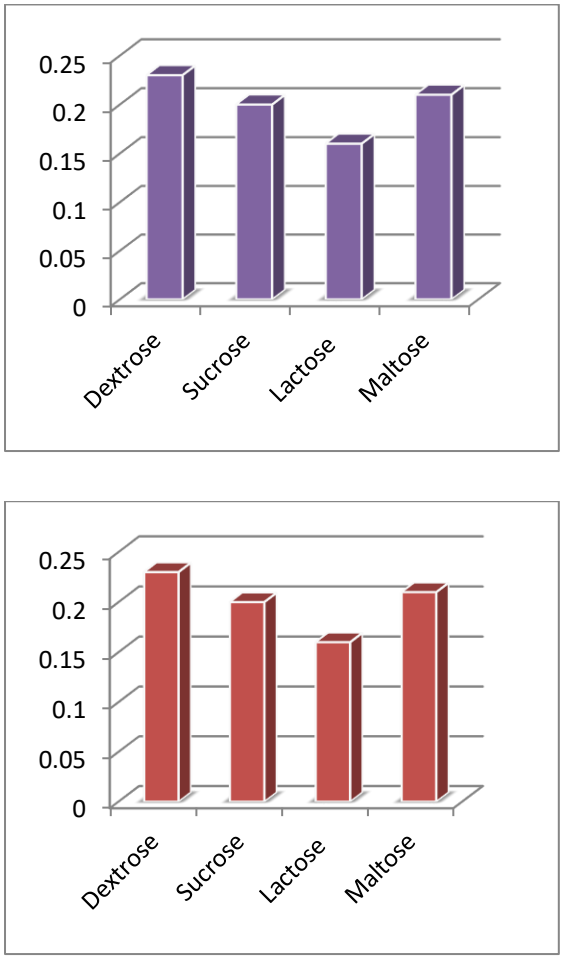


Fig-5 Effect of Carbon sources on the production of serotonin (optical density at 380 and 540 nm)

Optimization of C-sources in terms of concentration:

To optimize the medium an another set of experiment were performed by taking YMD media along with yeast extract + peptone (7:3) as a nitrogen source, pumpkin extract as a tryptophan source and different concentration of carbon sources (instead of manitol of YMD media) like 0.5 % dextrose, 1 % sucrose, 1.5 % lactose and 2 % maltose**.**

Table-1 Effect of different concentrations of carbon sources

Enzyme activity	OD at first day 380nm 540 nm			OD at 3rd day of incubation 380 nm 540 nm	
Salkowasayki Reagent assay	0.5% dextrose	0.20	0.15	0.42	0.30
	1%sucrose	0.22	0.16	0.32	0.26
	1.5 %lactose	0.16	0.13	0.29	0.20
	2 %maltose	0.19	0.13	0.23	0.18
Colorimetric assay with pumpkin extract	0.5 %dextrose	0.061	0.16	0.36	0.26
	1% sucrose	0.30	0.01	0.56	0.35
	1.5 % lactose	0.00	0.04	0.41	0.28
	2% maltose	.40	0.02	0.42	0.28

Optimization of C-sources in terms of different combinations and different concentration:

Media was also supplemented with different combinations and different concentration of different carbon sources like 0.5 % of dextrose + 0.5 % of lactose, 0.75 % dextrose + 0.25 % lactose, 0.25 % dextrose + 0.75 % lactose and 1 5 dextrose + 1 % lactose. After enzymatic assay it was observed that 0.75 of dextrose with 0.25 % of lactose were best for the production medium.

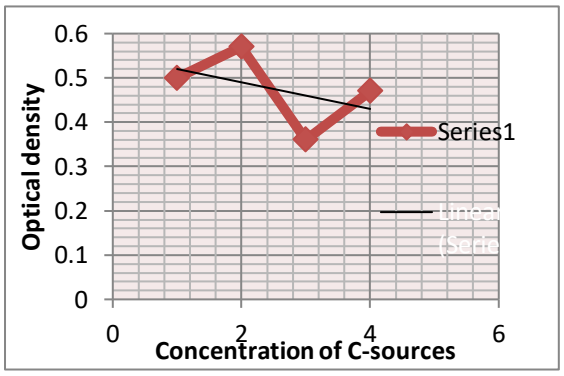


Fig-6 Effect of concentration of C-sources .

Optimization with Metal ions:

Most enzymes involved in production of bioactive compounds require high concentration of metal ions for both activity and stability, for example malic enzyme involved in metabolic reaction show highest activity in presence of magnesium, cobalt and manganese. Supplementation of metal ions ($FeSO_4$, $CuSO_4$, $CaCO_3$, $PbNO_3$) in cultivation medium (YMD medium) improved the bacterial growth as compare to that in YMD medium. $FeSO_4$ and $CaCO_3$ showed almost similar growth as compared with YMD medium. Among all the metal compounds $FeSO_4$ (0.1 g/L) gave maximum bacterial growth (3 times more than in YMD medium)

Table-2 Effect of different metal ions on medium

Enzyme activity		OD at first day		OD at 3rd day of incubation	
		380nm	540 nm	380nm	540 nm
Salkowasayki Reagent assay	$FeSO_4$	0.16	0.09	0.40	0.30
	$CuSO_4$	0.20	0.12	0.16	0.08
	$CaCO_3$	0.24	0.16	0.36	0.27
	$PbNO_3$	0.23	0.15	0.22	0.14
Colorimetric assay with pumpkin extract	$FeSO_4$	0.30	0.23	0.36	0.26
	$CuSO_4$	0.31	0.21	0.36	0.22
	$CaCO_3$	0.39	0.27	0.15	0.08
	$PbNO_3$	.030	0.25	0.20	0.12

Optimization with Metal ions in different concentrations:

In another set of experiment medium was supplemented with different concentration of metal ions like $FeSO_4$ + $CaCO_3$ (0.05 gm: 0.05 gm), $FeSO_4$ + $CaCO_3$ (0.005 gm +0.045 gm), $FeSO_4$ + $PbNO_3$ (0.05 gm: 0.05 gm), $FeSO_4$+ $CaCO_3$ (0.045: 0.005).

It was observed that combination and concentration of $FeSO_4$+ $CaCO_3$ (0.045: 0.005) was best as a metal ion supplement.

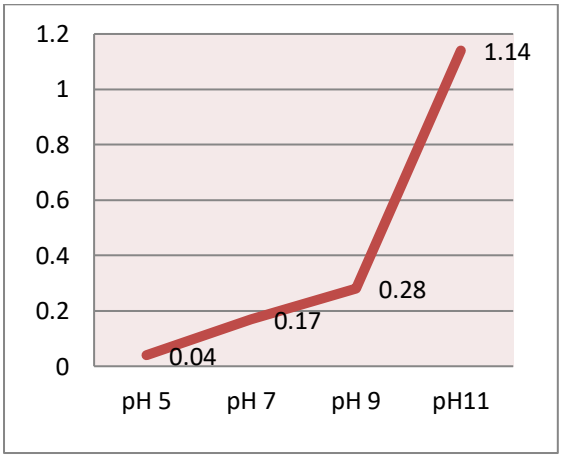
Table-3 Effect of different concentrations of metal ions on medium

Enzyme activity	OD at first day		OD at 3rd day of incubation	
		380nm 540 nm	380nn 540 nm	
	$FeSO_4$ + $CaCO_3$ (0.05 gm: 0.05 gm),	0.07	0.05	0.25	0.16
Salkowasayki Reagent assay	$FeSO_4$ + $CaCO_3$ (0.005 gm +0.045 gm)	0.13	0.10	0.30	0.20
	$FeSO_4$ + $PbNO_3$ (0.05 gm: 0.05 gm)	0.05	0.02	0.34	0.25
	$FeSO_4$+ $CaCO_3$ (0.045: 0.005).	0.06	0.03	0.50	0.20
Colorimetric assay with pumpkin extract	$FeSO_4$ + $CaCO_3$ (0.05 gm: 0.05 gm),	0.18	0.18	0.07	0.08
	$FeSO_4$ + $CaCO_3$ (0.005 gm +0.045 gm)	0.15	0.14	0.11	0.04
	$FeSO_4$ + $PbNO_3$ (0.05 gm: 0.05 gm)	0.15	0.12	0.09	0.07
	$FeSO_4$+ $CaCO_3$ (0.045: 0.005)	.039	0.27	0.04	0.02

Media optimization with pH:

Bacteria are specific to their environment. They evolve so they can grow in different areas. Within the human body and throughout the different environment different bacteria grow in different places and the pH in these areas differs. So their physiology and genetics are specific to pH. for bacteria to multiply in a culture the pH has to be the right level.

For example if we try to culture an unknown bacteria on one kind of media and it doesn't grow there or grows there it indicate the unknown species. Also if the pH level is inaccurate the culture won't grow and the media will be useless. On this basis we optimized the media in terms of pH to provide the suitable environment to bacteria to grow rapidly and to get high yield. We optimized pH range from 7 to 11 and observed that pH 11 was the best pH for this particular bacterium.



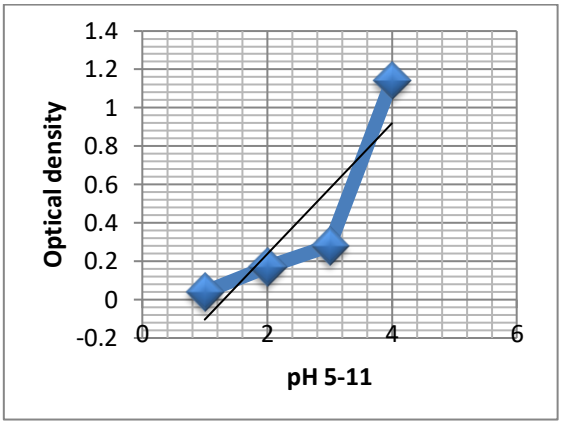


Fig-7 effect of pH on enzyme activity

Purification of enzyme:

Purification of enzymes was done by ammonium sulfate precipitation fallowed by dialysis. Purified enzyme showed maximum absorption 1.18 at 380 nm

CONCLUSION

The parameters optimized in these optimization steps can be helpful in future study for increasing the yield. These new approaches covered a way that we can also synthesized serotonin from the bacteria.

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