ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Characterisation of histidine decarboxylase from Enterobacter and Lactococcus species.

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ABSTRACT

Histidine decarboxylase is a metabolic enzyme involved in the conversion of histidine to histamine. Histamine is a biologically active amine which can bring about a range of physiological effects. Even then, the enzyme histidine decarboxylase hasn't been completely characterized due to its low availability and high instability. The various histidine decarboxylases that have been isolated and studied provide contrasting results with respect to the structure and involvement of coenzyme, adding to the complexity of the enzyme. The present study was carried out to isolate and purify histidine decarboxylase from the auxotrophic strains of *Enterobacterspp* and *Lactococcus spp*. The organisms were cultured and characterized using biochemical tests such as IMViC tests, catalase and oxidase test. The bacterial species was confirmed using these tests along withGram staining which indicated the presence of Gram-negative bacilli and Gram-positive cocci. The stepwise purification of the enzyme involved ammonium sulphate precipitation followed by ion exchange chromatography and gel permeation chromatography. The activity of the enzyme was studied using a spectroscopic method wherein the product histamine, was made to react with Alizarine red S and Nickel sulphate in presence of acetate buffer. A zymogram was obtained to indicate the presence of the active enzyme activity. The optimum pH and temperaturewere performed to obtain the optimum pH and temperature along with pH and thermo stability. The pH optimum was found to be 7 and optimum temperature was obtained at 47°C. The K_m and V_{max} were determined to be around 0.5 mM and 0.001 IU respectively. The enzyme was thus purified and studied for its activity and characteristics. This data obtained was further used to study enzyme inhibition which can prove a potential alternate to the side – effect driven fourth generation of antihistamines.

Keywords: *Histidine decarboxylase, Enterobacterspp, Lactococcus spp, histamine, Alizarine Red S, zymogram*

https://doi.org/10.33887/rjpbcs/2019.10.6.1

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ISSN: 0975-8585

INTRODUCTION

*Enterobacter spp*and *Lactococcus spp* are some of the common genus present ubiquitously in the environment. *Enterobacter* are opportunistic pathogens which can cause infections in immunocompromised patients. They are Gram negative bacilli which produce acetoin and can use citrate as a carbon source. *Lactococcus* belong to Lactic acid bacteria and are characteristically Gram-positive cocci in nature.

Histamine is a metabolically active, low molecular weight amine, which is naturally occurring in plants, animals and microbes. It is an imidazole derivative produced by the decarboxylation of the essential amino acid, histidine. This reaction is catalysed by the enzyme histidinedecarboxylase. Histamine is predominantly stored in mast cells, basophils, histaminocytes and histaminergic neurons of the brain.

Histidine decarboxylase is widely distributed in the gastrointestinal mucosa, mast cells, cerebrospinal fluid, bone marrow and certain actively growing tumors. They are known to be of two types based on the coenzymes employed in the reaction. They arepyruvoyl dependent enzymes and the pyridoxal dependent enzymes [6]. The histidinedecarboxylase of various organisms has been studied till date but the mechanism of action has not been clearly understood. Histidine decarboxylase also exhibits variation in their structure depending on the microenvironment of the organisms adding to the complexity of the study. The partial inhibition of the enzyme could be a possible option as an alternate to prevent the aftermath caused by antihistamines.

The present study aims at isolating, purifying and characterizing the enzyme histidine decarboxylase.Both *Enterobacter* and *Lactococcus*species as they are lactose fermenters, auxotrophic strains were used for HDC isolation in the study.

MATERIALS AND METHODS

Materials: Ammonium persulphate, bis-acrylamide, boric acid, bromophenol blue, citric acid, crystal violet, di-sodium hydrogen phosphate,disodium tetraborate, EDTA (Ethylene diamine tetra acetic acid), glacial acetic acid, grams iodine, hydrogen peroxide,potassium hydroxide, potassium sodium tartarate, safranin, sodium acetate, sodium carbonate, sodium chloride,sodium dihydrogen orthophosphate, sodium hydrogen carbonate were procured from **Fisher scientific**. Acrylamide, agarpowder, eosin methylene blue agar, glycerol, histidine, Kovac'sindolereagent, Mac Conkey agar, MRS (De Man, Rogosa and Sharpe) broth, MR-VP broth, nutrient agar, nutrient broth, Peptone, Simmon's citrate agar, sodium hydroxide, TEMED (N,N,N',N' – tetramethyl ethylene diamine), trisfree base, Wuster's reagent (N,N,N',N' – tetramethyl-p-phenylenediaminedihydro chloride) were procured from **Hi Media**. Alizarine red S was procured from **ChemiePvt Ltd**. Methyl red was procured from **Merck**. Cedar wood oil, tryptone were procured from **Nice Chemicals**. Ammonium sulphate, histamine, nickel sulphate, were procured from **S D Fine chem Ltd**. Carboxymethylsepharose, diethyl aminoethylsepharose, quercetin, sephadex G-100 were procured from **Sigma Aldrich**. α- naphthol was procured from **Thomas Baker**.

Bacterial source: The bacteria of interest was isolated from freshly fermented curd and garden soil, Bangalore, Karnataka, India.

Isolation of bacterial cultur: Serial dilutions including agar plating was employed to isolate the bacterial culture from the source. A small volume(1mL)/weight(1gm) of the sample was mixed 9mL of saline, thus, making it the dilution stock. The serial dilutions were then prepared by transferring a known volume of the dilution stock to the next dilution blank and so on. The dilutions were carried up to 10^{-6} and the aliquots were cultured on nutrient agar by spread plate technique. They were incubated for 24-48 hours at 37°Cfor 2hours [22].

Sub culturing of bacteria: Sub culturing is a microbial technique of transferring microbes from one growth media to another i.e., from a previous culture to a fresh growth media. De Man, Rogosa and sharp agar, abbreviated as MRS is a selective cultural media for the luxuriant grown of lactobacilli species as it suppresses the growth of the competing bacteria due to the presence of sodium acetate. The media facilitates growth of lactobacillus from oral cavity, dairy products, foods as well as other resources. MacConkey agar is both a selective and differential media to selectively isolate Gram-negative bacilli based on their ability to ferment

lactose. The presence of crystal violet and bile salts inhibit the growth of Gram-positive bacteria and the pH indicator, neutral red helps distinguish lactose fermenters from non-lactose fermenters [2]. Eosin methylene blue agar is also both a selective and differential media for coli forms as it contains dyes like eosin and methylene blue which are toxic to Gram positive bacteria. The coli form produces purplish black colonies on taking up methylene blue – eosin dye complex on lowering of the pH [12].

Biochemical test: These are a series of different tests performed in order to identify and differentiate bacteria.

IMViC test: It is a group of important tests performed to differentiate coli forms. The coli form group of bacteria includes both aerobic and facultative aerobic bacteria which are Gram negative and non-sporulating. The classical species include *Enterobacter* and *Escherichia.* IMViC stands for the first letter of each test in the series which includes Indole test, Methyl red test, Voges-Proskauer test and citrate utilization test. This test is mainly performed in order to distinguish *E. coli*from *Enterobacter aerogenes* [13,14]

Catalase test: It is a test performed to check the ability of the bacteria to produce the catalase enzyme. This enzyme helps in the breakdown of hydrogen peroxide to water and oxygen, which is produced as a result of aerobic respiration [5].

Oxidase test: This test is used to detect the presence of cytochrome C in mitochondria which produces the enzyme cytochrome C oxidase [8].

Gram staining: This is the most common technique used to differentiate bacteria into two large groups based on the composition of the cell wall i.e., Gram positive and Gram negative. This technique was developed by Hans Christian Gram and is the preliminary step used in the identification of bacteria [3].

Enzymology

Extraction of enzyme by sonication: The pure cultures of the characterized bacteria was further sub cultured in the broth and incubated for 24-48 hours at 37°C. The grown cultures were then subjected to sonication in order to obtain a cell-free extract. The cells were disrupted at a pulse rate of 0.5S and amplitude of 40% for 30 minutes, maintained at 25°C. The disrupted cells were subjected to centrifugation at 10000 rpm for 10 minutes to pellet out the cell debris.

Partial purification by ammonium sulphate salt precipitation: Ammonium sulfate fractionation was carried out by adding the small amount of powdered ammonium sulfate to crude extract by constant stirring over magnetic stirrer at 4°C to obtain 0 - 30% saturation. The solution allowed to stand for 30 minutes at 4°Cfollowed by centrifugation at 10,000 rpm for 20 minutes. The pellet obtained was dissolved in 5 ml of 10 mM sodium acetate buffer pH 5. The supernatant was collected and subjected to 30-60 %, 60-80% ammonium sulphate fractionation as described above, followed by centrifugation at 10,000 rpm for 20 minutes. The pellet was dissolved in 10 mM sodium acetate buffer pH 5. The fractions (0 - 30%, 30 - 60 % and 30 - 80%) were dialyzed against 10 mM sodium acetate buffer pH 5 for 8 hours, with two changes in the buffer. The dialysate was centrifuged at 10,000 rpm for 20 minutes to remove the insoluble residue and the supernatant was used for assay of the enzyme and protein content [20].

Purification of enzyme by CM – sepharose Ion exchange chromatography: The preswollen matrix was mixed with 50mM acetate buffer to prepare the slurry of CM – sepharosewhich was packed into column (10cm × 1.6cm) under gravity at room temperature. After packing, the column wasequilibrated with two volumes of the start buffer at a flow rate of 25 ml/hr. The upper surface of the CM-sepharose was protected by filter paper. The 30% and 80% ammonium sulphate precipitated fractions were loaded separately onto the column and the column was washed with two bed volumes of start buffer. The bound proteins were eluted with stepwise increase in the ionicstrength (0.1M, 0.3 M and 0.5 M NaCl). Fractions of 5 ml were collected. The enzyme histidine decarboxylase did not bind to the column and was eluted in the washings (CMS – I and II fractions). These fractions were further subjected to DEAE Sepharose column for anion exchange chromatography [20].

Purification of enzyme by DEAE – Sepharose Ion exchange chromatography: Prewash DEAE – Sepharosewith 0.5M HCl and then with 0.5M NaOH and filtered through the Buckner funnel. The matrix is then washed with 50mM acetate buffer ofpH 5 and was packed into column (10cm × 1.6cm) under gravity at room temperature. After packing, the column was equilibrated with two volumes of the start buffer at a flow rate of 25 ml/hr. The upper surface of the DEAE- Sepharose was protected by filter paper. The 30% ammonium sulphate precipitated fraction was loaded onto the column and the column washed with two bed volumes of start buffer. The bound proteins were eluted with stepwise increase in the ionic strength (0.1M, 0.3 M and 0.5 M NaCl). Fractions of 5 ml were collected. The enzyme histidine decarboxylase was bound to the column and was eluted in the washings (DEAE S – VI fraction). This fraction was further subjected to gel permeation chromatography usingSephadex G-100 column [20].

Purification of enzyme by Molecular exclusion chromatography: The DEAE S – VI fraction was filtered through Sephadex G-100 column (20cm × 0.8cm) previously equilibrated with 10mM acetate buffer of pH 5. Sephadex G – 100 gel was equilibrated with 10 mM sodium acetate buffer, pH 5 and packed into a column under gravity (200mm × 8mm). The column was equilibrated with two bed volumes of 10 mM sodium acetate buffer, pH 5 at a flow rate of 10 ml/hr. The DEAES fraction, DEAES-VI containing histidine decarboxylase activity was subjected to gel permeation chromatography separately using Sephadex G – 100. The proteins were eluted with start buffer and fractions of 2 ml were collected [20].

In-gel assay of enzyme: Separating gel was run with 10% polyacrylamide. They were electrophoresed for 1-1.5 hours with a constant current of 50V [21]. After electrophoresis, the gel was subjected to react with 1mM substrate, histidine and incubated at room temperature for 20 minutes. After incubation, the gel was treated with Alizarine Red S, Nickel Sulphate and Acetate buffer of pH 5 and molarity of 50mM in order to observe the bands.

Determination of pH stability: The pH stability of the enzyme was determined by incubating the enzyme in buffers of different pH in the range of 3.0–9.0 for 1 and 3 h, at 50 °C. Aliquots were withdrawn and enzyme activity was determined at pH 7.0 and 25 °C.

The different buffers that were used were acetate buffer, carbonate buffer, borate buffer, phosphate buffer and citrate buffer.

Determination of optimum pH: Effect of pH on HDC activity was determined by incubating and carrying out the reaction at various pH ranging from pH3 to pH9 by pre incubating the enzyme with Acetate buffer [1].

Determination of thermo stability of the enzyme: The thermal stability of the enzyme was determined by pre incubating the enzyme fraction at various temperatures between 0℃- 70℃ and then checking the enzyme for its activity [1].

Determination of optimum temperature: Optimum temperature for the activity of HDC was determined by conducting the enzyme assays at temperatures ranging from 25℃- 50℃ [1].

Determination of K_m and V_{max}: The kinetic constants of HDC were determined by incubating fixed amount of the enzyme with varying concentrations of the substrate, histidine by constructing LB- plot, MichaelisMenton plot, EadieHofstee plot and Hanes-Woolf plot [10].

RESULTS AND DISCUSSION

Previous studies reveal the isolation and purification of histidine decarboxylase from organisms like *Lactobacillus* 30a[19], *Morganellamorganii* AM -15[20], *Klebsiellaplanticola*, *Enterobacteraerogenes* [6], *Leuconostosoenos* 9204[18], *Tetragenococcusmuriaticus*[11]. Various techniques such as ammonium sulphate precipitation, affinity chromatography, PAGE, gel filtration chromatography and isoelectric focusing were employed in order to purify the enzyme to homogeneity and study its properties[20]. The isolation of bacteria was followed by its characterization. Histidine decarboxylase was purified and characterized from the obtained bacteria and was studied to decipher its kinetics and activity.

ISSN: 0975-8585

Isolation of Bacteria: The bacteria were isolated by culturing in nutrient broth medium and nutrient agar medium as these are basic media used for isolation and cultivation of bacteria which enhances the growth of even non- fastidious bacteria [Figure 1 and Figure 2].

Figure 1: Soil sample cultured on Nutrient Agar Media

Figure 2: Curd sample cultured in the Nutrient Broth media.

Sub-culturing of bacteria

MRS Agar: The curd sample was cultured on MRS agar to favour the luxuriant growth of lactic Acid bacteria. MRS media also acts as a selective media for Lactic Acid Bacteria by reducing the pH to 5.7 due to the presence of Tween 80 thus, inhibiting the growth of Streptococci spp and moulds [Figure 3].

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Figure 3: Curd sample sub-cultured on MRS Media

Mac Conkey Agar: Mac Conkey agar helps differentiate lactose fermenters from non - fermenters. Lactic acid bacteria and Enterobacter are said to be lacto fermenters and thus, the curd and soil sample weresubcultured onto Mac Conkey agar to confirm their ability of lactose fermentation and also distinguish them from other contaminating bacteria. The pink colouration of the culture indicates that the bacteria are lactose fermenters [Figure 4].

Figure 4: Curd sample on MacConkey Agar Media.

EMB agar: The soil sample was cultured onto the EMB Agar media in order to reconfirm the absence of *E. coli. E. coli*. strains develop a characteristic green metallic sheen on EMB Agar due to the rapid fermentation of lactose and production of strong acid which reduces pH of the media. The lack of green metallic sheen confirms the absence of E. coli and presence of Enterobacter [Figure 5].

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Figure 5: Soil sample on EMB Agar Media.

The *Enterobacter* and *Lactococcus*spp was identified using the biochemical tests and gram staining [Table 1]. The identified organisms were isolated and subjected to large scale culturing in order to obtain a greaterbiomass. This culture was subjected to sonication to obtain cell free extract from which the enzyme, histidine decarboxylasewas purified by ammonium sulphate precipitation, ion-exchange chromatography followed by molecular exclusion chromatography.

Table 1: Biochemical Tests and Gram staining of bacteria.

Biochemical Tests	Curd Sample	Soil Sample
Indole Test	Negative	Negative
Methyl red Test	Negative	Negative
VogesProskauer Test	Negative	Positive
Citrate Test	Negative	Positive
Catalase Test	Negative	Positive
Oxidase Test	Negative	Positive
Gram staining	Gram positive cocci	Gram negative Bacilli

Enzymology

Sonication – The crude enzyme was obtained in the form of cell free extract by subjecting the bacterial cultures to sonication and centrifugation in order to remove the cell debris. The time curve was obtained using these crude enzymes [Figure 6 and Figure 7]. Their activity and protein content were also estimated. The standard curve of histamine was used to deduce the value for product formation. 1 OD from the standard curve of histamine corresponds to 0.0287µmoles/g equivalent of histamine. The protein was estimated with reference to the standard BSA curve in which 1 OD corresponded to 0.789mg/g equivalent of BSA.

ISSN: 0975-8585

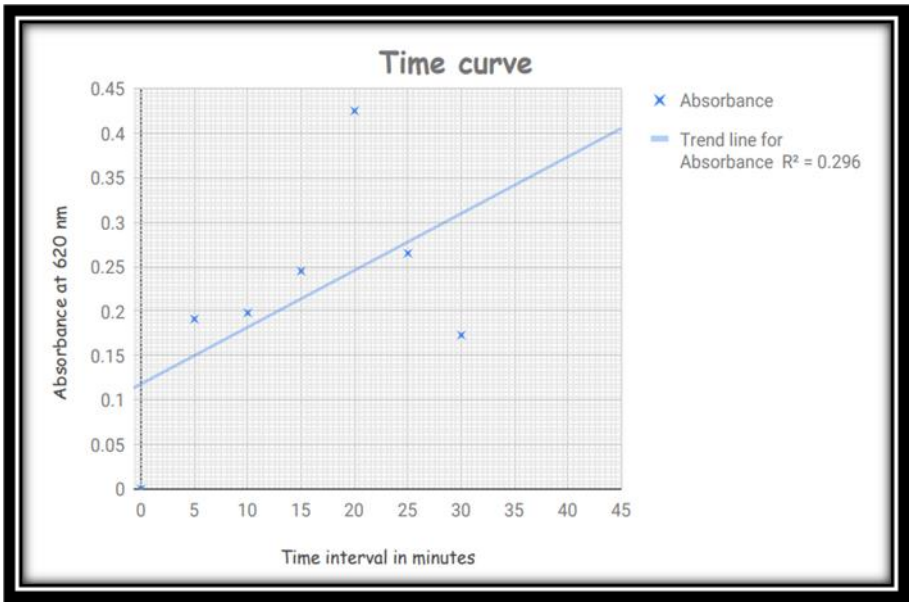

Figure 6:Time Curve for crude Enterobacter sample

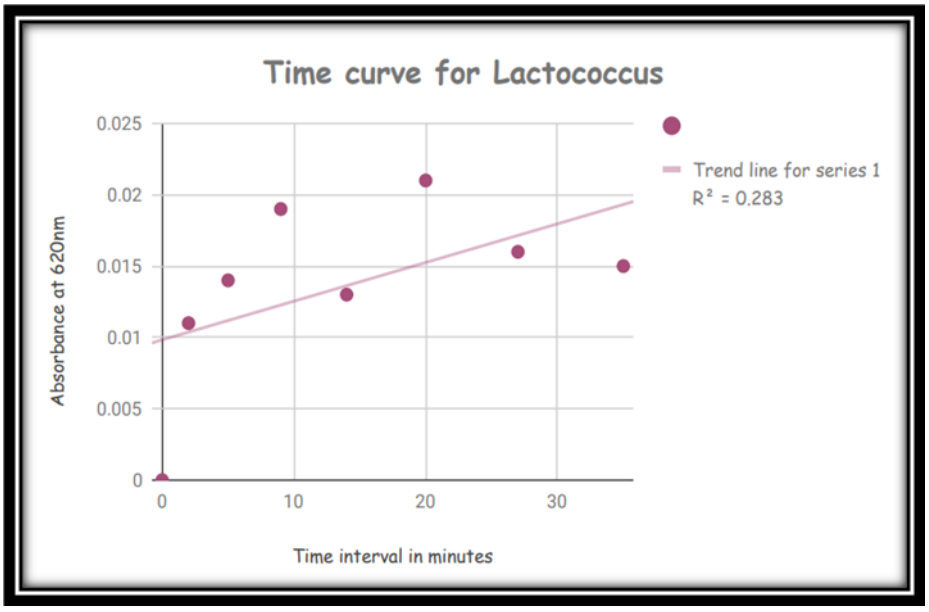

Figure 7: Time curve for crude Lactococcus sample.

Ammonium sulphate precipitation: The crude enzymes of both *Enterobacter spp.* and *Lactococcusspp* were subjected to 30%, 60% and 80% ammonium sulphate precipitation followed by dialysis. The activity and protein contentwere estimated. In case of *Enterobacter spp*., the 30% precipitated sample provided promising results whereas 30% and 80% precipitated samples of *Lactococcusspp*., proved to have higher activity. These samples having been promising were subjected to further purification.

Ion exchange chromatography: The ammonium sulphate precipitated samples were subjected to CM – Sepharose and DEAE Sepharose Ion exchange chromatography. The enzyme was eluted in the washings of CM Sepharose chromatography. The CMS – I and II fractions which were found to have the enzyme presence were pooled and subjected to DEAE Sepharose chromatography. The enzyme was then eluted in DEAE S –VI fraction as the enzyme was bound to the column and the increasing concentraction of saline brought about the elution of the enzyme. The Lactococcus sample wasn't subjected to further purification due to the lack of stability. The Enterobacter sample was further subjected to molecular exclusion chromatography in the Sephadex G-100 column.

ISSN: 0975-8585

Molecular Exclusion chromatography: The DEAE S -VI fraction of the Enterobacter was subjected to Sephadex G-100 column. The fractions obtained were then estimated for activity and protein. The enzyme was found to be eluted in the void volume as the enzyme had a larger molecular mass and thus couldn't enter the beads. Previous studies reveal that the enzyme could be eluted in the Sephacryl S-300 column [20]. [Table 2 and Table 3]

Table 2: The activity of HDC in case of Enterobacter at various steps of purification

Enzyme sample	Total Activity (IU)	Protein (mg)	Specific activity (IU/mg)	Fold Purity	Percentage Yield
Crude enzyme	42.476	567	0.07	1	100
30% Ammonium sulphate precipitation	0.011125	37.7475	0.0002947	0.00421	0.0261
Cation Exchange Chromatography	0.1019	51.187	0.00199	0.0284	0.239
Anion Exchange Chromatography	3.08875	154.087	0.02004	0.286	7.27
Gel Permeation Chromatography	12.430	120.815	0.10288	1.469	29.26

Table 3: The activity of HDC in case of Lactococcus at various steps of purification

Enzyme sample	Total Activity(IU)	Protein (mg)	Specific activity(IU/mg)	Fold Purity	Percentage Yield
Crude enzyme	2.01428	478.02	0.00421	1	100
30% Ammonium sulphate precipitation	0.004574	7.0911	0.000645	0.1532	0.227
80% Ammonium sulphate precipitation	0.08789	14.1822	0.006197	1.4719	4.363
Cation Exchange Chromatography of 30% Ammonium sulphate precipitation	0.18468	25.6853	0.007190	1.707	9.1685
Cation Exchange Chromatography of 80% Ammonium sulphate precipitation	0.09718	15.9271	0.00610	1.4489	4.824
Anion Exchange Chromatography of 30% Ammonium sulphate precipitation	0.3375	5.418	0.0622	14.774	16.755
Anion Exchange Chromatography of 80% Ammonium sulphate precipitation	0.44375	6.406	0.0692	16.437	22.030

In – gel assay: The HDC enzyme was detected in the zymogram, where they appeared as very light brown bands on a pink background when incubated with the substrate and on treatment with Alizarine Red S and acetate buffer. This indicates the presence of HDC in the purified samples [Figure 8].

ISSN: 0975-8585

Lane 1	**Gel permeation Sample**
Lane 2	**Anion exchange sample**
Lane 3	**Cation exchange sample**
Lane 4	**30% ammonium sulphate sample**
Lane 5	**Crude enzyme**

Figure 8: Zymogram of HDC activity

Determination of Stable and Optimum pH: HDC was found to exhibit its maximum activity in the citrate and borate buffers. The enzyme was found to be higly stable at a pH range of 4.5 – 7. The enzyme histidine decarboxylase was found to have a pH optimum of 7. Studies revealed the optimum pH of HDC isolated from *Lactobacillus 30a* to be 4.8 [19] whereas HDC of *Micrococcus* spp., has an optimum pH of 5.8[15]. The partially purified mammalian mast cells were found to have an optimum pH between 6 and 7 [7]. *M. morganii, K. planticola* and *E. aerogenes* were most active at pH of 6.5 [6]. The optimum pH of *C. perfringens* was observed to be 4.5 [16,17] and that of *Lactobacillus buchneri* was found to be 5.5 [9,16]. *Tetragenococcusmuriaticus* has an optimum pH ranging from 4.5 – 7.0 [11]. *Oenococcusoeni* was found to have pH optimumof 4.8 [4]. Studies revealed the optimum pH for HDC isolated from *Leuconostocoenos* 9204 was obtained at 4.8 when the concentration of histidine was kept constant at 25 $mmol^{-1}$ and0.2 mol l^{-1} ammonium acetate buffer was used [18].

Determination of the temperature stability and optimum temperature of the enzyme: The temperature stability studies showed decreased activity after 50℃ and before 20℃ indicating that the enzyme is stable at temperatures 20 ℃ - 50℃. The decrease in activity at higher temperatures may be due to the change in the conformation of the enzyme. The optimum temperature of the enzyme was said to be 47℃ as the enzyme showed its peak of activity at this temperature.

Determination of K_m and V_{max}: The K_m and V_{max}values of HDC were obtained by varying the substrate concentrations and the constants were determined by the MichaelisMenten plots in comparison to the reciprocal plots of Lineweaver Burk plot, EadieHofstee plot and Hanes Woolf plot. Low values of K_m indicate greater affinity of the enzyme to the substrate. The V_{max} determines the rate of the reaction when the substrate is at saturation. The K_m and V_{max} values obtained for HDC are tabulated (Table 4). *Morganella* AM-15 was observed to have a K_m value of 1.3 mM and V_{max} of 731 IU [20]. *Lactobacillus buchneri* revealed to have a K_m of 0.6 mM and V_{max} of 69 IU [9,16]. Studies on *C. perfringens* indicate a K_m of 0.3 mM and V_{max} of 7.010 [16,17]. HDC of *Micrococcus* spp., have a K_m of 0.8mM and V_{max} of approximately 25 IU[9,15,17]. HDC derived from *Lactobacillus 30a* has a K_m of 0.4 mM and $V_{max}$of 80IU [9,17]. *Tetragenococcusmuriaticus* produces HDC which has $K_m$of 0.74mM and V_{max} of 16.8 IU [11]. HDC isolated from *Oenococcusoeni* was found to have a K_m value of 0.33 mM and V_{max} of 17.8 IU [4]. Partially purified HDC obtained from mammalian mast cell is said to have a K_m value of 0.5mM [7]. The K_m value obtained in the present study was found to be around 0.5 mM and the $V_{max}$was found to be 0.001 IU [Table 4].

ISSN: 0975-8585

Table 4: Determination of K_m and V_{max} values of HDC

Name of the plot	K_m (mM)	V_{max} (IU)
MichaelisMentonPlot	0.55	0.00106
Lineweaver Burk Plot	0.588	0.0012
EadieHoftsee Plot	0.663	0.0011
HanesWoolf Plot	0.35	1.03

ACKNOWLEDGEMENT

The authors wish to acknowledge Department of Chemistry (PG Biochemistry) and the management of Mount Carmel College Autonomous, Bengaluru for funding this project and offering their facilities for the analysis.

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