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Isolation, purification and characterization of acid phosphatase from *Chara sp.*

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ABSTRACT

Algae are also very important ecologically because, used in production of many economical valuable products and they are beginning of the food chain for other animals. Whereas, ample of reports are available in morphological studies on fresh water algae throughout the globe. However, enzyme research of algal origin is ephemeral. Hydrolytic enzymes have great reasonable significance owing to their central role in biological processes. In this study, acid phosphatase enzyme characterized from *Chara sp.* fresh water alga isolated province Waghur River located near Skegaon, district Jalgaon, Maharashtra (India). The acid phosphatase is a monomer protein purified by ion-exchange and gel filtration to 3.87 fold with an apparent molecular mass 66 kDa on SDS PAGE. The purified acid phosphatase has an optimum pH of 4.0, and optimum temperature for the hydrolysis of p-Nitro phenyl phosphate at 50°C and the k_m and V_{max} 0.25 mM and 0.012 $\mu\text{mol min}^{-1}\text{mg}^{-1}$ respectively at the same conditions. The activation energy found to be 54.20 KJ/mole and Q_{10} value was 2.08 between 40 and 50°C and fairly stable at temperature up to 37°C. The activity of the enzyme enhanced by EDTA, Tween 20, Triton-X and Guaiacol and heavy metals Fe^{3+} , Cu^{2+} , Ca^{2+} , Mg^{2+} , Hg^{2+} and K^+ . The enzyme strongly inhibited by organic solvents, SDS, tween 20 and heavy metals Zn^{2+} . The present article reveals on bio-molecular characterization of acid phosphatase with kinetic studies.

Keywords: Acid phosphatase, kinetic studies and *Chara sp.*

INTRODUCTION

Algae are one of the primitive and most adaptive organisms on the earth. Nowadays, the importance of algae constantly increases in the whole world for their industrially and economically important products to fulfill the demands of increasing population. Due to the highly economically importance of algae very primordial and scanty of work are available on the algal hydrolytic enzymes. Acid phosphatase is one of them, which has been isolated from diverse group of organism from bacteria to higher vertebrates and higher cryptogams [1]. *Chara sp.* found in ponds lakes and river throughout the year at peak during late summer. In this report, the isolation, partial purification and characterization of an acid phosphatase from fresh water alga *Chara sp.* (CS).

MATERIALS AND METHODS

Materials The chemicals used of analytical grade purchased from Sigma Aldrich, USA; Himedia laboratories, Mumbai; Fischer Scientifics, India and Merck Chemicals, India.

Collection and Authentication of *Chara sp.* from Waghur River: the *Chara sp.* microalgae collected from the Waghur river at Sakegaon, brought to the laboratory, and kept in 4°C for further experimentation. Meanwhile, the collected algae identified by using standard monographs based on their morphological characters like appearance, reproductive organs, habitat *etc.* While in certain cases, special help taken from the expert phycologist Dr. Shantaram R. Mahajan, Bhusaval.

Extraction of enzyme from *Chara sp.* The enzyme was extracted from *Chara sp.* and subjected to intracellular enzyme, activity. The enzyme was extracted by using mortar and pestle *i.e.*, mechanical method. The macerated algae were mixed with chilled buffer and subjected to centrifugation at 4°C for 15 min's and the supernatant (CSCE) was used as intracellular enzymes.

Protein assay The protein concentration of soluble protein quantified by the method of Lowry *et al.* (1951) and using bovine serum albumin (BSA) as a standard substrate [2].

Phosphatase assay The p-nitrophenyl phosphate was use for measuring acid phosphatase quantitatively. In this assay, amount of released p-nitrophenyl measured at 405 nm. The acidic phosphatase reaction mixture contains 1.5 ml 3X sodium acetate incubation buffer (1M, pH 5.0), 0.2ml 1.5X para-nitrophenyl phosphate in water. Sodium carbonate was used to stop the reaction after 15 minutes of incubation at 37°C. Blank contained the same ingredients except the enzyme solution. The enzyme activities were expressed in unit per ml (U/ml) or $\mu\text{mole}/\text{min}/\text{ml}$.

Partial purification of acid phosphatase The CSCE subjected to partial purification by solvent precipitation or ammonium sulfate precipitation and further dialysis. The acid phosphatase fractionated by ammonium sulfate precipitation (CSAP) [3] and solvent precipitation (CSSP) [4]. Further, both the fractions subjected, to check stability.

Purification of the acid phosphatase

a) Gel filtration Chromatography The CSAP after dialysis with 20mM phosphate buffers the sample at pH 7.0, loaded on a previously equilibrated sephadex G-75 gel column [5]. The maximum active fraction abbreviated as CSGF.

b) Ion-exchange chromatography The purification of the CSGF by DEAE-cellulose column done starting by determination of buffer pH that is suitable for the ion exchange column. The experiment result showed that the enzyme was unable to modify the counter ion at pH range 5.0 – 8.0. The enzyme was able to change the counter ion above pH 8.0 that was at pH 8.6, so the Tris buffer 50 mm with pH 8.6, used as an initial buffer. While the gradient of sodium chloride and Tris 50 mm pH 8.6 buffer solution used for ablation. The protein pattern (A280) obtained from DEAE-cellulose column chromatography and the highest peaks (CAP1) subjected for phosphatase activity and the fraction ranges of highest peak are analyzed for phosphatase enzyme.

Characterization of CAP1 The CAP1 characterized for optimum pH, temperature, effect of metals, detergents and solvent on enzyme activity. Molecular weight was determined by SDS-PAGE.

Effect of temperature on CAP1 activity The optimal temperature for SOIF action was determined by incubating the assay mixture described above at different temperatures between 4°C and 85°C for 15 minute at 50°C. The relationship between Energy of activation (E_a) and temperature, formulated empirically by an integrated form of the Arrhenius equation [6].

Effect of pH on CAP1 activity In order to find the pH profiling of SIGF, sodium acetate buffer (pH 3.0-5.0), potassium phosphate buffer (pH 5.0-7.0), Tris-HCl buffer (pH 7.0-9.0) and glycine-NaOH buffer (pH 9.0-12.0) selected. The concentration of all buffers is 50 mM. The assay mixture (1.5ml each buffer, 0.2ml 50 mM substrate and 0.1ml of enzyme solution) was incubated at optimum temperature for 15 min in different pH values of above buffers and the enzyme activity was determined by spectrophotometric enzyme assay.

Effect of substrate on CAP1 activity Determination of CAP1 activity in different substrate concentration from 5 to 60 mM p-nitrophenyl phosphate, studied. The kinetic parameters like K_m and V_{max} of enzyme preparation were determined using pNPP substrate. The values are determined by using the Michaelis-Menten plot and Lineweaver-Burk plot [7].

Effect of solvent on CAP1 activity

To determine the effect of various solvents as possible activators or inhibitors on the partially purified acid phosphatase, the enzyme solution were pre-incubated with 100 μ l solvent at 37 °C for 5 min with the compounds and then the activity was assayed. The substrate added to the medium and incubated at standard conditions.

Effect of detergents on CAP1 activity

To determine the effect of various detergents as possible activators or inhibitors or the purified acid phosphatase, the enzyme solution pre-incubated, with 100 μ l detergents (1mM) at 37°C for 5 min with the compounds and then the activity determine. The substrate added to the medium and incubated at standard conditions.

Effect of metals on CAP1 activity

To determine the effect of various metals as possible activators or inhibitors or the purified acid phosphatase, the enzyme solution pre-incubated with 100 μ l of 1 mM metal solution at 37 °C for 5 min with the compounds and then analyzed for enzyme activity. The substrate added to the medium and incubated at standard conditions.

Thermal inactivation of CSGF The thermal inactivation of the enzyme was determined at 37 and 55°C for a period of 10 to 120 min. the enzyme incubated in 100 mM buffer at optimum pH. Aliquots removed at intervals and immediately quantified for enzyme activity.

Molecular weight determination by Polyacrylamide gel electrophoresis (PAGE) Electrophoresis carried out by the method of Laemmli (1970) [8] on (10% w/v) acrylamide gels under denaturing and non-denaturing conditions. In denaturing conditions, a sample of CAP1 incubated for 5 min at 100°C with SDS PAGE sample loading buffer containing 2-mercaptoethanol. For non-denaturing conditions, samples had been mixed but before running with sample buffer without 2 mercaptoethanol and SDS. Gels were stained with Coomassie brilliant blue R 250. The molecular mass standard markers GeNe™ SDS PAGE kit containing 66.0 kDa., 43.0 kDa., 29.0 kDa., and 14.3 kDa were used.

RESULTS

Partial purification of acid phosphatase

a) Precipitation of CSCE by salt precipitation and solvent precipitation

Fractional precipitation of CSCE with ammonium sulfate The CSCE shows the presence of two iso enzymes since, 20% ammonium sulfate precipitation shows higher specific activity than 40%. Although the biggest portion of acid phosphatase enzyme precipitated at 80%, saturation had 177.39% higher specific activity compared to the crude enzyme with 15.62% recovery. For further purification 80%, saturated acid phosphatase (CSAP) used.

The result of solvent precipitation indicated that as the solvent ratio increases the extracted protein content and enzyme activity also increased (table 1) and the precipitated acid phosphatase (CSSP).

Table 1 Enzyme activity of CSCE with CSSP

Solvent and enzyme ratio	CS acid phosphatase		
	EA (U/ml)	PC μ g/ml	SA (U/mg)
CSCE	2.91	1.02	2.85
CSSP1 (1:1)	1.09	0.24	4.54
CSSP2 (1:2)	2.59	0.42	6.18

EA= Enzyme activity, PC= protein concentration, SA = Specific activity

Stability of CSAP and CSSP2 The results of comparative stability in between salt precipitate and solvent precipitate presented in Table 2. The loss of enzyme activity occurred during storage of precipitates at 4°C for a week. The loss of *Chara* acid phosphatase activity was highest in the case of enzyme fraction obtained by acetone

precipitate (CSAP1) i.e, 100 % while in case of salt precipitation (CSSP1) only 17.41 % of acid phosphatase activity was lost in a week.

Table 2 Stability of CSAP and CSSP2

Technique	Acid phosphatase activity (units/min)			Half life
	Initial activity	Activity after a week	Loss of activity (%)	
CSAP	ASP (60%)	10.45	8.63	17.41
CSSP2	AP (1:2)	8.64	0.97	99.03

The enzyme extracted with ammonium sulfate precipitation showed maximum activity and storage stability as compared with acetone-precipitated fraction of acid phosphatase; hence, the ammonium sulfate precipitated fraction of enzymes subjected to chromatographic separation of acid phosphatase. It can thus be stated that the solvent precipitation is suitable for enzyme precipitation for characterization of enzyme in terms of molecular weight determination, but not suitable for studying enzyme application and enzyme kinetics.

Purification of acid phosphatase

Gel filtration Chromatography The dialyzed and CSAP 16.552mg loaded on sephadex G-100 column (20 X 1.5 cm). The CSCE high protein content observed in eight fractions, 2, 7, 15, 21, 26, 29, 34 and 38. The protein rich fractions subjected to phosphatase assay, where in, 14-16 fraction of CS showed acidic phosphatase activity and abbreviated as CSGF. The results also indicated that there might be the presence of more than one acid phosphatase in CS. Further, CSGF subjected to ion exchange chromatography.

Ion-exchange chromatography the phosphatase activity in the fraction numbers 5 - 7 fraction of CSCE. The total enzyme activity of fractioned samples from 6-7 is 12.01 units. After ion exchange, the CS acid phosphatase named CAP1.

Purification scheme of CAP1 from *Chara sp.* The method employed for purification of acid phosphatase was salt precipitation, dialysis, gel filtration and ion exchange chromatography. The table 3 shows recovery and fold of purification of acid phosphatase enzyme. The crude extract of CS contained total 281.93 units of enzyme, out of which 121.70 units (12 ml) of the enzyme recovered by ammonium sulfate and dialysis. The 81.12 units (8 ml) of enzyme subjected to gel filtration out of which 22.44 units (6 ml) of enzyme obtained. Further 18.70 units (5 ml) of enzyme were loaded on the ion exchange column out of which only 12.01 units of enzyme recovered with 3.87 fold purification and 426% of the recovery. The specific activity of CAP1 is 11.53 units/mg.

Table 3 Purification scheme of CSCE

Technique	Total enzyme (U)	Total protein concentration (mg)	Specific activity (U/mg)	Purification folds	Yield (%)
CSCE	281.93	94.50	2.98	1	100
CSAP	121.70	24.82	4.91	1.64	43.16
CSGF	22.44	3.12	7.18	2.41	7.96
CAP1	12.01	1.04	11.53	3.87	4.26

Effect of Temperature on CAP1 The CAP1 is most active at a temperature between 30°C and 60°C. It retained more than 77 % activity till 60°C, where in the activity dropped rapidly below 30°C and above 60°C and showed maximum activity at 50°C. Above 50°C, the enzyme activity declined rapidly as the temperature was increased; only the enzyme was completely inactivated at 80°C in CS (figure 1). The effect of temperature on the CAP1 showed that it is stable up to 80°C with temperature optima at 50°C. The activation energy (Ea) found to be 54.20 KJ mol⁻¹ and Q10 value was 2.08 between 40 and 50°C (optimum temperature) for CSGF.

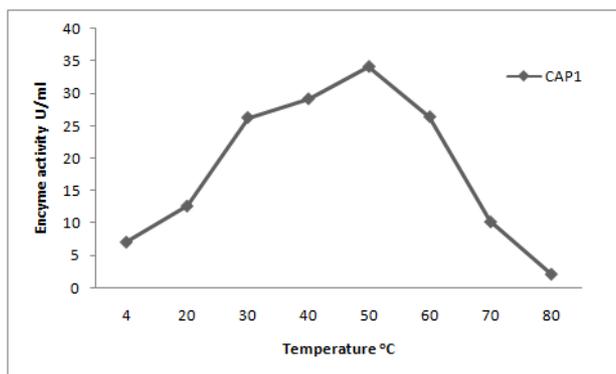


Figure 1 Effect of Temperature on CAP1

Effect of pH on CAP1 The CAP1 optimum pH observed at 4.0 with enzyme activity 2.75 U/ml, most stability in the pH range 4.0 – 6.0, and its activity decreased sharply above pH 5.0 retaining. The effect of pH on the CAP1 showed that it is stable in a range of 4.0 -6.0 with close pH optima of 4.0 (figure 2).

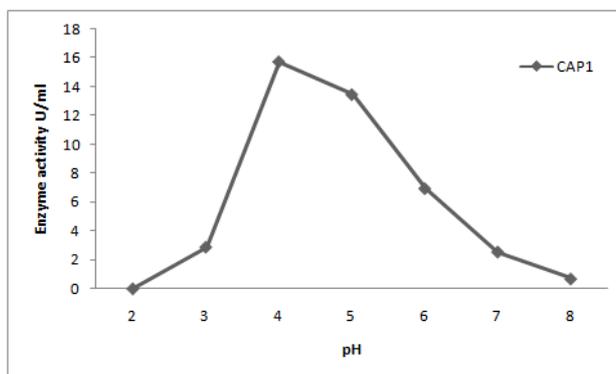


Figure 2 Effect of pH on CAP1

Effect of substrate on CAP1 activity The K_m value for pNPP obtained, from the acid phosphatase of both algae was within the same magnitude. When the activity of the enzyme measured at various concentrations of pNPP substrate, a double-reciprocal plot gave a straight line (Figure 3) that allowed the determination of K_m and V_{max} values of 0.25 mM and $0.012 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for CAP1.

Table 4 Kinetics of CAP1

Calculation method	Michaelis-Menten	Lineweaver-Burk	Hofstee	Eadie
V_{max}	0.012	0.0121	0.012	0.0124
K_m	0.25	0.238	0.23	0.236

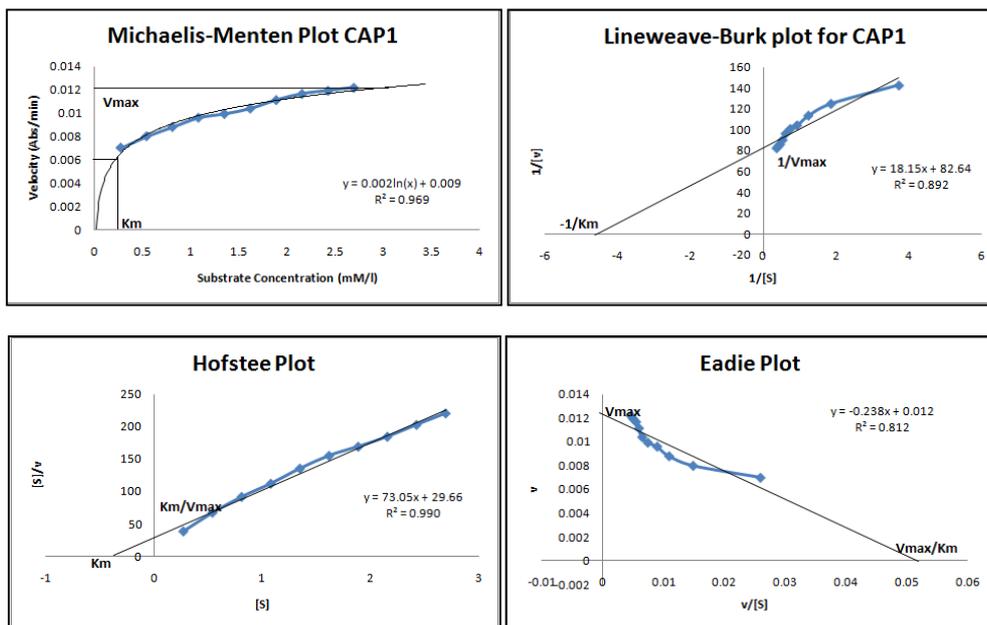


Figure 3 K_m and V_{max} of CAP1, M-M plot, L-B plot, Hofstee Plot and Eadie Plot

Effect of solvent on CAP1 activity The CAP1 activated by Acetone, butanol, isoamyl alcohol and inhibited by Chloroform. The effect of CAP1 activity on tested solvents are given in figure 4.

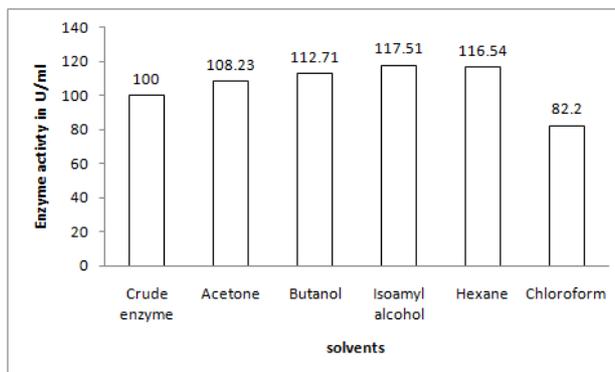


Figure 4 Effect of organic solvents on CAP1

Effect of detergents and chelator on CAP1 The influence of various detergents on the CAP1 activities studied (Figure 5). The SOIF activity inhibited by SDS and stimulated by EDTA, Tween 20, Triton-X and Guaiacol.

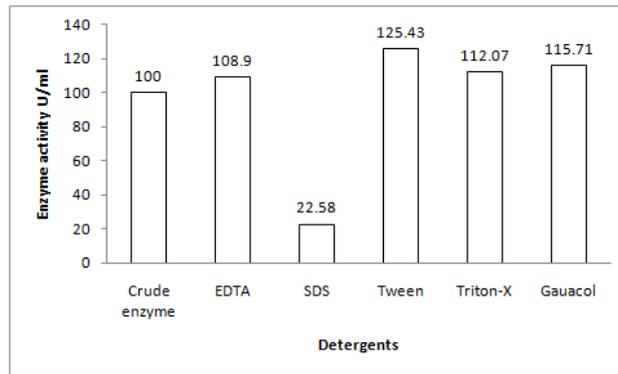


Figure 5 Effect of detergents and chelator on CAP1

Effect of metals on CAP1 activity The purified CAP1, sensitive to ions at various degrees, depending on ion nature and isoenzymes. SOIF, activity enhanced by Fe^{3+} , Cu^{2+} , Ca^{2+} , Mg^{2+} , Hg^{2+} , K^+ and inhibited by Zn^{2+} (figure 6).

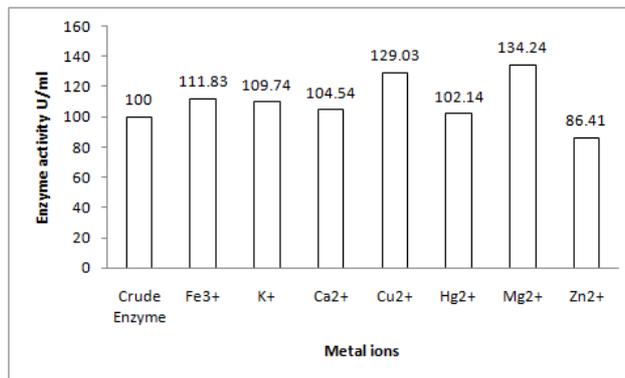


Figure 6 Effect of metal ions on CAP1

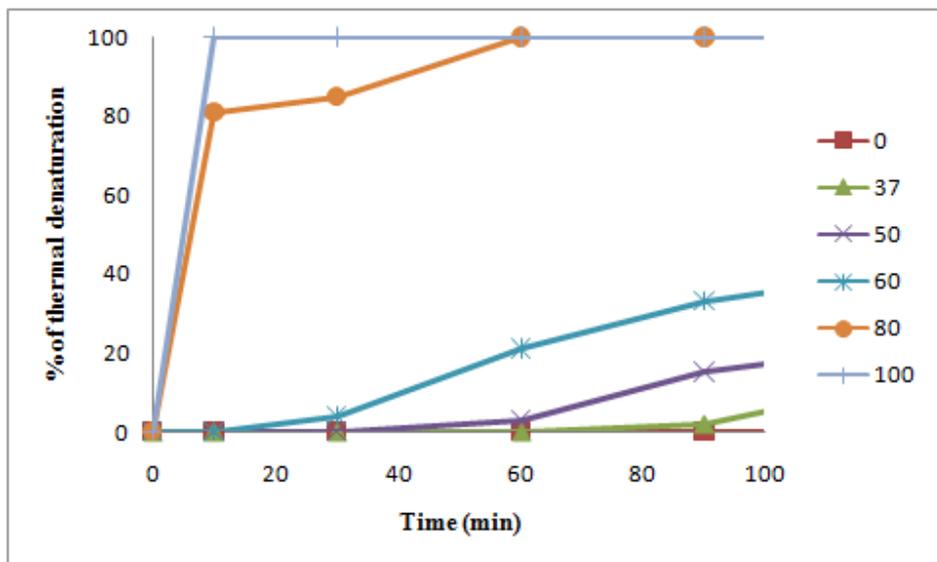


Figure 7 Percentage of thermal inactivation of CAP1

Thermal inactivation of CAP1 The thermal inactivation studies indicated that CAP1 remained completely stable for 120 min at 4 °C (figure 7). However, the SOGF were less stable and lost 21% of their hydrolytic activity after 120 min of incubation, at their temperature optima. The enzymes were fairly stable at temperature up to 37°C. Above 50°C, their activities declined rapidly as the temperature was increased. This conduct appears to be common for plant acid phosphatase. Although, acid phosphatase from soybean seeds showed maximum catalytic activity at 60°C. This enzyme lost its complete activity at 68°C after 10 min [9].

Molecular weight determination by Polyacrylamide gel electrophoresis (PAGE) After SDS-PAGE analysis under reducing conditions, single bands were observed for acid phosphatase CAP1 (Figure 8 and table 5) and their apparent molecular weight were estimated to be 66 kDa.

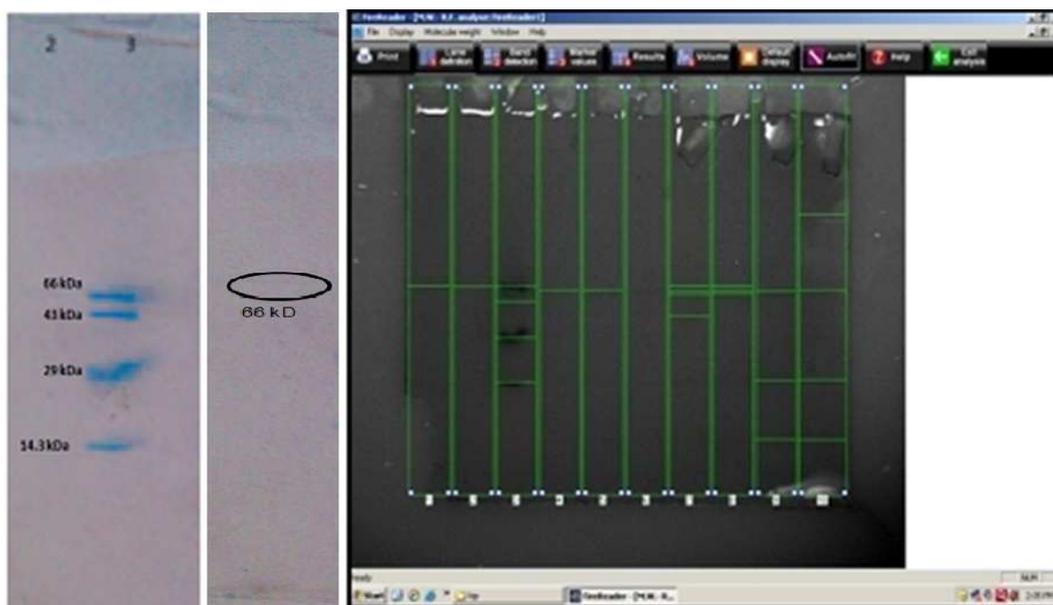


Figure 8 SDS-PAGE of CAP1 Lane 3- Marker Proteins, Lane 2 – CAP1

Table 5 Molecular weight determination of SOIF by SDS PAGE

Molecular weight (kDa)	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
Band 1	67.48	66.00	66.00	59.41	59.41		67.48	67.48	57.79	177.29
Band 2			43.00				57.79	59.41	14.93	59.41
Band 3			29.00				53.10	53.86		15.14
Band 4			14.30				32.36			

Note: B= Band, L = Lane number and kDa = Kilo Dalton, Lane 2- CAP1, Lane 3- Marker Proteins

DISCUSSION

The fresh water *Chara sp.* strain collected from Waghur River at Sakegaon from Jalgaon district, Maharashtra (India). The isolated enzyme screened for the presence of acid phosphatase. The method employed for purification of acid phosphatase was salt precipitation, dialysis, gel filtration and ion exchange chromatography. The purification technique of acid phosphatase showed that the specific activity 11.53 units/mg for CAP1. This value is higher than reported for *Vigna aconitifolia* [10], *Trichoderma harianum* [11] and *Vigna mungao* [4]. Nevertheless, these values are more depressed than acid phosphatase reported from the wheat germ [12] and fresh water algae *Scenedesmus obliquus* [1]. The comparison of biochemical properties of isolated acid phosphatase and referred acid phosphatase given in table 6.

Table 6 Comparison of biochemical properties of isolated acid phosphatase and referred acid phosphatase

Organism name	pH	Temperature	MW (kDa)	Km (mM)	Vmax ($\mu\text{mol}/\text{min}/\text{mg}$)	Activators	Inhibitors	References
<i>Chara sp.</i>	4.0	50	66	0.25	0.012	Fe ³⁺ , Cu ²⁺ , Ca ²⁺ , Mg ²⁺ , Hg ²⁺ and K ⁺	Zn ²⁺	
<i>Scenedesmus obliquus</i>	5.0	50	59.41	0.24	0.020	Hg ²⁺ , Fe ³⁺ , Cu ²⁺ , and Ca ²⁺	Zn ²⁺ , Mg ²⁺ , Hg ²⁺ and K ⁺	1
<i>Pseudokirchneriella subcapitata</i>	5.0	47	--	0.27	0.037	--	Hg ²⁺	13
<i>Rohu Fish Liver</i>	5.0	40	50	0.25	1.1	--	P, F, Va, Mo and Ta	14
<i>Hypericum perforatum L.</i>	5.0	15	57	0.03	9.3	Cu ²⁺	Mo	15
<i>Artemisia vulgaris pollen</i>	5.4	--	73-76	0.16		Tween 20 and Triton X-100	P, Py, F, Hg ²⁺ , Cu ²⁺ and Zn ²⁺	16
<i>Cladophora glomerata</i>	4.5	--	--	0.27	0.037	--	P, Mo, Zn ²⁺ and I	17
<i>Ochromonas danica</i>	4.8	--	--	0.33	--	EDTA	Co ²⁺ , Zn ²⁺ , Hg ²⁺ , Fe ³⁺ , Ar, and Ta	18
<i>Aspergillus niger</i> ITCC 7782.10	4.0	60	33 and 67	0.28 and 0.72	1.21 and 1.35	SDS, Ta and Cu ²⁺	UV, Hg ²⁺ , Co ²⁺ and Fe ³⁺	19
<i>Vigna radiata</i> Seeds	5.5	50	29	0.3	1.33	--	P, Va	20

Ar = arsenate, P = phosphates, Py = Pyrophosphate, F= fluoride, Va = vanadate, Mo = molybdate and Ta = tartarat

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