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ENZYMATIC STUDY OF FRESH WATER MACRO AND MICRO ALGAE ISOLATED FROM JALGAON, MAHARASHTRA

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ABSTRACT

The phenotypic, phylogenetic, geographical diversity and sustainability ability of micro-algae capable of producing enzymes provides a challenge for the accurate determination of biotechnological potential. For production of economically important products like agar, alginates, biodiesel, bio-fuel and medicinally important products algae extensively employed. Although, so lengthily for enzyme production. The modified methods developed to facilitate fast and reliable screening of enzyme production. Experiments carried out on nine macro algae and ten micro-algal strains isolated from Jalgaon region, Maharashtra (India) and two micro-algal strains obtained from BIT Ranchi, an approach found to provide a more reliable measure for enzyme production. Out of 21 algae, 5 strains are positive for lipase, 20 for phosphatase, 06 for amylase, 07 for gelatinase, 05 for urease and 19 for catalase. This is an important escalation for isolation and screening of enzyme production from microalgae.

KEYWORDS: ***microalgae, lipase, phosphatase, urease, amylase and gelatinase***

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1. INTRODUCTION

Production and usage of the enzymes are decisive in the bio-processing industry. Enzyme costs can reduce by finding the optimum conditions for their production [1]. The production of hydrolytic enzymes related to the available substrates in their habitat. Therefore, an augment in the concentration of a meticulous substrate can stimulate organisms to produce enzymes specifically for utilization of that substrate. Due to increase in contamination or pollutions, wastewater contains large amounts of organic matters. This organic matter contains large quantity of complex mixture of polymeric compounds, hydrolyzed by extracellular enzymes [2]. Microorganisms produce enzymes in response to their carbon and energy needs as the soluble hydrolysis products needed for further metabolic activities. Algae is also one of the most abundant and adaptive organism that have developed the ability to grow in diverse habitats. In natural habitat, algae may synthesize different enzymes to deal with organic matter found in water. Extracellular enzymes produced by microorganism and used to break down polymers in the external environment [3,4]. Algae are important organisms in aquatic environments because of their ecological roles, which include biogeochemical cycling of matter (including oxygen generation and nitrogen fixation), contributing to the structural maintenance and biodiversity of aquatic communities and higher living organisms. There have been increasing incidents of algal blooms, and this phenomenon is of economic and ecological concern worldwide. Extracellular enzymes may be an important factor contributing to the success of cyanobacteria in aquatic ecosystems, but the studies on cyanobacteria and microalgae are infrequent. Although it is quite difficult to cultivate, especially, macroalgae in axenic culture and to determine the number of the enzymes produced by that particular algae. The primary aim of this study is to screen fresh water microalgae for the presence of industrially important enzymes like lipase phosphatase, urease, amylase, catalase, laccase and gelatinase. The investigation of extracellular enzymes may in better understanding of nutrient strategy on the aquatic environment. Therefore, this work proposes to investigate the profile of a range of extracellular enzymes of algae. This study intends to contribute to knowledge of extracellular enzymes, green algae produce and how they respond to environmental changes such as reduced key nutrient conditions. The results may lead to the understanding of metabolism of cyanobacteria and microalgae with respect to the breakdown and assimilation of various enzyme substrates. This study undertook experiments on 9 macroalgae and 10 strains of microalgae. Strains cultured in suitable modified medium incorporated with substrate for specific enzymes. The extracellular enzymatic activities measured by detecting their products. The protocols, general experimental conditions and the methods evolved for the detection algal extracellular enzyme described in this report. The analysis resulted helps in biotechnology approaches to improve the uses of algal enzymes in industrial field.

2. MATERIALS AND METHODS

2.1 Collection and cultivation of macro-algae

Algae collected in the sterile container from various sources and brought to the laboratory quickly. Further, it subjected to the standard isolation procedures.

2.2 Isolation and cultivation of microalgae

Algae cultivated by modifying methods used for algal cultivation [5,6,7,8]. Media selected based on results obtained in the laboratory conditions. Macroalgae cultivated in NPK medium [9] in closed and open systems, whereas microalgae cultivated in TAP and Chu's medium [10] in a closed system at room temperature under 3 CFL lights (15 V) 16-8 photoperiod.

2.3 Screening of Algae for enzyme production

The collected algae tested for enzyme production of different enzymes like lipase phosphatase, urease, amylase, catalase, laccase and gelatinase. The enzyme producer dogged by simple method used for microbial enzyme screening [11,12]. The algae inoculated in media having a substrate for respective enzymes, for example palmatic acid, olive oil and Tween 80 for lipase, hydrogen peroxide for catalase, p-nitrophenyl phosphate for phosphatase, and urea for urease, gelatin for gelatinase and starch for amylases. The liquid media used for macroalgae and solid media contain agar used for microalgae screening. To reduce the time period for screening well method used, in which a well prepared in media containing substrate with the help of borer and 0.5µl of microbial culture inoculated in that well.

a) Lipase (E.C. 3.1.1.3)

Lipids are high molecular weight compounds possessing large quantities of stored energy. The two common lipids catabolized by most of the organisms, are the triglycerides (triacylglycerols) and phospholipids. Triglycerides hydrolyzed by enzyme called lipases, into glycerol and free fatty acid molecules. Lipase hydrolyzes the detergent polyoxyethylene sorbitan monooleate (Tween 80) into oleic acid, palmitic acid or polyoxyethylene sorbitol or palmitic acid into palmititae and free fatty acid. Palmitic acid in methanol and distilled water (0.1: 9.9) solution at pH 7.2 inoculated with algal samples contains phenolphthalein indicator and pH adjusted to 8.2. Due to hydrolysis of palmitic acid, free fatty acids produced and thus the pH decreases, hence color changes from pink to colorless between 3 to 6 hours or 1 to 2 days depending upon the ability of hydrolysis.

b) Phosphatase (E.C. 3.1.3.2)

Phosphatase is the enzyme responsible for liberation of inorganic phosphate from organic phosphate esters. The algal samples inoculated in medium contains p-nitrophenyl phosphate and due to hydrolysis of the PNP, p-nitrophenol liberated and color changes from pale

yellow to yellow which is a positive test for phosphatase enzyme.

c) Amylase (E.C. 3.2.1.1)

The algae inoculated in M342 medium, contains1% starch and incubated for two days. After 2 days of incubation at 16-8 photoperiod then 1 ml of iodine solution added, formation of colorless zone around the colonies is the positive test for amylase production. Iodine can't form blue complex with hydrolyzed dextrin and maltose, since color zone around the inoculated organism is positive test for starch hydrolysis.

d) Gelatinase (E.C. 3.4.24.45)

Gelatinase enzyme is responsible for the hydrolysis of gelatin. After 24 hs of incubation, gelatinase containing TAP medium treated with trichloroacetic acid, which precipitate with unhydrolysed gelatin and forms a white color precipitate. The gelatinase producing organisms show a zone of gelatin hydrolysis.

e) Urease (E.C. 3.5.1.5)

Some Organisms able to produce an enzyme called urease that attacks nitrogen and carbon bond, in amide compounds such as urea, forming the product ammonia, CO_2, and water. Urease activity detected by inoculating algal samples in M342 medium, containing 1% urea pH 7.8 and pH indicator such as phenolphthalein. When urea hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in pH causes the indicator to change from colorless to pink at pH 8.2 and is a positive test for urea hydrolysis. Failure to develop pink color is a negative test.

f) Catalase (E.C. 1.11.1.6)

Catalase is an enzyme responsible for hydrolysis of H_2O_2 oxygen and water and protects algae from O_2 toxic products. To determine the catalase producing algae the algae inoculated in M342 and TAP media contains 3% hydrogen peroxide and within 2 min oxygen evolved and bubble formation occurs due to the action of catalase on hydrogen peroxide and the level of catalase present detected.

2.4 Quantitative estimation of enzymes

The primary screening methods validated by the quantitative enzyme assay lipase [13,14,15], acid phosphatase [16], amylase [17, 18], gelatinase [19], urease [20] and catalase [21]. The enzyme activity of all the enzymes is expressed in terms of unit/ml or µmole/min/ml.

RESULTS AND DISCUSSION

3.1 Collection and cultivation of macro-algae

In the present study, the algal samples collected from Tapi river, at Bhusawal site and Hatnur dam sites, Waghur river in Sakegaon site and Waradshim, Girna river at Jalgaon site, lakes like Hartala, Welhala, Maherun, rain water ditches. Forty-eight algae collected from various sources and brought into the laboratory. On the basis of separation achieved by manual method, 9 algae selected on the basis of its purity and subjected to further studies, washed with distilled water for 4-5 times and inoculated in Chu medium till identification. Efforts made to cultivate them in an open and closed system. The selected algae identified by using keys given by Edward and Sigee [22] monographs with the help of phycologist Dr. S.R.Mahajan. The lists of collected algae reported in table 1. The efforts made to cultivate the above-mentioned algae in artificial open tub system and in laboratory a closed system. The macro algae cultivated in algal culture media, Beneck's liquid medium, Baristol medium, Chu 10 media and NPK medium. Out of 9, four *viz*, *Chara, Cladophora, Pithophora* and *Oedogonium* easily cultivated in an open pond system in NPK medium without necessity to maintain to any conditions in an outdoor location. Whereas in closed system some amount of success observed for the preliminary day of incubation. From the algae growth observation in various media used for cultivation, it found that the nutritional requirements of macro-algae less as compared to microalgae, since they show prominent growth in rainwater and NPK medium for a few days. Later on as the macro-algae growth increases, they provide nutrients and suitable environment for the growth of microalgae. As the growth of macroalgae increased, the growth of microalgae also observed in cultures, which in turn resulted in reduced growth of macroalgae. While cultivating macroalgae, large amount of epiphytic growth observed on macroalgae. It may be due to the microbial association between epiphytes and macro algae. Obtained results conclude that the reduction in macroalgae growth may be due to the increase of microalgae contamination. The open pond system has plenty of disadvantages over the closed pond system since; it is quite difficult to maintain physical parameters essential for the growth of macroalgae. Similarly, micro algal contamination easily prevented in closed systems. The system and media used for cultivation of macro-algae in open and closed system noted in table 1. This macro alga does not contain only microalgae epiphytes other than also plenty of fungi and bacterial contamination detected in culture flask. Since, further efforts to isolate epiphytic microalgae from cultivated macroalgae cultures.

Table 1
Sample, Name and location, Success rate of Cultivation of Macro-algae in NPK medium

Sr.No.	Algae	Location	Cultivation system	SR
1.	*Cladophora*	Waghur river, Sakegaon	Closed and open	H
2.	*Enteromorpha*	Tapi river, Bhusawal	Closed	N
3.	*Hydrodictyon*	Tapi river, Bhusawal	Closed and open	N
4.	*Mougeotia*	Waghur river, Sakegaon	Open	N
5.	*Oedogonium*	Waghur river, Wardshim	Open	N
6.	*Pithophora*	Waghur river, Sakegaon	Closed and open	H
7.	*Spirogyra*	Tapi river, Bhusawal	Closed	N
8.	*Chara sp.*	Waghur river, Sakegaon	Open	H
9.	*Nitella*	Waghur river, Sakegaon	Closed and open	H

SR = Success rate, H = High, N= Negligible

3.2 Isolation and cultivation of microalgae from macro algae cultures

Micro-algae isolated from, cultivated macro-algae and inoculated in TAP medium, Chu's medium and bold basal medium. Micro-algae purified by strike plate technique in axenic culture and named as S1 to S10 (table 2). Meanwhile, two pure cultures of microalgae obtained from BIT, Ranchi and denoted as RB1(*Chlorella sp.*) and RB2 (*Chlamydomonas sp.*). During isolation of microalgae by strike plate techniques most of them show optimum growth on TAP medium. Therefore, TAP medium used to cultivate microalgae with some microbial contaminants. To prevent the microbial contaminants, various antibacterial and antifungal agents used. The isolated microalgae cultivated in TAP medium and maintained by sub culturing them for 2 months. Several literature report have already indicated that algae has high nutritive value as these cultures may act as favorable nutrient medium for the growth of microorganisms. To justify the above statement, similar work observed on the effect of red and green algal extract on hyphal growth of *Arbuscular mycorrhizal* fungi, and micorrhizal development and growth of Papaya and Passion fruit [23]. Therefore, to find out the enzyme producing algae so it is necessary to remove the contaminants from culture. To get rid of these problems, the antibiotic concentrations standardized to reduce the growth of contaminants and flourishing growth of desired algae.

Table 2
Isolated algae with their occurrence and growth on artificial medium

Sr.No.	Abbreviation (Species)	Media used for cultivation	Location
1.	S1 (*Chlorella sorokinian*)	TAP	Waghur river
2.	S2 (*Chlamydomonas sp.*)	TAP	Waghur river
3.	S3	TAP	Waghur river
4.	S4	TAP	Tapi river
5.	S5	TAP	Tapi river
6.	S6 (*Scenedesmus obliquus*)	TAP	Tapi River
7.	S7	TAP	Waghur river
8.	S8	TAP	Tapi river
9.	S9	M342	Waghur river
10.	S10	TAP	Tapi River
11.	RB1 (*Chlorella sp. CB4*)	TAP	BIT, Ranchi
12.	RB2(*Chlamydomonas sp.CRP7*)	TAP	BIT, Ranchi

3.3 Screening of algae for enzyme production

The selected macroalgae and microalgae isolates screened for the presence of the activities of industrially important enzymes by adding their substrates to the above-mentioned media. Enzymatic studies revealed that collected algae contained few of these substances and the results tabulated in Table 3. Out of 21 tested algae, 6 are positive for amylase, 5 for lipase, 20 for phosphatase, 5 for urease, 7 for gelatinase and 19 for catalase. From above tested algae the microalgae enzymes more accurate than macro algal enzymes, because they are free from other contaminations. The macro algal culture contains a large share of algae since, the enzyme activity considered for macro algae activity. Further, the macroalgae activity validated by the quantitative enzymatic assay.

a) Lipase

Lipase enzyme is responsible for the hydrolysis of triglycerides and algae are rich sources of triglycerides. Out of 21screened algae 5 are lipase positive for palmitic acid hydrolysis, *Chara, Hydrodictyon* and *Spirogyra*, S1, S6, (Figure 1), whereas, in literature only a single report is obtained. Lipase purified and characterized for the first time except *S. platensis*. The lipase purified about 375-fold with a specific activity of 45 U/mg protein. The molecular weight and pI of purified lipase found as 45 kDa and 5.9, respectively. *S. platensis* lipase is monomeric and specifically for 3-position in the ester bond [24].

b) Phosphatase

Phosphatase enzymes believed to have an essential function in nutrient dynamics in aquatic habitats since they promote the degradation of complex phosphate compounds into orthophosphate and organic moieties. Intended for phosphatase, after 4 hs of incubation, 20 algae exhibited phosphatase positive and 1 *Spirogyra* phosphatase negative (Figure 2). Elsewhere, the phosphatase enzyme isolated and purified from *Pseudokirchneriella subcapitata* [25], *Microcystis aeruginosa* and *Chlorella pyrenoidosa* [26], *Stigeoclonium tenue* [27], *Cladophora glomerata* [28], *Ulva lactuca* [29] and *Ochromonas danica* [30].

Table 3
Screening of macroalgae and microalgae for enzyme production

Algae	Enzymes					
	Lipase	Phosphatase	Amylase	Gelatinase	Urease	Catalase
Chara	++	++	-	+	+	++
Hydrodictyon	+	+	-	++	-	-
Enteromorpha	-	+	-	-	+	++
Nitella	-	++	-	-	-	-
Spirogyra	+	-	+	++	-	+
Cladophora	-	+	-	-	-	+
Pithophora	-	+	-	-	-	+
Mougeotia	-	+	-	-	-	+
Oedogonium	-	+	+	+	-	+
S1	+	++	+	-	-	+
S2	-	+	-	-	-	+
S3	-	+	+	-	+	+
S4	-	+	-	-	-	+
S5	-	+	-	-	-	++
S6	++	+++	+	+	-	+
S7	-	+	+	-	-	+
S8	-	+	-	-	+	+
S9	-	+	-	-	-	+
S10	-	+	-	-	+	+
RB1	-	+	-	++	-	+
RB2	-	+	-	++	-	+

(++ highest enzyme activity, + moderate enzyme activity, - negative test for enzyme activity, tremendous enzyme activity and enzyme activity differentiated on the basis of time for substrate utilization or hydrolysis and intensity of indicator dye.)

c) Amylase

The presence of amylase determined after 2 days of incubation in the presence of 16-8 photoperiod. Out of the 21 tested algae, 15 algae are negative for amylase except *Spirogyra, Oedogonium*, S1, S3, S6 and S7 (Figure 3). What important to note is that instead of autotrophic nature this algae synthesize amylase. Other strains do not produce amylase. Amylase is least metabolic essential enzymes that found in algae, because they are autotrophic. Although, starch metabolizing enzymes like 2 forms of amylase and 2 forms of α-glucan phosphorylase identified in crude extracts of the unicellular green alga *Dunaliella marina* [31] and α-amylase from marine algae *Stoechospermum marginatum* [32]. Cold adapted amylase and protease also isolated from new *Streptomyces* 4 alga Antarctic Strain [33].

d) Gelatinase

The gelatin liquefaction or zone of hydrolysis, which produced due to the hydrolysis of gelatin (Figure 4) observed in 7 strains of algae. Out of 21 selected algae 4 macroalgae (*Chara, Hydrodictyon, Spirogyra* and *Oedogonium*) and 3 microalgae (S6, RB1 and RB2) are positive for gelatinase and the other gelatin negative. No literature recorded observed on the gelatinase activity of algae. Probably, we are the first group to report the presence of gelatinase in algae.

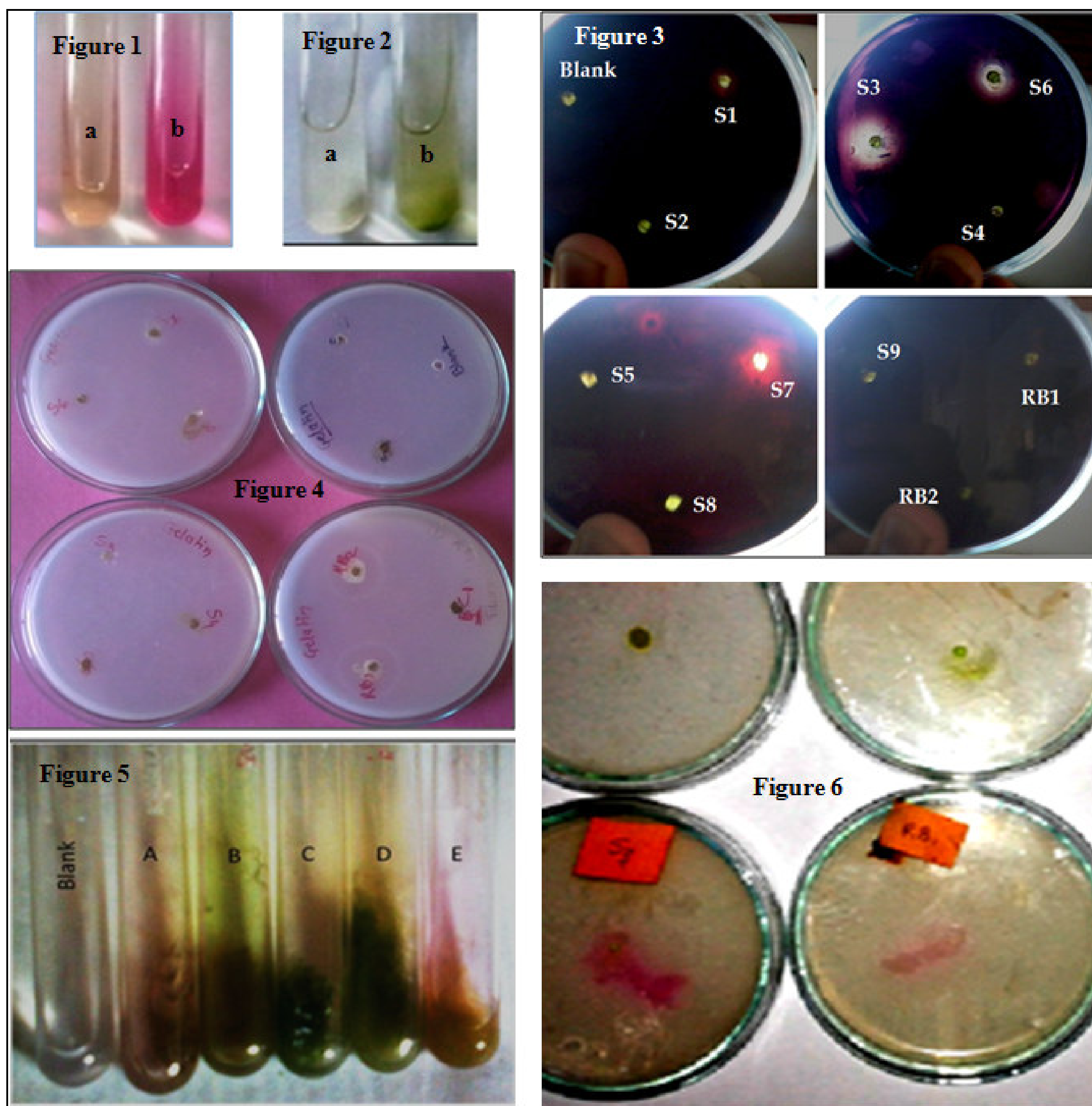

Figure 1

Screening of Lipase producing microalgae (a) S6 showing positive test for lipase production and (b) S7 showing negative test for lipase production. Figure 2: Screening of microalgae for production of phosphatase (a) blank represent negative test for phosphatase production and (b) S1 showing positive test for phosphates production. Figure 3: Screening of microalgae for amylase production (S1, S3, S6 and S7 are positive for amylase production). Figure 4: Screening of microalgae for Gelatinase production (S6, RB1 and RB2 are positive for gelatinase production). Figure 5: Screening of macroalgae for urease production, (Chara and Enteromorpha are positive for urease). Figure 6: Screening of microalgae for urease production (S3, S8 and S10 are positive for urease).

e) Urease

The algae use urea as nitrogen source and used for metabolic reaction. Urease is the enzyme responsible for the hydrolysis of urea. After 2 days of incubation 2 macroalgae (*Chara* and *Enteromorpha*) (Figure 5), and 3 microalgae S3, S8 and S10 (figure 6) are urease positive and remaining 16 algae urease negative. The presence of urease as urea-hydrolyzing enzyme in *A. doliolum* and *A. nidulans* [34], as reported in blue-green algal species *Phormidium luridum*, *Plectonema calothricoides* [35], *Aphanocapsa* 6308 [36], and *Spirulina maxima* [37].

f) Catalase

Catalase is one of the essential or general enzymes in algae. In the studies three *Chara*, *Enteromorpha* and S5 are highly catalase positive, while other are least catalase positive. However, the *Hydrodictyon* and *Nitella* are catalase negative. The first report on algal catalase

determined in blue green algae [38]. Seasonal variation in catalase activity of *Rhizoclonium riparium, Enteromorpha intestinalis, Lola capillaries* and *Ulva lactuca, Catenella repens, Polysiphonia mollis* and *Gelidiella acerosa* in salinity of Sunderban estuary, India [39]. A Heterotetrameric catalase isolated and purified from a desiccation tolerant Cyanobacterium *Lyngbya arboricola* [40]. Similarly, catalase activity determined in *Pseudokirchneriella subcapitata* [41] *Galaxea fascicularis* [42].

3.5 Quantitative estimation of enzymes

The obtained results, matches with the outcome of primary screening, which authenticate the primary screening methods, except in case of S1 phosphatase activity.

Table 4
Quantitative estimation of enzymes of macroalgae and microalgae for enzyme production

Algae	Enzyme activity (Units/ml = µmole/min/ml)					
	Lipase	Phosphatase	Amylase	Gelatinase	Urease	Catalase
Chara	1.98	3.74	ND	0.94	39.0	0.254
Hydrodictyon	0.07	1.47	ND	1.72	ND	0.51
Enteromorpha	ND	1.11	ND	ND	27.0	0.236
Nitella	ND	3.21	ND	ND	ND	ND
Spirogyra	0.21	2.51	0.042	1.59	ND	0.63
Cladophora	ND	1.87	ND	ND	ND	0.71
Pithophora	ND	1.68	ND	ND	ND	0.54
Mougeotia	ND	1.42	ND	ND	ND	0.78
Oedogonium	ND	0.97	0.035	0.91	ND	0.49
S1	0.13	0.41	0.027	ND	ND	0.91
S2	ND	2.65	ND	ND	ND	0.63
S3	ND	1.19	0.013	ND	25.0	0.54
S4	ND	2.59	ND	ND	ND	0.75
S5	ND	2.96	ND	ND	ND	0.221
S6	2.42	4.26	0.009	1.07	ND	0.97
S7	ND	0.88	0.014	ND	ND	0.11
S8	ND	0.67	ND	ND	19.0	0.84
S9	ND	0.92	ND	ND	ND	0.36
S10	ND	0.93	ND	ND	15.3	0.99
RB1	ND	1.09	ND	1.67	ND	1.02
RB2	ND	0.88	ND	1.51	ND	0.45

ND= not detected

3. CONCLUSION

A comprehensive solution is to develop simple and rapid method for screening of algae for enzyme production. The aim of developing simple method for enzyme screening, such information is essential to understand the metabolic processes of fresh water algae. The overwhelming conclusion that emerges from these studies is that algae also have potential of producing different enzymes on basis of substrate availability and used for production of industrially important enzymes for applied uses. However, previous studies had not shown the diversity of algae with respect to enzyme production.

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