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STANDARDIZATION OF pH FAVOURING AUGMENTED GROWTH AND BIOMASS PRODUCTION OF FRESH WATER MICRO ALGAL SPECIES OF *Coccomyxa,* IN BOLDS BASAL MEDIUM

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ABSTRACT

The production and maintenance of axenic cultures of desired microalgae, the renewable source of diverse groups of compounds, seems to be a hectic effort. Recognizing the ability of *Coccomyxa* species to accumulate and produce high value added products such as β -carotene and luetin, the culturing of the members of genus has attained commercial interest. Researchers report that modification in the culture condition can result in pure cultures of desired species and thereby reduction in the expenditure associated with microalgal cultivation. However no such reports are available on the optimum growth conditions of *Coccomyxa* species, especially for culturing them in Bolds Basal (BB) medium. The present work has been attempted with the intention of finding out the optimum pH favouring the augmented growth of *Coccomyxa* in BB medium. For fulfilling this target, treatment sets were maintained in triplicate with control and treatment sets in which the pH of culture medium was adjusted from 3.0 to12.0, with a gradation of 0.5. The magnitude of changes like pH, temperature, conductivity and resistivity of the culture medium and the growth attributes of micro algal species like cell count, cell size, turbidity and biomass were worked out daily for a period of 1 week. The optimum pH favoring increased growth of *Coccomyxa* species was noted to be in acidic range (pH 4 -4.5).

KEYWORDS: *Coccomyxa*, pH, Bolds Basal Medium.

Microalgae are a group of photosynthetic organisms seen in heterogeneous habitats. The unique properties like higher photosynthetic efficiency, rapid multiplication rate and nominal nutritional requirement make them a promising tool for multi oriented research activities. Several R & D efforts were carried out worldwide for exploiting the potentialities of microalgae, which include extraction of bioactive compounds for pharmacy, food and feed industry, waste water treatment, pollution control, carbon sequestration etc. Moreover the biomass from these processes serves as an ecofriendly feed stock for biofuel production and also for the generation of a wide range of other valuable products.

Risk of developing cultures without contamination is one among the major constraints in initiating research using microalgae. Lack of sufficient light intensity, proper aeration and mixing and contamination due to cyanobacteria, bacteria etc. are some of the difficulties faced during micro algal cultivation. Moreover few micro-algal members tend to dominate in culture systems, in spite of the desired microalgae present in the inoculum and to this category include species of *Chlorella, Dunaliella, Botryococcus, Scenedesmus*etc.

Many research activities are undertaken to develop protocols for large scale cultivation of micro algal members, including open ponds and closed photobioreactors. As the growth preferences of microalgal members vary in accordance with species characteristics, a unified protocol for the mass multiplication of micro algae is inappropriate. It is reported that species specific modification of culture conditions can contribute to the multiplication of micro algal members to a greater extent (Moheimani,2012 and Khatoon *et al*. 2014).

The environmental factors such as pH, temperature, light, mixing regime and nutrients have marked influence on the photosynthesis and cellular metabolism of microalgae. Beherens (1996) pointed that modification of these parameters can have profound influence on biomass production, fatty acid content and production of other bioactive metabolites. However the optimal and tolerated ranges of parameters tends to be species specific and may vary. pH, the hydrogen ion concentration of a solution directly or indirectly affects the enzyme activity and metabolic processes of organisms associated with it(Juneja *et al*. 2013). Although all micro algal members show excellent growth at the neutral ranges, the optimum pH at which maximum growth occurs may vary (Ying *et al*. 2014).Richmond and Becker (1986) stated that subjecting the microalgal culture to a temporary alteration of environmental factors such as pH can decrease unwanted organisms and can improve the culture densities. Several researchers reported the same

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(Visviki and Santikul;2000,Moheimani,2012,Al-shatri *et al.*2014, Khatoon *et al*.2014).

The *Coccomyxa* species cultured in BB medium (pH 6.6) was highly prone to contamination from other microalgal species. This has also hindered the production of biomass in adequate quantities for continued research. As no references are available on the optimized growth of *Coccomyxa* sp. in BB medium, the present study is undertaken with an objective to evaluate their growth performances in BB medium at varying ranges of pH. This has been attempted not only for overcoming contamination, but also for maximizing biomass production and thereby ensuring their utilization for various value added purposes.

MATERIALS AND METHODS

For the study, treatment sets were maintained in triplicate with 50 ml each of BB medium taken in 100 ml sterilized conical flasks. Using 0.1 N NaOH and 0.05 N HCl, the pH of culture medium was adjusted from 3.0 - 12.0, with a gradation of 0.5.After adjusting the pH, 5.0 ml each of pure cultures of *Coccomyxa*, which were maintained in extreme aseptic conditions, were added to respective conical flasks. Control sets were also maintained in original pH of the culture medium (pH 6.6).The entire treatment sets were kept under illumination during day time. The parameters like pH, temperature, conductivity and resistivity of the culture medium and the growth attributes like turbidity, cell count, cell size and biomass associated with micro algal members in the treatment sets were monitored. Every day after observation, the altered pH of the culture medium was readjusted. The biomass estimation was carried out on the initial and final days of treatment. Monitoring of the treatment sets were carried out for a period of 1 week. The median values of triplicate data were taken for compilation of the results.

RESULTS AND DISCUSSION

Results of turbidity, number of cells and biomass of *Coccomyxa* species in response to different ranges of pH are given in Tables 1a – c. The changes associated with turbidity, cell count and biomass of the microalgal members under different ranges of pH are represented in.

In the present work, throughout the treatment period, the pH showed a tendency to move towards the neutral range from the pre adjusted range. This is indicative of the adaptation of the micro algal members to survive in modified environments by altering the pH to neutral levels. Dubinsky *et al.*(1974) stated that the algal growth leads to obvious changes in culture medium, either by increasing the lower pH or decreasing the higher pH. Al-shatri *et al.* (2014) pointed that the pH variation observed in the culture medium may be due to the nature of the metabolities secreted by microalgae in response to the nutrients in culture medium. Hence like other microalgal members, *Coccomyxa* species also showed the tendency to alter the pH to neutral levels in culture conditions. In the control set, the pH value ranged from 6.7 on first day to 6.74 on the seventh day.

Upon comparison of the data pertaining to the temperature (^{0}C) of the culture medium, higher temperature was noticed on the fifth and sixth day (30.9) and lower temperature on the second day of treatment (29.0). Fernandez *et al*. (2013) reported that the optimal temperature range of freshwater microalgae is 25-35^{0}C and can also tolerate temperature up to 40^{0}C for short period. Temperature range of the culture observed during the study was almost within the reported ranges to sustain the growth of micro algal members.

As a major growth parameter, the maximum turbidity (NTU) was observed in pH 5(6.9)on seventh day and the minimum was recorded in pH 3(1.4) on first day. In control set, turbidity ranged from 1.2on first day to 5.9 on the seventh day. Throughout the treatment set, at higher alkaline pH ranges, especially above pH 9 and 10, the formation of precipitation was noticed and as a result higher turbidity values were obtained. There are reports regarding precipitate formation in the microalgal cultures, if pH exceeds value of 9 (Sirisansaneeyakul *et al*. 2011). While compiling the results of major growth parameters like cell count, turbidity and biomass for suggesting optimum pH at which maximum growth attained, the pH ranges above 8.5 was discarded in the present study.Table.1

The estimation of cell count ($\times 10^4$ cells/ml)at regular intervals provide information regarding to the growth status of microalgae under varying treatment conditions. Here the maximum cell count was noticed in acidic pH 5.5 on seventh day (27.5) and minimum in pH 12 on the seventh day (4.25). The cell count in control set ranged from 11.25on the first day to 22.5 on the seventh day. Gross (2000) worked on the ecophysiology of algae growing in acidic environments and stated that the low

external pH condition necessitates the microalgae to expend energy to maintain neutral pH in the cytosol, by means of suitable biochemical systems, which can resist the proton gradient across the plasma membrane for cell function. Guckert and Cooksey (1990) worked on triglyceride accumulation and fatty acid profile changes of *Chlorella* CHLORI using BB medium in alkaline pH ranges (6.7 to 10.4).During the morphological observations on pH 10-10.4,they noticed that in the autosporangial stage, the flexibility of the cell wall of mother cells increased, which prevents the rupturing of the cells and inhibits autospore release and thus increases the time for cell cycle completion and as a result lower cell count in alkaline ranges. Similar observations were also noticed by Malis-Arad and MC Gowan (1982 a,b). Here in the treatment set also lower cell counts were observed in higher alkaline ranges.Table.2

Table 1: Comparison of the turbidity difference noticed in cultures of *Coccomyxa* sp.

Experimental set	1st Day	2nd Day	3rd Day	4th Day	5th Day	7th Day	8th Day	Median
Control	1.2	1.6	1.4	5.1	5.8	5.4	5.9	5.1
pH-3	1.4	1.4	1.8	5.2	6	5.1	6.2	5.1
pH-3.5	1.8	2	1.9	5.1	6.1	5.1	6.1	5.1
pH-4	1.6	1.7	1.8	5.3	6.3	5.2	6.6	5.2
pH-4.5	2.3	1.9	2	5.1	6.3	5	6.7	5
pH-5	2	2	2.2	5.3	6.4	5.1	6.9	5.1
pH-5.5	2	1.8	2.2	5	5.8	4.9	6.7	4.9
pH-6	2.2	2	2.2	5	5.8	5	6.4	5
pH-6.5	2.3	1.9	1.9	5	6	4.8	6.2	4.8
pH-7	2.3	1.2	2.3	5.4	6	4.7	6.2	4.7
pH-7.5	2.2	1.8	2.3	5.5	6.2	4.8	6.5	4.8
pH-8	2.2	1.4	2	5.4	6.2	5.1	6.3	5.1
pH-8.5	2.2	1.8	1.8	5.1	6.1	5	6.5	5
pH-9	2.2	1.8	2.4	5.8	6.5	5.1	6.5	5.1
pH-9.5	2.4	2.8	3	5.9	6.6	5.2	6.7	5.2
pH-10	3	3.3	3.3	6	6.8	5.5	6.5	5.5
pH10.5	3	3.6	3.3	5.8	6.1	5.5	6.3	5.5
pH-11	3.1	3.4	2.8	5.8	6.5	5.2	6.3	5.2
pH-11.5	3.6	3.5	2.9	5.9	6.5	5.3	6.4	5.3
pH-12	4.4	3.9	3	6.1	6.6	5.3	6.6	5.3

The shrinkage or abnormal increase in the cell size of the microalgae owing to the addition of 0.1 N NaOH and 0.05 N HCl for maintaining in particular pH in the treatment sets has been monitored through micrometry (µm).Visviki and Santikul (2000) while working on the effects of hydrogen ions on the growth and ultra-structure of *Chlamydomonas applanata* under varying ranges of pH (1.4 to 8.4) noticed excessive mucilage production, abnormal cell division and cell death in pH 3.4and large single cells than the controls cells with increased pyrenoidal volume, thicker cell walls in pH 4.4.Here in the treatment sets in all the pH ranges, including the extreme acidic and alkaline ranges, no significant decrease or abnormal increase in cell size was observed. Furthermore the cell size cannot be taken as a major parameter of growth as the undivided cells are likely to appear in large size, which will then be subjected to division and multiplication and the newly formed cells as a result of division will appear to be small (Menon and Harilal; 2016).

Conductivity (µS) is directly related to the amount of dissolved ions present in the culture medium. During the treatment period, maximum conductivity was observed at pH 12 (4899) and the minimum at pH 6 (796.9).Conductivity increase in control set has ranged from 801on the first day to 819 on the seventh day. From the initial day to final day of treatment, it was observed that the conductivity value had a rise more rapidly in all treatment sets especially in higher acidic and alkaline ranges than the control set. This rapid increase in the conductivity values can be due to the increased

concentrations of ions in the culture medium by the addition of 0.1 N NaOH and 0.05 N HCl to maintain the particular pH ranges.

Table 2: Comparison of the cell count difference noticed in cultures of *Coccomyxa* sp.

Experimental set	1st Day	2nd Day	3rd Day	4th Day	5th Day	7th Day	1st Day	Median
Control	11.25	12.5	13.5	16.75	17.5	15.5	22.5	15.5
pH-3	7	9.75	10	16.75	16.5	21.5	21.5	16.5
pH-3.5	5.5	9.25	14.5	20.25	18	10.25	23.25	14.5
pH-4	7	9.75	13.25	18.5	16.25	19.75	23.5	16.25
pH-4.5	10	12.5	11.75	18.5	19	25.75	27	18.5
pH-5	9.75	7.25	13	21.75	14.5	24.5	26.5	14.5
pH-5.5	8.25	6.5	10.25	17.25	15.25	23	27.5	15.25
pH-6	8.25	6.5	12.25	16.5	14.5	17.5	24.75	14.5
pH-6.5	12.5	12.75	10.5	20.75	19	15.25	23.25	15.25
pH-7	11.5	11.5	11.25	16.75	15.5	19	23.25	15.5
pH-7.5	9	9	8.25	16	19	19.75	23.75	16
pH-8	10.25	8.25	12.25	15.75	19.25	21.5	19.75	15.75
pH-8.5	10.5	10.75	12	13.75	16.25	21.25	17.75	13.75
pH-9	12.75	10.5	11.75	13.5	12.75	11.75	10.5	11.75
pH-9.5	10.25	10.75	10.75	9.5	12.5	10.75	10.25	10.75
pH-10	7.5	11.25	11.75	9	9.75	9.75	9.25	9.75
pH10.5	10	10	11	8.75	9	9.25	5	9.25
pH-11	8.75	9.5	10.75	8	7.25	7.75	5.75	8
pH-11.5	8	9.5	9	10	5.75	6.5	5.5	8
pH-12	8	8.5	10.75	11.25	5.25	6	4.25	8

The maximum resistivity (kΩ) was observed in pH 6 (1.23) and6.5 (1.23) on the first day and minimum in pH 12(0.2) on the seventh day. The resistivity of the control set ranged from 1.22 on the first day to 1.19 on the seventh day. Resistivity of the cultures were found to be high in neutral ranges and low at high alkaline ranges, which are noted to be in accordance with conductivity in all treatment sets of microalgae maintained at varying pH.

In the present study, the maximum biomass value (gm) was noticed in pH 4(0.022) and minimum in pH 6.5(0.01).In the control set, biomass obtained was 0.009.The treatment sets above pH 9showed precipitate formation. Throughout the experimentation, the biomass estimated from the treatment sets above pH 9 was high, compared to other pH ranges and is mainly due to precipitate formation. The growth was also reduced in progressive days of the treatment sets, evidenced by low cell count and the white coloured precipitate formation.Table 3.

Table 3. Comparison of the biomass difference noticed in cultures of *Coccomyxa* sp.

Experimental set	Median	Experimental set	Median
Control	0.009	**pH-7.5**	0.014
pH-3	0.012	**pH-8**	0.014
pH-3.5	0.017	**pH-8.5**	0.018
pH-4	0.022	**pH-9**	0.024
pH-4.5	0.014	**pH-9.5**	0.038
pH-5	0.011	**pH-10**	0.03
pH-5.5	0.014	**pH10.5**	0.049
pH-6	0.011	**pH-11**	0.048
pH-6.5	0.01	**pH-11.5**	0.038
pH-7	0.018	**pH-12**	0.052

Various literatures recommended that the maximum algal growth occurs around neutral pH (pH 7 to7.6) and the optimum pH was the initial culture pH at which microalgae adapted to grow (Visviki and Santikul;2000 and Hansen, 2002).Rai and Rajashekhar (2014) also stated that microalgal members preferred near neutral to alkaline pH. Vaquero et al. (2012) isolated *Coccomyxa onubensis* from the acidic environment having low pH (between 1.7 and 3.1) and cultured in the pH 2.5 in the k9 medium having chemical composition based on the natural environment for copper mediated biomass enhancement and luetin production. Casal et al. (2011)worked on *Coccomyxa acidophila* in acidic medium (pH < 2.5) and noticed that acidophile microalgae growth rates will be low when compared to other common microalgal members, at the same time the acidic condition prevents the contamination owing to un desired microorganisms. Apart from these observations, in the present study, the growth rate of *Coccomyxa* species was higher in the acidic ranges while compared to the neutral and alkaline ranges.

For confirmation of the suitable pH range in which maximum growth and multiplication of *Coccomyxa* occurred, the median values of triplicate data for a period of seven days pertaining to cell count, turbidity and biomass were analyzed and represented (figures 1a-1b).Upon analyzing major growth parameters, *Coccomyxa* species showed highest cell count in pH 4.5 and turbidity and biomass in pH 4,which indicates the optimum pH requirements of *Coccomyxa* for augmented growth ranges from 4–4.5 in BB medium.

The selection of the suitable candidate that can withstand extreme acidic pH is an essential prerequisite for carbon sequestration efforts, wherein the influx of carbon dioxide/flue gas changes the pH of the culture medium to acidic ranges. Thus the ability of *Coccomyxa* species to withstand and reproduce in acidic conditions can be effectively utilized for micro algae based carbon sequestration programmes.

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