Antibiotic sensitivity assay for *Spirulina*: In relation to marker selection for genetic improvement

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Accepted : March, 2009

SUMMARY

Spirulina is a model organism in mass and outdoor cultivation of algae biomass as a source of protein, chemicals and nutrition. There is urgent need to improve food quality of *Spirulina* for human consumption. It's a need to develop selectable marker system for genetical improvement to introduce new genes. For this purpose the sensitivity of *Spirulina platensis* was tested against 5 different antibiotics (Kanamycin, Streptomycin, Ampicillin, Hygromycin and Chloramphenicol) with varying concentration of 25µg/ml to 1600µg/ml. *S. platensis* showed sensitivity to all antibiotics but maximum inhibition was found with Chloramphenicol. Thus the Chloramphenicol will be best marker for selection for further studies and the chlorophyll *a* concentration for sensitivity assay.

Key words : Spirulina, Transformation, Antibiotic sensitivity, Chloramphenicol

Spirulina is a multicellular, filamentous, unbranched, helical cyanobacterium and belongs to family Oscillatoriaceae with a length of 200- 300µm and a breath of 5-10 µm. *Spirulina's* nutritional qualities are truly "one-of-a-kind", with its structure consisting of nearly 71 per cent of total protein. *Spirulina* represents the highest natural source of protein ever discovered which is superior to all standard plant protein, such as that of legumes (Ciferri, 1983; Babadzhanov *et al*., 2004).

There have not been any approaches available related to the reproducible and stable gene transfer system for *Spirulina* platensis (Vacchhani and Vonshak,1997). This situation blocks the development of new strains and new utilization of this economic species through biotechnology. Introducing a selectable marker gene helps to screen transformants. However, the selectable marker assay for suitable gene transfer in *S.platensis* has not been properly documented. In the present study, the sensitivity of *S.platensis* to 5 antibiotics was examined in order to pick out one or more suitable selectable markers for further gene transfer of this alga with suitable sensitive assay method.

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MATERIALS AND METHODS

Growth and maintenance of strain:

Selected *Spirulina* strain was grown and maintained in Zarrouk medium(Zarrouk,1966), modified by Ogawa and Terui (1970).The cultures were allowed to grow at 25±1ºC under continuous illumination by using cool, white fluorescent tubes with approx light intensity of 3000 Lux, above the surface of culture vessel.

Antibiotics treatment:

5 days old actively grown culture was taken and transferred in 50ml fresh medium containing antibiotics (Kanamycin- 200, 400, 800, 1600 µg/ml ; Streptomycin- 100, 200, 300, 500µg/ml ; Ampicillin- 100, 200, 300, 500µg/ml; Hygromycin- 50, 100, 200, 400µg/ml ; Chloramphenicol- 25, 50, 100, 200µg/ml) with 5µg/ml concentration of chlorophyll a. It is then incubated under continuous illumination at 27± 2^{0}C. Samples were used for further studies from 0 to 5 days. Assay run in triplicates.

Estimation of protein :

Protein content was estimated by the method of Lowry *et al.* (1951) with slight modification by Singh and Singh (1997). To 0.5ml of cell suspension, 0.5ml of 1 N sodium hydroxide was added and mixture was placed in boiling water bath (100^{0}C) for 10 min. Mixture was allowed to cool before the addition of 2.5ml of reagent (it contains 50ml of 5% Na_2CO_3, 1ml of 1%Na-K tartarate and 1ml of 0.5% $CuSO_4.7H_2O$). This mixture was thoroughly shaken and left at room temperature for 10-15min. Then 0.5ml of 1N Folin-ciocalteau reagent was added and mixture was centrifuged. Supernatant was taken and after 15 min. of color reaction, color intensity was read

at 660nm in a spectrophotometer (Cary 5000, Australia). Amount of protein was estimated with standard calibration curve prepared by using BSA (5µg/ml -100µg/ml).

***Chlorophyll extraction*:**

Chlorophyll was extracted by method of Kratz and Myers(1955). 2ml of culture was taken and centrifuged at 6000 rpm for 2 min. Pellets were taken and mixed with 5ml of methanol. The mixture was heated at 60ºC in waterbath for 2-3 min. Then it was centrifuged at 6000 rpm for 2 min, supernatant was collected and O.D. was taken at 665nm.Chlorophyll a concentration was calculated using factor 12.5.

Measurement of photosynthetic pigments from whole cell scan:

The cells were scanned daily (350-750nm) in a UV-visible spectrophotometer (Cary 5000, Australia) by using light path of one centimeter. 3ml of suspension was used every time for recording of the absorption spectra. A quantification of the photosynthetic pigments was done by using the formula of Astier *et al*. (1979) as mentioned below-

Phycocyanin= $(0.16\times A_{622}-0.06\times A_{678})\times 10^{-3}$µg/ml

Chlorophyll *a*= $(14.5\times A_{678}-0.56\times A_{622})10^{-3}$µg/ml

β-Carotenoid= $(7.6\times A_{480}-3.6\times A_{510})\times 10^{-3}$µg/ml

Ratio of phycocyanin to chlorophyll a (PC/Chl a) was calculated.

RESULTS AND DISCUSSION

Fig. 1, 2 and 3 clearly indicate that all the antibiotics added in the culture showed the growth in terms of chlorophyll *a*, phycocyanin and protein inhibited, indicating that the antibiotics are toxic for *Spirulina*. Out of all antibiotics the chloramphenicol was most effective even at very low concentration of 25 µg/ ml in all cases.

From the protein concentration data at day 5th this inhibition varied depending upon each antibiotic, kanamycin (200-1600 µg/ml), streptomycin (100-500 µg/ml), ampicillin (100-500 µg/ml), hygromycin (50-400µg/ml) and chloramphenicol (25-200µg/ml).

Kanamycin, streptomycin and ampicillin did not exhibit potent suppression on protein concentration fig 1(a,b,c) whereas hygromycin and chloramphenicol antibiotics showed almost complete inhibition at such a low concentration of 50 µg/ml and 25µg/ml Fig. 1(d,e) was 82% and 81%, respectively and was significant. The per cent decline in chlorophyll and phycocyanin concentration was higher in chloramphenicol than the hygromycin Fig. 2 and 3.

Loss in chlorophyll concentration was faster than the phycocyanin. Chlorophyll synthesis and photosynthesis were first affected by antibiotics. These results showed that *Spirulina* was sensitive for hygromycin and chloramphenicol antibiotics.

Kanamycin resistance is one of the most frequently used selectable markers for obtaining transgenic plants (Nap *et al.*, 1992) and the generally applied concentration is between 200-1600 µg/ml. It was also reported in case of *Synechocystis* spp. PCC 6803 (Metz *et al.*,1989), *Synechococcus* spp. PCC 7492 (Vander *et al.*, 1990). Kanamycin, neomycin are aminoglycosidic antibiotic, can be modified and then inactivated by neomycin phosphotransferase II (NPT II), a kind of aminoglycoside-modifying enzyme (Dong *et al.*, 1995).However *Spirulina* spp. used in experiment did not exhibit sensitivity to kanamycin, the result implies that gene *nptII* encoding NPTII was not suited as the marker gene for *Spirulina* transformation. Low level resistance against streptomycin shown in results may be due to alteration in *strC* or *strB* genes responsible for ribosomal proteins. It is not due to alteration in aminoglycoside transport system, inadequate membrane potential, lipopolysaccharide modification because inhibition found less or more in all concentration of antibiotics (Mingeot-Leclercq *et al.*, 1999). Ampicillin is also frequently used selective marker both for eukaryotes and prokaryotes (Sambrook *et al.*, 1989). Ampicillin is also reported as selective marker in case of *Synecococcus* sp. PCC 7002, PCC 7492 (Murphy *et al.*, 1990; Luque *et al.*, 1992).However, in present study selected strain of Spirulina did not showed high sensitivity. It seems that ampicillin is not suited as the selectable marker although it can be used as marker for unicellular cyanobacteria and it is also a temperature sensitive antibiotic.

The aminocyclitol antibiotic hygromycin B is thought to have a dual effect on translation by inducing misreading of aminoacyl-tRNAs as well as impairing translocation. Of particular interest is the fact that hygromycin B has been shown to protect N7 of G1494 from dimethyl sulfate modification and to significantly enhance the modification of A1408 at N1. These sites are proximal to the sites that confer resistance to hygromycin B. These sites occur in the decoding center of the ribosome, as expected from their effects on miscoding. Thus, hygromycin B has been proposed to distort the ribosomal A site, thereby inhibiting translation.

Chloramphenicol inhibits translation on a 50S ribosomal subunit at the peptidyl transferase step, that is, it is elongation inhibitor (Sambrook *et al.*, 1989). Cm activity can be removed by acetylation, which is catalyzed by Chloramphenicol acetyltransferase (CAT) encoded by

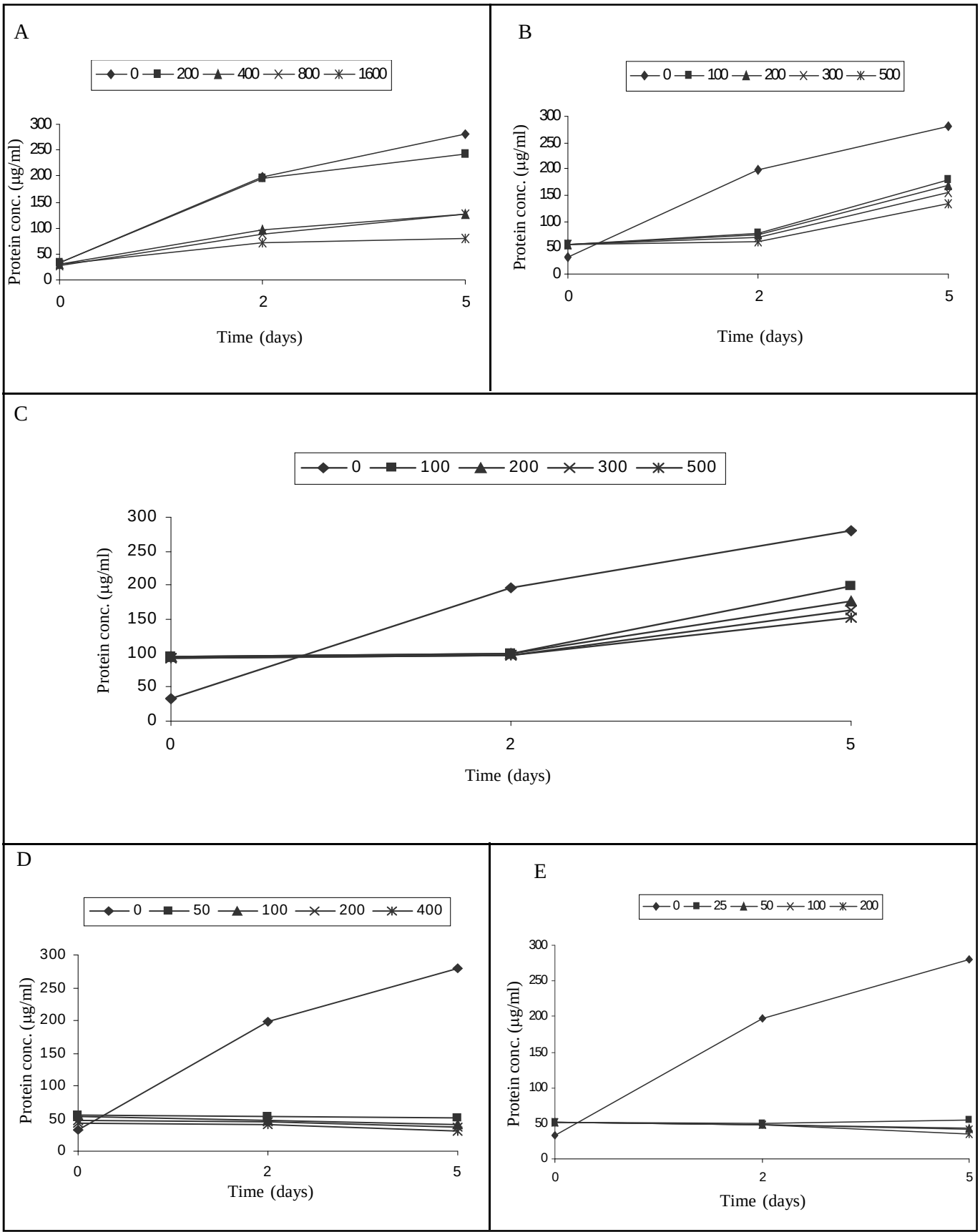

Fig. 1 : Effect of different antibiotics conc. on total protein content of *Spirulina* strain. A- Kanamycin, B- Streptomycin, C- Ampicillin, D- Hygromycin, E- Chloramphenicol.

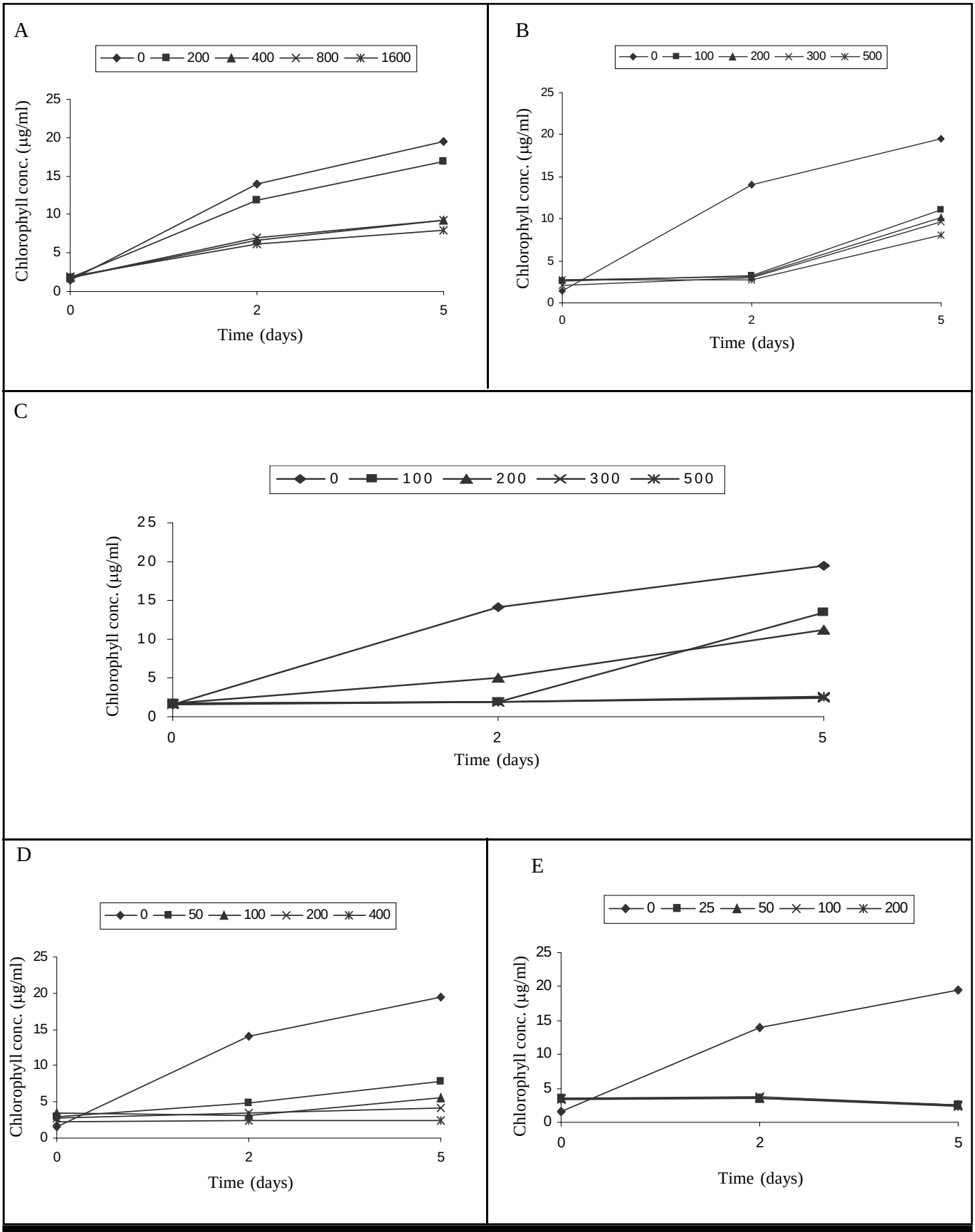

Fig. 2 : Effect of different antibiotics conc. on chlorophyll *a* content of *Spirulina* strain
A- Kanamycin, B- Streptomycin, C- Ampicillin, D- Hygromycin, E- Chloramphenicol

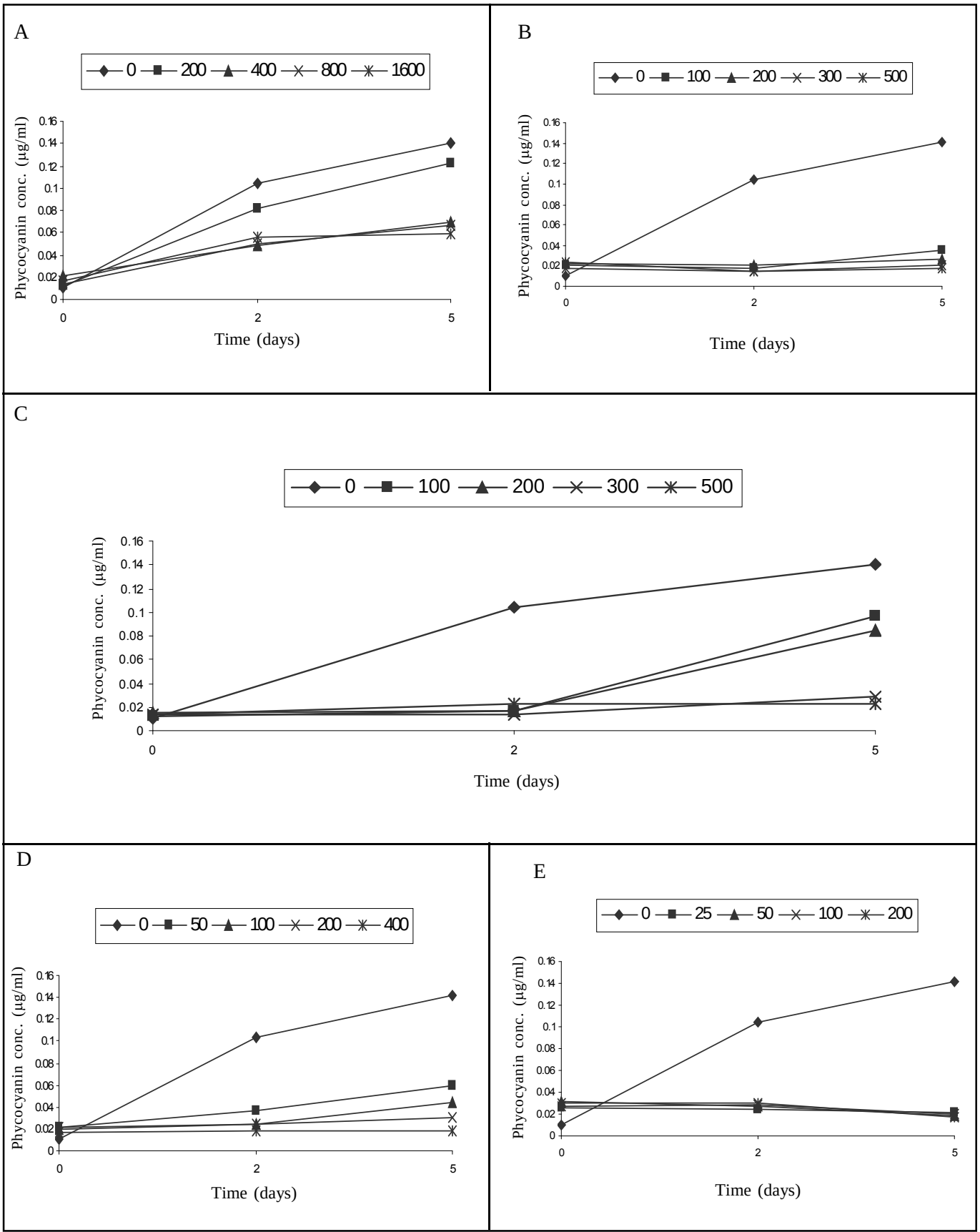

Fig. 3 : Effect of different antibiotics conc. on phycocyanin content of *Spirulina* strain
A- Kanamycin, B- Streptomycin, C- Ampicillin, D- Hygromycin, E- Chloramphenicol

cat gene. Thus the *cat* could confer the resistance of transformed cell to Chloramphenicol in a medium and the untransformed cells would be killed. It was reported that Chloramphenicol was once used as selectable marker in cyanobacterium (Metz *et al.*, 1989; Thiel and Poo, 1989).Thiel and Poo (1989) succeeded in transforming the *cat* gene into a filamentous cyanobacterium *Anabaena* sp. M131 by electroporation for the first time, in which 25µg/ml of chloramphenicol was applied to screen the transformants.

Present study showed sensitivity of selected strain of *Spirulina* towards the chloramphenicol and hygromycin but chloramphenicol sensitivity was much higher than the hygromycin. Therefore, much emphasis given to the chloramphenicol and the protein concentration as an index for growth and chlorophyll concentration for sensitivity, at the place of traditionally used parameters (algal biomass/ wet weight/ dry weight) because in these methods it is difficult to distinguish between live and dead cell mass. No reports were till yet available about screening for selection of transformation marker for *Spirulina platensis* with the selected sensitivity assay.

Acknowledgment:

Authors acknowledge UGC for financial assistance for research project.

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