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Annual Report
to
NASA CELSS Program
(Cooperative Agreement NCC 2-231)

UTILIZATION OF NON-CONVENTIONAL SYSTEMS FOR CONVERSION OF
BIOMASS TO FOOD COMPONENTS

Potential for Utilization of Algae in Engineered Foods

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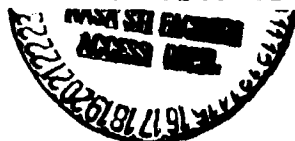
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March 1985

(NASA-CR-176257) UTILIZATION OF
NON-CONVENTIONAL SYSTEMS FOR CONVERSION OF
BIOMASS TO FOOD COMPONENTS: POTENTIAL FOR
UTILIZATION OF ALGAE IN ENGINEERED FOODS
Annual Report (Massachusetts Inst. of Tech.) G3/51

M86-1183J

Unclas
15264



INTRODUCTION

The challenge of producing safe, nutritious, and acceptable fabricated foods in a space habitat (Controlled Ecological Life Support System = CELSS) requires conversion of algal biomass cells into protein (SCP = Single Cell Protein) as a possible food component (Karel, 1980; Karel and Kamarei, 1984). The role of algae in CELSS is rather unique. High photosynthetic activity and lack of need for "organic" carbon or nitrogen substrate, make algae systems suitable candidates for a primary oxygen regenerating system within CELSS (Lachance, 1968; Waslien et al., 1978; Clement, 1975; Santillan, 1982; Casey and Lubitz, 1963). Other possible algal functions include water purification, waste processing, nitrogen fixation, and removal of volatile gases. Cultivation of algae for above purposes will result in large quantity generation of algae biomass from which nutrients can be isolated and utilized as food component for the space crew.

Nutritional and safety considerations of SCP consumption have been addressed to a limited extent by Omstedt et al. (1973); Bourges et al. (1971); Mitsuda (1973); Kraut et al. (1966); and recently by Tuse (1984). There are 3 genera of algae (Scenedesmus, Spirulina, and Chlorella) with relatively known nutritional safety record. However consumption of unrefined algae by humans may cause side effects such as gastrointestinal disorders and allergic responses (Waslien, 1975).

The variations in the reported nutrient content of algae

reflects to a great extent the composition and environmental conditions of the cultivation media (Clement et al., 1967; Lipinsky and Litchfield, 1970; Waslien, 1975; Becker, 1981; Santillan, 1982; and Piorreck et al., 1984).

Review of the literature also shows that while the proteins are the major component of the three mentioned algae genera the nutrition value of the "whole algae" is far less than animal proteins such as egg and milk (Becker, 1981; Chen and Pepler, 1978).

Direct consumption of single cell biomass, without purification, is not feasible in the amount which would be of any significance to biomass recycling. This is because of physiological concerns (e.g. nondigestive components of cell wall and disturbing carbohydrates, excess nucleic acids, unknown toxins and allergens, etc.) as well as organoleptic concerns (unpleasant flavor, color, and texture). To utilize the generated algal biomass, as a food component in space, one should first overcome the physiological and organoleptical concerns due to undesirable or potentially undesirable components in the algae system. To accomplish this goal and to enhance the nutritional value and acceptability of fabricated foods containing algae, attempts were made to recover the algae proteins in a relatively pure form. Major reviews of work on algae for human consumption either on earth or in space, stress the need for developing appropriate food technology for this raw material, and all

acknowledge the complete absence of an adequate existing knowledge base for this purpose (Waslien et al., 1978; Lachance, 1968; Rha et al., 1975; Litchfield, 1977; Cooney et al., 1980). For this purpose, we developed a methodology to eliminate or largely reduce three known undesirable components of algae system (cell walls, nucleic acids, and pigments and lipids) and obtained "algae protein isolate" with a reasonably high yield.

MATERIALS AND METHODS

Source of Algae:

Green algae (Scenedesmus obliquus) were grown in the Constant Cell Density Apparatus (CCDA) at Martin Marietta Lab (Baltimore, MD). In this continuous culture apparatus, photosynthetic parameters can be constantly controlled and monitored and algae can be harvested on demand.

Algae were grown at 32°C and pH 7.0 up to cell density of 0.55 mg (dry weight) per milliliter. The source of nitrogen was KNO₃ (2.0g/l) in the growth media and the source of carbon was 2% CO₂ in air. To harvest the algae, overflow from the culturing apparatus was collected in 4-liter containers (4°C). When the cells settled down, the supernatant was discarded and the remaining concentrated cell suspension was transferred to one liter bottles and centrifuged (7000 g) for 30 minutes. The pellet ("packed cells") was washed 3-4 times with distilled water to remove traces of growth media.

Cell Wall Rupture:

To study the cell components and to isolate the algal proteins, the first task was to break the cell wall of the intact algae packed cells (fraction #1). For this purpose, we tried three physical methods namely: shear of freeze dried algae cells (using krups type 203 coffee mill, up to 8 min at room temperature), sonication of 3% algae solution (Branson sonifier cell disruptor 200, up to 24 min at 0°C), and homogenization of 7.6% algae solution (Sorvall omni-mixer, with 40% 450-500 μ glass beads, 16,000 RPM, up to 30 min at 0°C). Cell wall rupture was followed by direct microscopic inspection of the resultant solution.

Upon freeze drying of above solution (Vitrus Co., Gardiner, NY), we obtained algal flour (fraction #2) which was used, for determination of major nutritional composition.

Composition Determination:

All composition determinations were done on duplicate samples and calculations of recovery (% yield) of various algae fractions were based on the initial weight of the algae flour (freeze dried broken cells). Moisture and ash determination were carried out according to A.O.A.C. (1980) methods.

For protein determination, nitrogen was determined according to the modified Kjeldahl method (A.O.A.C., 1980) as summarized in fig. 1. Protein concentration was then calculated by multiplying total nitrogen content by 6.25.

Total nitrogen also includes the nitrogen from nucleic acids as the prime interfering source. Therefore, we measured initially the total nucleic acid (RNA & DNA) concentration in various fractions and then subtracted these values from the total protein to obtain the actual protein concentration. Knowledge of nucleic acid concentration was also important for reduction of these undesirable constituents in the final products. Determination of Deoxyribo Nucleic Acid (DNA) and Ribo Nucleic Acid (RNA) in various fractions was accomplished by modified procedure of Schmidt and Thannhauser (1945) and similar to those described by Smillie and Krotkov (1960).

For this purpose each algal fraction (20 mg for RNA and 40 mg for DNA) was initially extracted with cold methanol (10 ml, 3x), centrifuged (10,000 RPM, 4 min, 4°C), extracted with cold 5% TCA (10 ml, 2x), centrifuged, and finally extracted with cold and then boiling ethanol (10 ml) to produce "algae residue". The algae residue was dried (37°C), powdered, and used for determination of DNA and RNA as summarized in fig. 2A and 2B.

Purified DNA (from calf thymus, sigma, St. Louis, MO) and RNA (from bakers yeast, Sigma, ST. Louis, MO) were treated similarly to "algae residue" for preparation of standards. For preparation of diphenylamine reagent, 1 gram of purified diphenylamine (recrystallized from boiling hexane to a white crystalline product) was dissolved in 100 ml glacial acetic acid and 2.75 ml of sulfuric acid. For preparation of Orcinol reagent, 1 gram of

purified orcinol (dissolved in boiling benzene, decolorized with charcoal, and crystallized to a perfectly white crystalline product) was dissolved in 100ml HCl containing 0.5 g FeCl₃.

Total pigments and lipids were determined by extraction with boiling ethanol in a Soxhelt apparatus as summarized in fig. 3. The obtained values, under above conditions, were assumed to be the highest extractable values and were used as reference for extraction treatments. For routine extraction of pigments and lipids, we, however, submerged thimbles containing 200 mg algae protein concentrate in 75 ml absolute ethanol at various temperatures (20,0, and -20°C). This solution was gently mixed until equilibrium was reached (usually overnight). Solvent could then be decolorized (with 1-2 hours) with 10 g charcoal at the same temperatures.

Total carbohydrates were calculated from the weight difference of all above components with that of the total original weight.

Nucleic Acid Reduction:

Considering the overall conditions and limitations of space habitat we decided to use an enzymatic method for reduction of RNA and DNA, using extracellular RNase and DNase (both from bovine pancreas, Sigma, St. Louis, MO). To obtain the optimum reaction conditions for enzymatic treatment of algal nucleic acids, initially pure RNA and DNA were treated with RNase and DNase according to the method of Worthington (Millipore Corp.,

Bedford, MA) and under the following conditions:

For RNase treatment: 1 ml of 1% RNA in 0.1M sodium acetate (pH 5.0 and/or 7.4) was mixed with 1 ml of RNase in 0.1 M sodium acetate at concentrations of 0, 2.5, 10 and 50 $\mu\text{g/ml}$. Upon incubation of the mixture at 37°C for 0, 4, and 8 min, the reaction was stopped with 1 ml of 25% PCA and ice bath (5 min). Following the centrifugation (5000 RPM, 10 min, 4°C) of the cold mixture, 0.1 ml of the supernatant was mixed with 2.9 ml water and increase in absorption at 260 nm, due to liberation of mono and oligo nucleotides, was measured.

For DNase treatment: 2.5 ml of 0.004% DNA (in 200 ml 6.25 mM MgSO_4 , 25 ml 1.0 M sodium acetate, and 25 ml H_2O) at pH 5.0 and/or 7.4 was mixed with 0.5 ml DNase in 0.15 M NaCl at concentrations of 0, 25, 50, 100, and 200 $\mu\text{g/ml}$. Upon incubation of the mixture at 25°C for 0, 5, and 10 min, the reaction was stopped with 1.5 ml 25% PCA and ice bath (5 min). Following centrifugation (5000 RPM, 10 min, 4°C) of the cold mixture, absorption at 260 nm was determined. Based on the obtained optimum conditions for enzymatic treatment of "pure" RNA and DNA and with a similar treatment, freshly-prepared (i.e., non freeze-dried) algae crude protein (fraction #4) was treated separately with both enzymes. Since upon enzyme inactivation with acid, the supernatant was slightly greenish and chlorophyll has an absorption peak at 260 nm, we determined DNA and RNA concentration directly in the Pellet (i.e. protein concentrate or fraction #6) of the control and enzyme treated samples.

RESULTS & DISCUSSION

Cell wall consists mainly of complex polysaccharides and murein, the typical structural macromolecules of bacterial cell walls (Soeder, 1978) and if left intact, is an obstacle to digestability of cell components. To remove the cell wall it is possible to use chemical methods which include the use of urea, guanidine, sodium hydroxide (Mitsuda et al., 1969; Huang and Rna, 1971), alkaline biocarbonate buffer (Tannenbaum et al., 1966), acetic acid, oxalic acid, citric acid (Samerjima et al., 1971) methanolic hydrogen chloride (Tamura et al., 1972) and ethanol acetone (Lee et al., 1979). It is also possible to use enzymatic methods which include incubation of active cells at the optimum temperature to induce the lytic reaction of endogenous enzymes or addition of enzymes to lyse the cell wall (Hedenskog et al., 1969; Maul et al., 1970; Castro et al., 1971; Carenberg and Heden, 1970).

Although each of above methods has its own advantages, we, however, due to the potential limitations and restraints of CELSS, focused on simple physical methods for cell wall disruption. The physical methods generally involve the rupture of cells with a high-pressure press, freeze-thaw treatments, and sonication, as well as high-speed ball mill grinding, high pressure homogenization, and high-speed mixing (Hedenskog and Mogren, 1973; Lee et al., 1979; Cunningham et al., 1975; Dunnill and Lilly, 1975).

Comparison of physical cell wall disruption methods by direct Scanning Electron Microscopy (SEM) showed that, under our experimental conditions, homogenization with glass beads results in more disruption than the other methods. Consequently, homogenization was chosen as our method for breakage of algae cell walls. Fig. 4 shows the comparison of the microscopic inspection of intact algae cells with homogenized (30 min) cells. Upon completion of above stage and removal of beads with sieve #40 (420 μ openings), we obtained a homogeneous mixture of cell wall fragments, various released cytoplasmic proteins and cell organelles, nucleic acids, and pigments, from which we were interested in separation, concentration, and isolation of proteins. The utilization of the remaining components could be the subject of additional research activities in the area of by-product management.

Major nutritional components of algae flour (freeze dried broken cells) are shown in Table 1. Our values are in good agreement with those reported in the literature (Becker, 1981; Jurkovic et al., 1983; Piorreck et al., 1984) indicating that continuous algal culture results in algal biomass similar to batch-type products.

Fig. 5 shows the stepwise procedure for preparation of various algal fractions. Adjustment of broken cell suspension to pH 10.0 was based on the known pH-solubility curves for plant proteins (Wolf, 1978) and according to the methods of the

Hedenskog and Mogron (1973) and Hedenskog (1978). At this pH, proteins and associated molecules are soluble and therefore, upon centrifugation, undesirable cell walls (containing murein and disturbing carbohydrates), along with some fragments and organelles are excluded from the product. This results in algal crude protein (Fraction #4) which theoretically have higher digestibility values. At this stage, attempts were made to remove another undesirable component of the algal system i.e. nucleic acids. Nucleic acids, insoluble below pH 4.5, if ingested directly, lead to the elevation of blood uric acid levels as a result of their degradation to the purine bases, adenine and guanine, and the in vivo oxidation of these latter. Uric acid with extremely low water solubility cannot be further degraded and is only partially excreted. This leads to gout, to diseases affecting the joints and to the formation of "stones" in, for example, the kidneys and the bladder (Hudson, 1980). Reduction of the levels of nucleic acids is thus of special importance.

The generally accepted safe level of nucleic acid intake in the human is 2 grams per day (Scrimshaw, 1975), and since the total concentration of nucleic acids (RNA & DNA) of the studied algae* is found to be 6.0%, this would limit, the algal consump-

*Results of purified RNA and DNA (Standards) measurements are presented in the appendix.

tion to about 33 grams per day. Thus, if algal proteins are to be used as a major ingredient in foods in space habitats, reduction of nucleic acids is an essential step.

There are numerous methods for reduction of nucleic acid content in cell suspensions and in cell homogenate. These methods have been reviewed by Sinskey and Tannenbaum (1975), Litchfield (1977), Chen and Pepler (1978), Hedenskog (1978) and Gierhart and Potter (1978). Cell suspensions have been treated with acid (Pepler, 1970), aqueous ammonia (Ayukawa et al., 1971; Akin and Chao, 1973), heat shock/pancreatic RNase (Castro et al., 1971), heat shock/ Na_2HPO_4 solution (Canepa et al., 1972), MeOH/HCl mixtures (Tamura et al., 1972), heat shock (Tannenbaum, 1973), EtOH/HCl mixture (Akin and Chao, 1974) heat shock/pH 5.0-5.5 (Akin, 1974), heat shock/carboxylic anion (Sinskey and Tannenbaum, 1975), and NaOH or aqueous ammonia (Viikari and Linko, 1977).

Reduction of nucleic acids in cell homogenates have been reported with NaCl 3%/50°C - pH 5.6 (Lindblom and Morgan, 1974), pH 6.0/80°C (Vannanuvat and Kinsella, 1975), high temperature-low alkali, or low temperature-high alkali (Newell et al., 1975), 100°C/pH 6-8 (Robbins, 1976), extracellular RNase (Fazakerley, 1976), and succinylation after cell disruption (Shetty and Kinsella, 1979).

Enzymatic treatment results in depolymerization of nucleic acids into mono and oligo nucleotides and since the latter are

soluble at lower pH's, acidification of the algal crude protein (to pH 4.0) will result in exclusion of undesirable nucleotides (along with soluble peptides and free lipids) from protein concentrate into supernatant.

Treatment of pure RNA with RNase showed that optimum condition at 37°C for maximum absorption (260 nm) are: pH 5.0, Enzyme/substrate ratio of 1/200 (w/w) and reaction time of 6 min. Similarly, the optimum conditions for DNase at 25°C are: pH 5.0, Enzyme/substrate ratio of 1/1 (w/w) and reaction time of 10 min (appendix).

When the algal crude protein (#4) was treated under above optimum conditions, results showed 96.5% decrease in RNA concentration and 79% decrease in DNA concentration.

Table 2A shows the comparison of RNA and DNA concentrations in various algae fractions before and after enzymatic treatment. Reduction of total nucleic acids concentration of algae protein concentrate (#6) to 0.81% increases the safe consumption level of this protein from approximately 20 g (non-treated) to approximately 250 g per day. Similarly reduction of total nucleic acid concentration of protein isolate (#8) to 1.04% increases the safe consumption level of this protein from 15 g to 190 g per day (Table 2B).

Extraction of high concentration of pigments (mostly chlorophyll a) and lipids from the algae protein concentrate (fraction #6) results in removal of almost all green color and consequently the algae protein isolate (fraction #8) has a "light olive color"

upon freeze drying. Removal of the pigments and lipids enhances the acceptability of algal products from the color and flavor points of view, but causes denaturation of algae proteins (regardless of ethanol extraction temperature) and therefore water insolubility of the final product. This side effect may result in loss of some useful functional properties during the following fabrication processes. Yield (%), protein concentration, and protein recovery (%) of various algae fractions is represented in Table 3. Although the total recovery and protein concentration of protein isolate seem rather reasonable, attempts should be made to increase these values.

Above results show that green algae can be processed and upgraded (with a reasonable yield) to algal protein isolate by removing 3 major undesirable cell components (cell wall, nucleic acids, and pigments and lipids). This will enhance the potential nutritional and organoleptic acceptability of algae products while some functional properties may be lost and need to be restored or avoided. Further research on optimization of each processing step and consequently improving the yield and quality of final product (upon protein characterization) is needed before any study on nutritional, toxicological, and technological aspects is planned. The latter studies are of prime importance and should be carried out before the actual utilization of algae as a protein source in space habitat.

SUMMARY

The major nutritional components of the green algae (Scenedesmus obliquus) grown in a Constant Cell Density Apparatus were determined. Suitable methodology to prepare proteins from which three major undesirable components of these cells (i.e., cell walls, nucleic acids, and pigments) were either removed or substantially reduced was developed. Results showed that processing of green algae to protein isolate enhances its potential nutritional and organoleptic acceptability as a diet component in Controlled Ecological Life Support System.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. Richard Radmer and Paul Behrens for supplying them with the algae used in this study. This work was supported by the NASA CELSS Program.

The authors express their appreciation to Drs. Robert MacElroy and Melvin Averner of the Ames Research Center of NASA for their technical review and advice.

The expert assistance of Erika Hartweg from EM facility of Biology Department (MIT) for preparation of SEM Pictures is appreciated.

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Table 1) Nutritional composition of algae flour (freeze dried broken cells). Solid content of whole algal cell prior to freeze drying was found to be 23% .

Proteins	52.6%
Nucleic Acids:	
RNA	4.3%
DNA	1.7%
Pigments & Lipids	15.0%
Carbohydrates	16.5-18.5%
Ash	7-8%
Moisture Content	1-2%
Total	98.1-102.1%

Table 2A) Concentration of RNA and DNA in various algal fractions before and after enzymatic (RNase & DNase) treatment.

fraction #	% RNA		%DNA		total N.A. reduction upon Enz. treatment %
	before	after	before	after	
2 (algae flour)	4.3	-	1.7	-	-
4 (algal crude protein)	6.8	-	2.0	-	-
6 (algal protein concentrate)	7.4	0.26	2.6	0.55	92
8 (algal protein isolate)	9.9	(0.35)	3.3	(0.69)	92

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Table 2B) Amount of algae (grams) containing safe consumption level of nucleic acids i.e., 2g/day (Scrimshaw, 1975).

<u>fraction #</u>	<u>Before</u> <u>Enz. treatment</u>	<u>After</u> <u>Enz. treatment</u>
2 (algae flour)	33	-
6 (algal protein concentrate)	20	247
8 (algal protein isolate)	15	190

Table 3) Yield (%), protein concentration (%), and protein recovery (%) of various algae fractions without enzymatic treatment.

<u>fraction #</u>	<u>yield(%)</u>	<u>Protein concentration(%)</u>	<u>Protein Recovery (%)</u>
2 (algae flour)	100	52.6	100
4 (algal crude protein)	65	57.6	71.2
6 (algal protein concentrate)	51.6	53.4	52.4
8 (algal protein isolate)	36.6	70.5	49.1

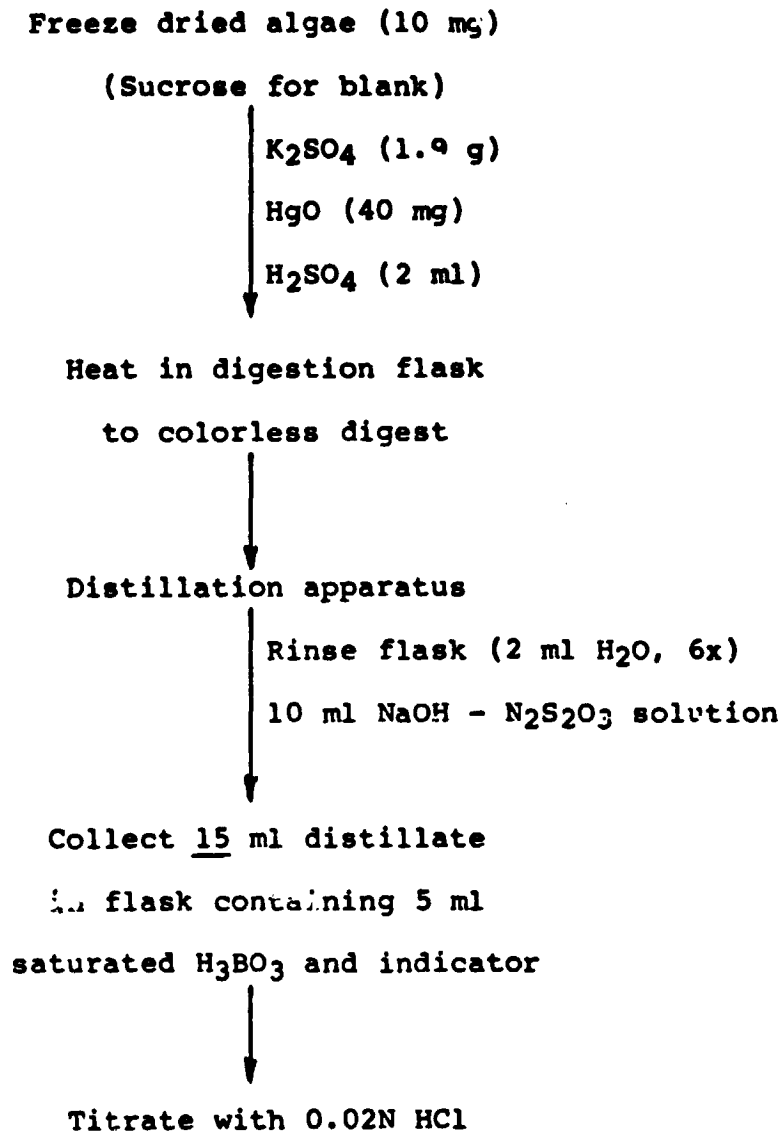


Fig. 1) Modified microkjeldahl procedure for "N" determination in various algae fractions. %N = (ml HCl - ml blank) x normality x 14.007 x 100/mg freeze dried algae.

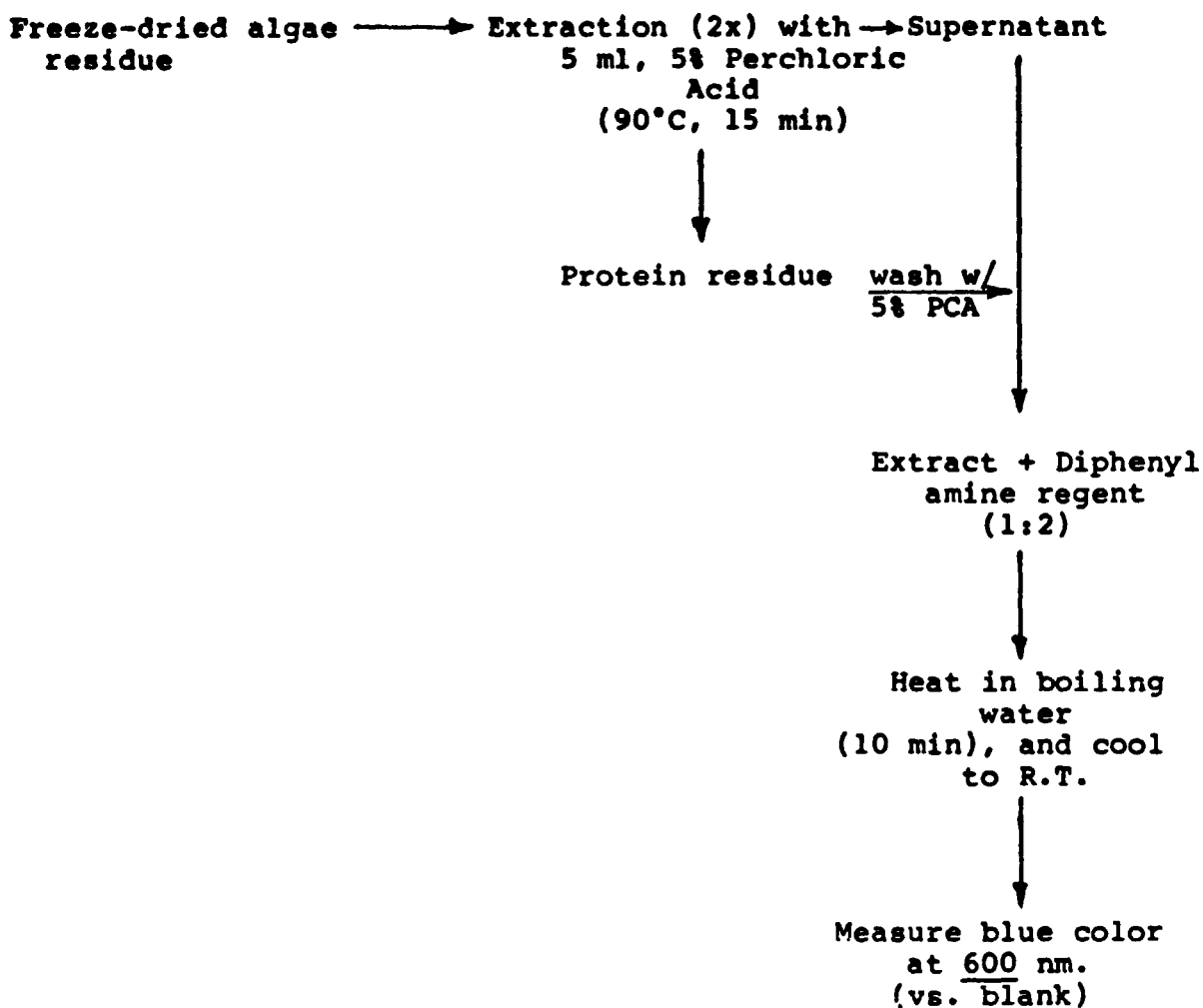


Fig 2A) the determination procedure for "DNA" in various algae fractions

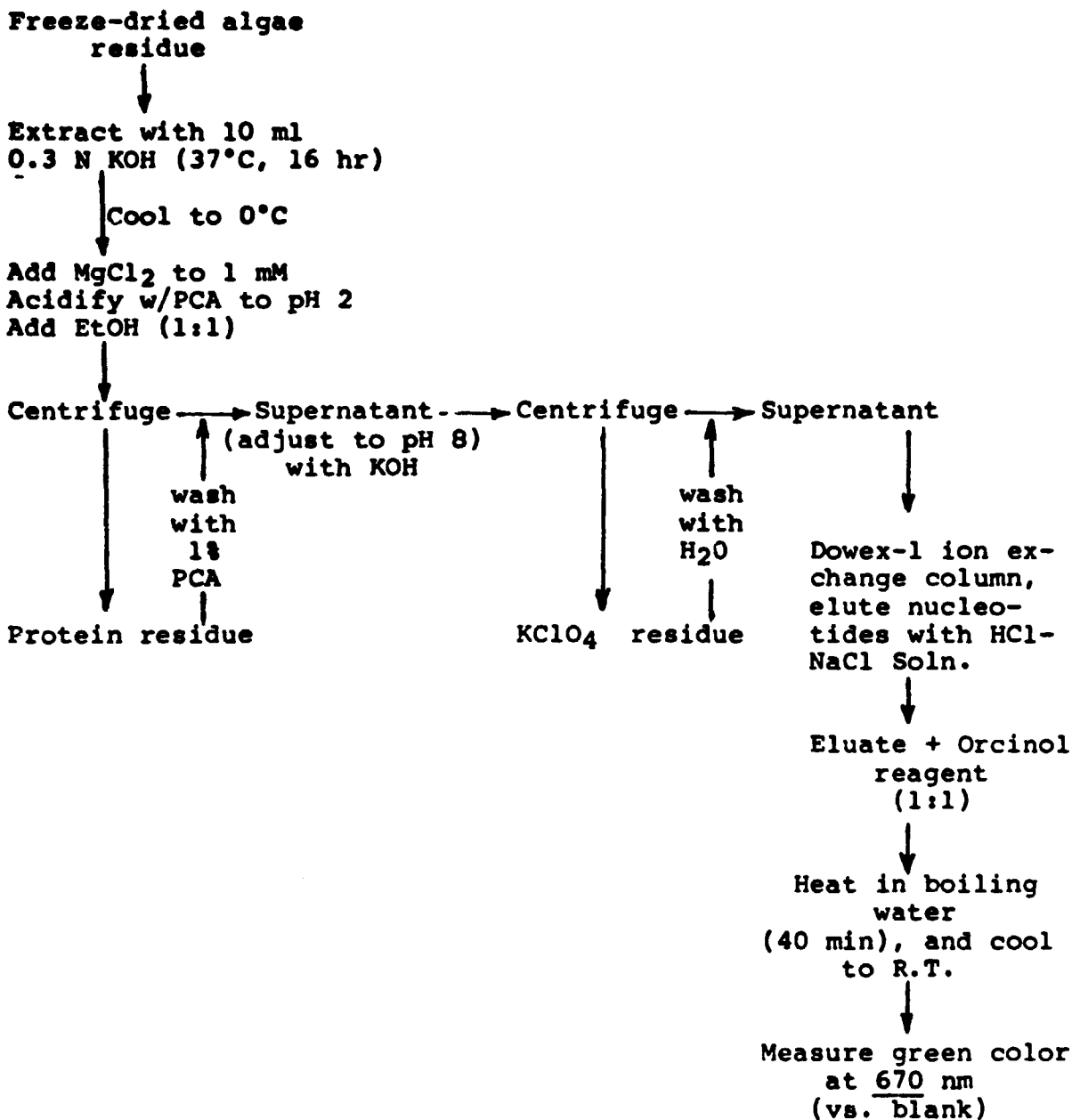


Fig 2B) The determination procedure for "RNA" in various algae fractions.

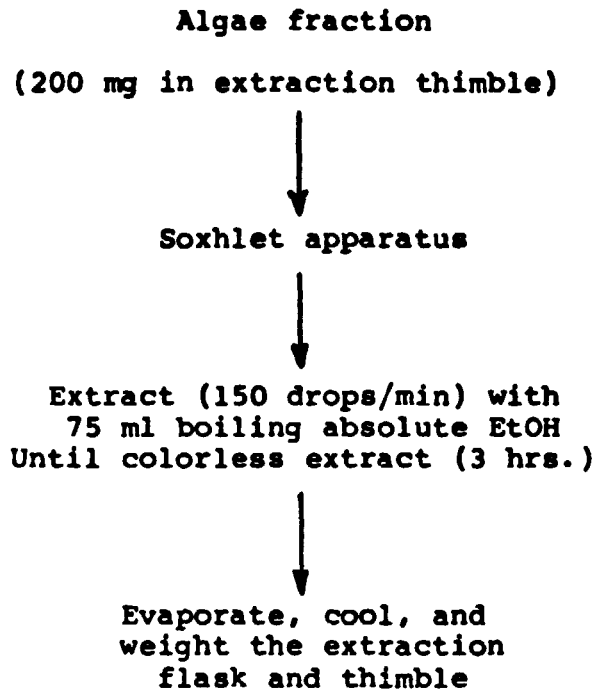


Fig 3) The determination procedure for total pigments and lipids in various algae fractions. % total pigments and lipids = wt. of extract (mg) x 100/wt. of algae (mg).

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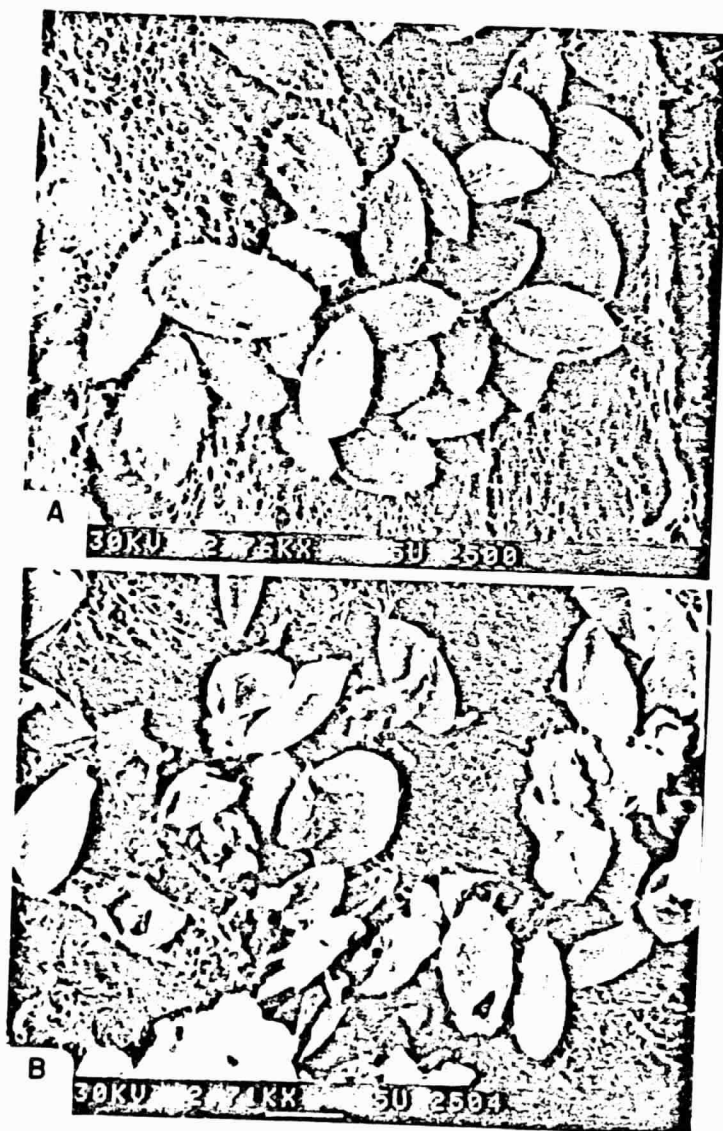


Fig 4) Scanning Electron Micrograph of Green algae (Scenedesmus obliquus) cells (A) and homogenized cells for 30 min (B).

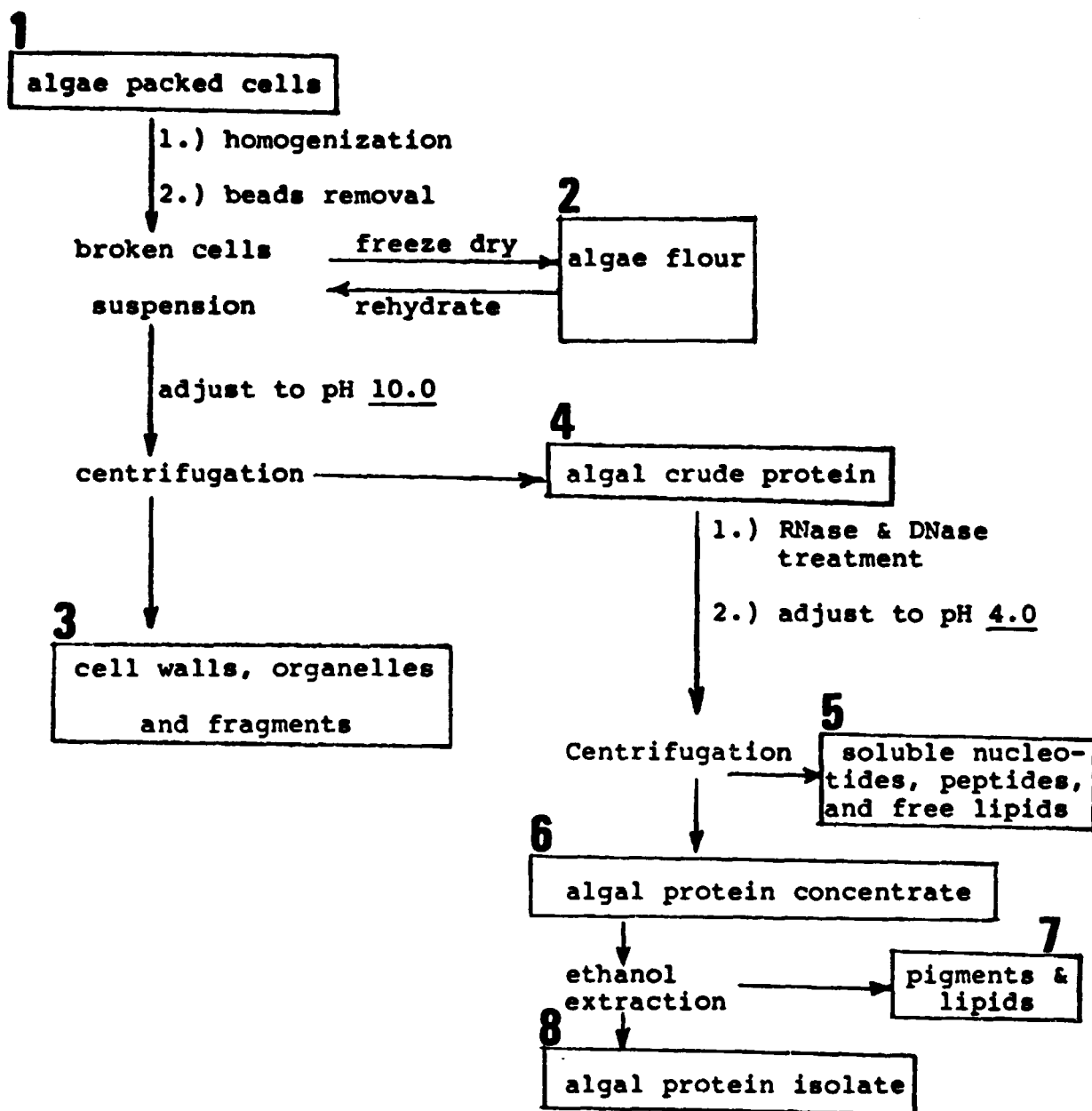


Fig 5) Stepwise procedure for preparation of various algae fractions

APPENDIX

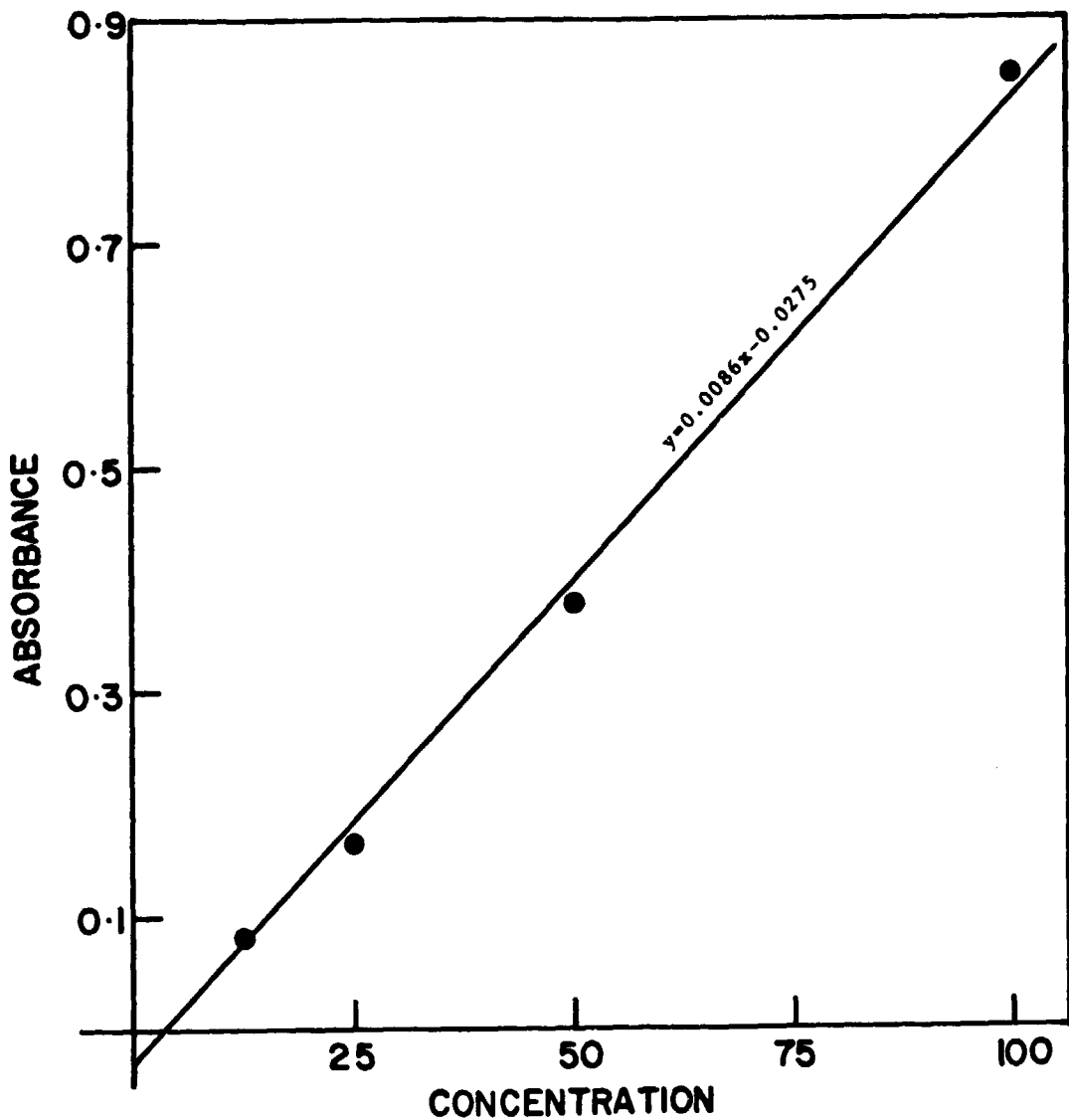


Fig. 1) Calibration Curve and regression line for standard RNA concentration (µg/ml) at 670 nm (not alkaline treatment). After alkaline treatment (0.3 N KOH, 16 hrs. at 37°C), absorption value increases by 29%. Upon ion exchange chromatography (Dowex-1), however, values are decreased by 29%.

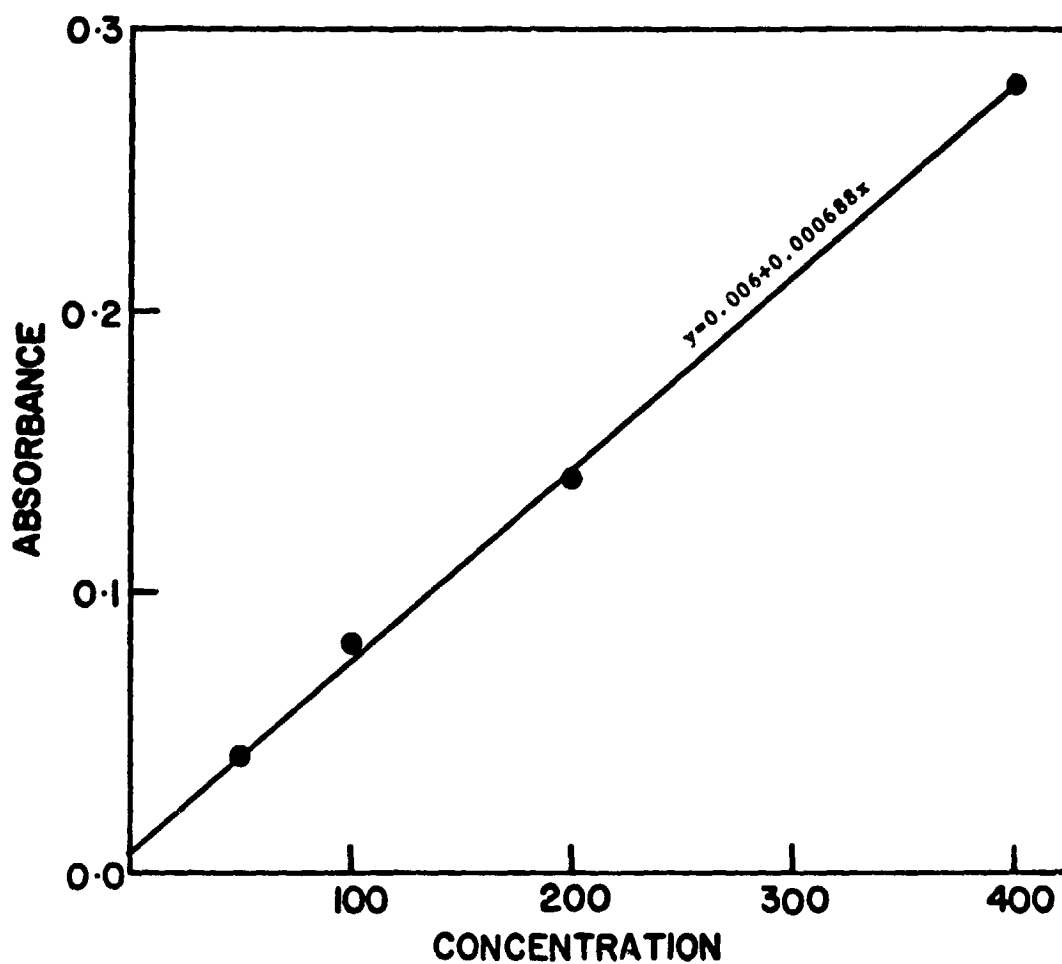


Fig. 2) Calibration Curve and regression line for standard DNA Concentration ($\mu\text{g/ml}$) at 600 nm.

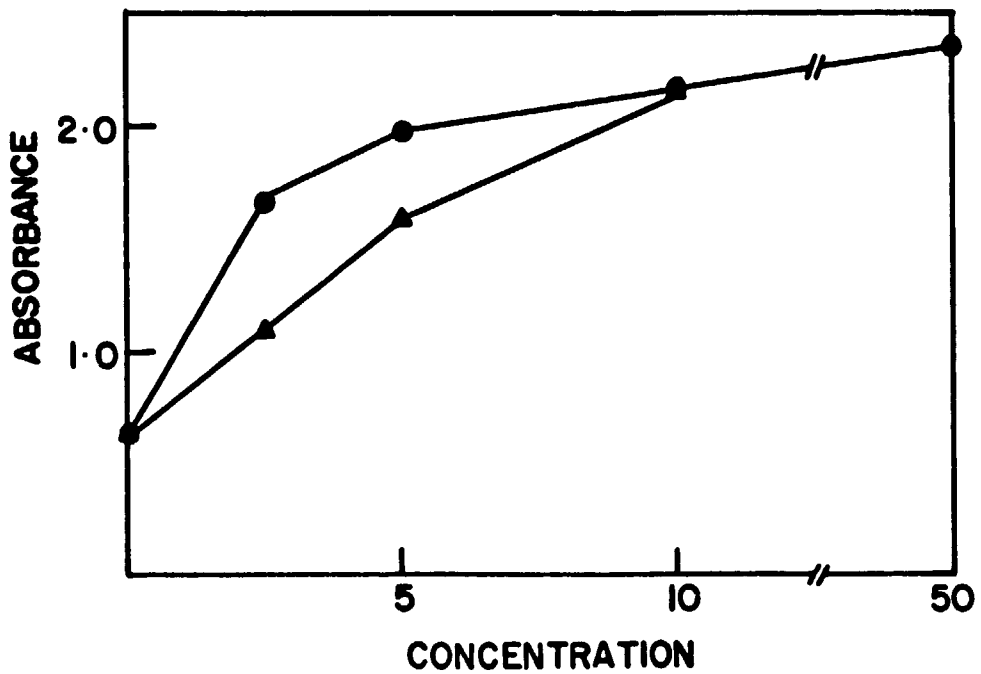


Fig. 3) Absorption intensities of RNA monomers (260 nm) upon hydrolysis of standard RNA (1%) by various concentrations of RNase ($\mu\text{g/ml}$); pH 5.0, temp. = 37°C

- 8 min reaction time
- ▲ 4 min reaction time