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Green Algae as a Way to Utilize Phosphorus Waste

Assel Tleukeyeva[1*], Radoslaw Pankiewicz[2], Akmaral Issayeva[3], Nuradin Alibayev[1], Zhanbolat Tleukeyev[1]

[1] M. Auezov South Kazakhstan University, Shymkent, Kazakhstan
[2] Adam Mickiewicz University, Poznan, Poland
[3] Shymkent University, Shymkent, Kazakhstan
* Corresponding author's e-mail: aseltleukeyeva@mail.ru

ABSTRACT
The possibility of using phosphorus-containing wastewater as a raw material for the cultivation of the green algae strain Chlorella vulgaris ASLI-1 can represent an effective processing of phosphorus-containing by-products. A laboratory experiment was made to study the effect of the concentration of phosphorus-containing wastewater on the biomass density of the green alga strain *Chlorella vulgaris* ASLI-1. Three weeks after sowing, we measured the biomass density of algae in various components of the phosphorus-containing wastewater. Compared to the control (distilled water), the addition of phosphorus-containing wastes did not adversely affect the culture of green algae, with the exception of a 20% medium where algal cells were discolored and had a low biomass density, 104 CFU. However, more research is needed to better study the response of green algae to phosphorus-containing waste, to determine the amount of phosphorus in cells and solution. In addition, evaluate the agronomic efficiency of the *Chlorella vulgaris* ASLI-1 strain, cultivated on phosphorus-containing waste, when applying fertilizers for growing vegetables.

Key words: *Chlorella vulgaris*, phosphorus-containing wastewater, algae, phosphorus- containing waste, agriculture

INTRODUCTION

The cultivation of crops in agriculture is largely dependent on phosphorus, which is widely recognized as an important macronutrient [Marschner, 2012]. In most cases, phosphorus fertilizers are produced from mineral rocks, economically justified reserves of which may be exhausted in the coming decades [Childers, 2011]. In this regard, it becomes necessary to introduce innovative methods for the processing of phosphorus-containing waste [Koppelaar and Weikard, 2013]. In Switzerland, for example, the amount of phosphorus-containing waste (i.e. 9600 tons) exceeds the annual amount of phosphorus provided by fertilizers (i.e. 4200 tons) [Mayer, 2019]. Taking the example of calcium phosphite, a by-product of the production of hypophosphite and phosphine, about 300 tons of phosphorus are dumped in Switzerland every year. In terms of phosphorus reserves, Kazakhstan ranks fourth in the world. The intensive development of the phosphorus industry in the 70–80s of the last century led to the gradual production of phosphorites with a high content of the target component (more than 28% P_2O_5) and, accordingly, the accumulation of refractory ores of complex mineral and chemical compositions [Issayeva A.U., 2020]. The complex composition of phosphorites and close physicochemical properties of phosphate minerals and host rocks do not allow the use of known enrichment methods and obtaining concentrates of the required quality [Piperd, 2013]. This, in turn, leads to a decrease in the quality of traditional phosphorus fertilizers and the formation of a significant amount of waste. As a rule, this waste, consisting of empty and enclosing rocks, is not utilized, and only small amounts are processed into building materials [Yang, 2013]. The ability to reuse phosphites as a phosphorus fertilizer in agricultural systems can be a valuable option for optimizing the phosphorus cycle in the environment.

Phosphite itself cannot be used as a fertilizer, since this form of phosphorus is ineffective in terms of plant nutrition [Gómez-Merino, 2015. Ratjen, 2009]. Phosphite is stored in plant cell vacuoles similarly to phosphate, but cannot be metabolized as a source of phosphorus [Lambers, 2013]. Phosphite prevents plant biochemical mechanisms responsible for phosphorus starvation, which subsequently leads to a negative effect on plant growth [Arai, 2007]. MacIntire et al. a toxic effect was observed for various crops (millet, red clover, ryegrass and soybeans) fertilized with phosphites, while a positive effect was observed for subsequent crops (alfalfa, ryegrass and soybeans), a result attributed to phosphite oxidation in the soil. It has been established that phosphite can be oxidized to phosphorus by soil microorganisms [Beauchemin, 1999], which means that this type of phosphorus can be a valuable source for agricultural crops. In fact, phosphite oxidation is the only way to improve phosphite as a source of phosphorus for crops, given that its oxidation does not occur naturally in plants [Bünemann, 2004]. Since the kinetics of the abiotic reaction is very slow, microbial activity is the main driver of phosphite oxidation [De Silva, 1994]. The ability of certain microbial strains to oxidize phosphites to readily assimilable forms of phosphorus is a hereditary feature of the evolution of life on Earth [Delorme, 2000], thus, phosphite oxidizing microorganisms are likely to be present in soils [Eichler-Löbermann, 2007]. Other studies have found that phosphite may still be toxic to some microorganisms (eg, *Phytophtora infestans*), and for this reason it is widely used as a fungicide [Fageria, 2007].

Although the efficiency of phosphite oxidation by individual strains of microbes, including soil microbes, has already been, only one study has clarified the ability of microflora to oxidize phosphite in various types of soils. In this regard, it becomes necessary to convert numerous polluting waste into useful resources by means of chemical and biological methods, as well as to introduce waste-free technologies that do not violate the ecological situation [Issayeva, 2021]. The purpose of this study is the possibility of using waste products from the production of phosphorus fertilizers for the cultivation of the *Chlorella vulgaris* ASLI-1 algae strain, which is promising for use as biofertilizer in agriculture.

OBJECTS AND METHODS

The objects of research were phosphorus-containing aqueous solutions taken from the phosphorus-containing waste storage area within the city of Shymkent (Figure 1). In addition, a strain of green microalgae *Chlorella vulgaris* ASLI-1 isolated from the Koshkar-ata river (Shymkent) was used in the studies.

The following types of crops were used as test plants, such as:

- Strawberry (*Fragária*) is a genus of perennial herbaceous plants of the rose family (*Rosaceae*).
- Pumpkin (*Cucurbita*) is a genus of herbaceous plants of the Pumpkin family (*Cucurbitaceae*).
- Cultivated soybean (*Glycine max*) is an annual herb, a species of the genus Soybean (*Glycine*) of the legume family.

Sampling was carried out in accordance with GOST 31861–2012. Chromatographic methods using a Dionex ICS-1600 ion chromatograph were used to determine the composition of ions of various metals in samples, according to GOST 31867–2012. Isolation and cultivation of

Figure 1. Places of sampling of phosphorus-containing waste (coordinates: 42.2702947, 69.7241641,2747)

A – a sample taken from a pit at the site of storage of phosphorus-containing sludge (spontaneously combustible waste),

B – a sample taken from a pit at the place of storage of phosphorus-containing sludge (after storage),

C – a sample taken from a concrete tank after washing the sludge.

microorganisms was carried out on specific media: heterotrophs were grown on RPA medium (1 dm^3 fish-peptone broth, 10 g glucose), micromycetes on Czapek's medium (g/l: distilled water – 1.0, $NaNO_3$ – 2.0, K_2HPO_4 – 1.0, $MgSO_4$ – 0.5, KCl – 0.5, $FeSO_4$ – traces), nitrogen fixers – on Ashby's medium (g/l: distilled water – 1.0, sucrose or mannitol – 20.0, K_2HPO_4 – 0.2, $MgSO_4 \cdot 7H_2O$ – 0.2, NaCl – 0.2, K_2SO_4 – 0.1, $CaCO_3$ – 5.0), *Chlorella vulgaris*- on Knop's medium (g/l: Ca $(NO_3)_2$ – 0, 25, $MgSO_4 \times 7H_2O$ – 0.06, KH_2PO_4 – 0.06, KCl – 0.08, $FeCl_3$ – one drop of 1% solution).

For microbiological sowing, the methods of Koch, Novogrudskyi, "depleting sowing", limiting tenfold dilutions were used. Culture media and microbiological glassware were sterilized according to the conditions in a bacteriological autoclave (SPGA-100-I-HH). Cultivation of microorganisms was carried out in a thermostat with a programmable temperature (TC 1/80). All microbiological inoculations were carried out in a sterile microbiological box. Distilled water for the preparation of media was prepared on a water distiller (AE-10MO). Weighing of reagents was carried out on an analytical balance (ScoutPro). Microscopy was carried out using light microscopes "Biomed-5" (Russia), "Tayuda" (Japan). When preparing percentage solutions for the cultivation of algae, GOST 4517–2016 was used. The cultivation of algae was carried out in glass flasks with a volume of 500 ml with the supply of carbon dioxide.

The analysis of the strain for non-pathogenicity and non-allergenicity was carried out at Nutritest LLP in accordance with GOST R 51921–2011. The analysis for non-pathogenicity is carried out on yolk and blood agar to detect potentially pathogenic signs (GOST 33379–2015). The study of the virulence of the culture was carried out by the generally accepted method (Birger MO, 1982) on 8 groups of animals (12 mice, including 6 females and 6 males) at concentrations from 10^3 to 10^{11} CFU.

Allergenic action on the sensitizing effect was carried out on guinea pigs, which were injected with the culture in doses of 10^3, 10^4, 10^5, 10^6 CFU/one animal. Saline was used as a control. The reaction was measured after 10 days by the diameter of the erythrema.

The site of the irritating action was carried out on rabbits, into the conjunctiva of the eyes of which a culture in a volume of $1x10^9$ CFU/cm^3 was introduced. All analyzes for pathogenicity were carried out on the basis of Nutritest LLP.

Statistical processing of the results was performed by calculating the arithmetic mean and the standard deviation. All determinations were carried out in 3-and 5-fold repetitions. The data was processed using an IBM Pentium personal computer based on Excel application software packages (GOST R ISO 5725–2).

RESULTS AND DISCUSSION

Characteristics of phosphorus-containing sludge and wastewater

During the operation of the phosphorus processing plant, production wastes were stored outside the city in compliance with all environmental requirements for the protective zone. In the period after the restructuring of the USSR in the 90s, the production of phosphate fertilizers was suspended, with the gradual bankruptcy of the enterprise. On the other hand, the increased rates of urbanization led to the expansion of the city's boundaries, as a result of which the waste storage site turned out to be within the city, surrounded by settlements. At present, the storage site for phosphorus-containing waste, with a volume of more than 50.0 million tons, occupies about 16 hectares of land, posing a serious threat to the environment and public health.

At the same time, after using the main components to obtain a number of phosphorus- containing fertilizers, a complex of valuable components remains in the waste. The presence of such elements as, wt%: P – 7.29; F – 3.16; Fe – 0.48, Mn – 0.1, K – 0.28, which are necessary for constructive plant nutrition.

In addition, in the original phosphorites, the presence of rare earth elements in the amount of 0.04–0.06% was revealed, in phosphogypsum the concentration increased to 0.15–0.4%, incl. cesium – 43.48–52%; neodymium – 14–16%; lanthanum – 24–27%; praseodymium – 4–6%; samarium – 2.3–2.5%; gadolinium – 1.1–1.3%; europium – 0.1–0.2%. In poor phosphoric sludge, REEs are in the form of various oxides: lanthanum oxide La_2O_3 – 0.008%, praseodymium oxide Pr_2O_3 – 0.001%, neodymium oxide Nd_2O_3 – 0.008%, samarium oxide Sm_2O_3 – 0.001%, gadolinium oxide Gd_2O_3, dysprosium oxide – 0.0005% Dy_2O_3 – 0.002%, yttrium oxide

Y_2O_3 – 0.22%, ytterbium oxide Yb_2O_3 – 0.001%. The total content of gallium Ga, germanium Gr, and scandium Sn compounds in the milk is up to 0.1% [Tleukeyeva A., 2021].

Phosphorus-containing waters are formed as a result of the collection of residual water after the flooding of spontaneously combustible sludge and storm water seeping through the slag. The content of biogenic elements such as potassium and phosphorus in the initial solid sediments suggests their presence in aqueous solutions. Therefore, the next step in the research was to conduct a chemical and microbiological examination of wastewater.

As a result of chromatographic analyzes, it was found that the composition of samples A, B, C is similar and differs in the quantitative characteristics of the ingredients contained (Table 1). The largest amount of sodium, potassium, ammonium, phosphate, chloride, sulfate, and fluoride ions was found in sample C. In all three samples, potassium and phosphorus ions occupy the most part.

The hydrobiological analysis of the samples showed the presence of green monadic and diatoms; Amoeba limax was found in a significant amount, which indicates a lack of oxygen and the predominance of biogenic elements in the wastewater composition (Table 2). The *Chlorella vulgaris* population was found only in sample B.

Microbiological examination revealed that the microflora of samples A and B were identical, coccoid bacteria of streptococcal configuration were abundantly represented in them. The total number of heterotrophs is 3.1 × 10^9 CFU/ml and 3.3 × 10^9 CFU/ml, respectively. The microflora of sample B showed the presence of coccoid bacteria forming large round convex colonies with a smooth and smooth edge, 2–8 mm in diameter, red, opaque and homogeneous, the total number of heterotrophs was 1.1 × 10^9 CFU. The dominant group of microorganisms is represented by the species *Bacillus megaterium var. phosphaticum*. In addition, the samples revealed sarcinogenic species of bacteria and actinomycetes. Nitrogen-fixing microorganisms are represented by oligonitrophilic species; *Azotobacter vinelandii* is found in small quantities.

Table 1. Chemical composition of water samples from «Kainar» LLP

Name	Sample A	Sample B	Sample C
pH, unit	5.1	5.1	5.8
Cations, mg/dm^3			
Li^+	0.83	1.31	2.82
Na^+	109.6	1575.3	5398.9
NH_4^+	19.2	39.8	225.8
K^+	325.0	6041.9	21139.2
Ca^{2+}	285.2	44.0	61.4
Mg^{2+}	142.1	219.1	772.8
Anions, mg/dm^3			
F^-	24.4	54	403.0
Cl^-	38.1	636.8	3233.5
NO_3^-	1.7	41.9	12.8
PO_4^{3-}	2959.2	12319.2	66385.8
SO_4^{2-}	174.3	4747.9	11330.7

Characteristics of the *Chlorella vulgaris* ASLI-1 algae strain

The algae strain *Chlorella vulgaris* ASLI-1 was isolated from the Koshkar-ata river (Shymkent, Turkestan region) and cultivated on Myers agar medium at a temperature of 25°C and an incubation period of 15 days. Algae cells are round, rarely ellipsoid, green color, sizes from 3 to 15 microns, gram negative. Reproduction occurs by autospores, are acid unstable, have a cellulose shell. Colonies are round, smooth, yellow-green in the beginning, later a rich green hue. The culture showed the following physiological and biochemical properties: photoautotrophs, the type of catabolism, photosynthesis, free-living, aerotolerant, the sun's rays are necessary for reproduction, the optimum temperature is 23–35 °C at a pH of 7–11.

According to the results of a study for non-pathogenicity of the culture, it was revealed that the strain does not show signs of lecithinase and hemolytic activity on yolk and blood agar. The results of the study of the virulence of the culture are shown in Table 3.

The results of the experiments showed that with the intra-abdominal injection of the culture at a dose of 10^9 CFU, three animals fell ill. After oral infection at a dose of 10^{11} CFU, 2 mice

Table 2. Organisms-hydrobionts of waters LLP "Kainar"

No.	Hydrobionts	Sample A	Sample B	Sample C
1	*Amoeba limax*	3	3	3
2	*Navicula* sp.	2	2	0
3	*Meridion circulare*	3	3	0
4	*Diatoma* sp.	2	0	2
5	*Chlamydomonas* sp.	4	0	4
6	*Chlorella vulgaris*	0	2	0
7	*Aspidisca* sp.	0	1	0

Table 3. Results of the study of acute toxicity of the culture of *Chlorella vulgaris* ASLI-1with intra-abdominal and oral administration

No.	Number of animals in the experiment	Route of administration	Dose CFU/ml	Animals fell ill	Animals died	Animals survived
1	12	Intraperitoneal	10^3	0	0	12
2	12	Intraperitoneal	10^5	0	0	12
3	12	Intraperitoneal	10^7	0	0	12
4	12	Intraperitoneal	10^9	3	0	12
Control	12	Intraperitoneal	Physical solution	0	0	12
5	12	Orally	10^5	0	0	12
6	12	Orally	10^7	0	0	12
7	12	Orally	10^9	0	0	12
8	12	Orally	10^{11}	2	0	12
Control	12	Orally	Physical solution	0	0	12

became ill. 24 hours after the introduction of the culture, they were noted: lethargy, loss of appetite, stool thinning, tousled coat. On the 2nd day after infection, all mice recovered. The results of morphological changes in internal organs after dissection showed that culture does not affect their changes. The organs are clean, smooth, easily detachable, no adhesions are noticed.

The average allergenic dose of the culture was 7.8×10^5 CFU per animal, i.e. the strain has practically no allergenic effect. When studying the site of irritation at a dose of 1×10^9 CFU/cm^3, a weak positive reaction was observed in the form of an injection of the vessels of the sclera and cornea, mucous secretions from the eyes. On the third day of observations, the above phenomena in all animals were completely stopped and the next 5 days, no deviations from the norm were observed. Thus, the culture has a mild local irritant effect.

Cultivation of *Chlorella vulgaris* ASLI-1 under model conditions using phosphorus-containing wastewater.

In the course of laboratory experiments carried out on the test crops, it was found that the *Chlorella vulgaris* ASLI-1 strain at a dose of 30×10^9 CFU applied to the soil increases the yield of strawberries by 23.1 ± 1.9%, pumpkin by 23.2 ± 0.9% and soybeans by 24.3 ± 1.7%. In addition, it was noted that inoculation of a culture of green algae directly onto plant seeds increases the fungistatistical effect of plants [28].

As a result of cultivation of the *Chlorella vulgaris* ASLI-1 strain under model conditions using different concentrations of phosphorus-containing waters, the stimulating effect of the composition of sample B was established, as can be seen in Figure 2. It was found that the microalga strain actively gains biomass in 5% and 10% sample solutions B.

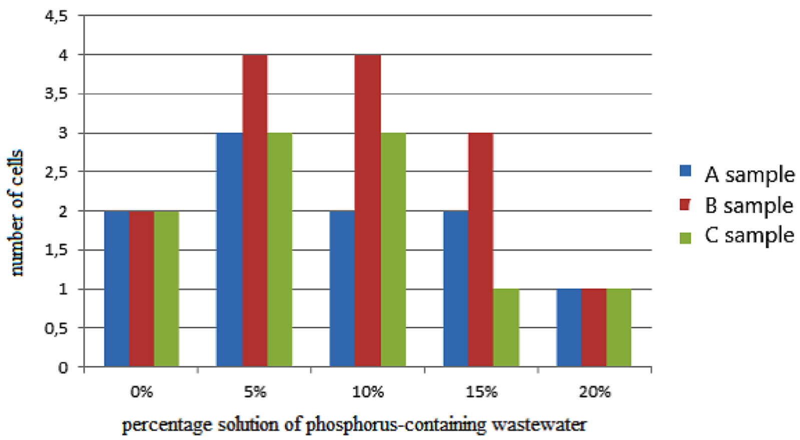

Figure 2. Results of cultivation of Chlorella vulgaris ASLI-1 on phosphorus-containing waters of Shymkent
Designation: 0 – absent, 1 – single, 2 – rare, 3 – many, 4 – abundant

When spectrophotometric determination of the optical density of the biomass of the culture of *Chlorella vulgaris* ASLI-1, it was found at a wavelength of 490 nm, the highest density in the samples was 5% (0.67 optical density units) and 10% (0.92 optical density units) of sample B solution, respectively.

CONCLUSION

According to the results of the study, it can be noted that the chemical composition of phosphorus-containing wastewater is identical and differs only in the quantitative characteristics of the ingredients. Ions of phosphates, sulfates and chlorides predominate. The hydrobiological composition is depleted, but the presence of biogenic elements in the form of phosphates and potassium contributes to the eutrophication of water, which is an indicator for the cultivation of green algae. The microflora of waters is characterized by the predominance of forms of microorganisms participating in the biogeochemical cycle of phosphorus in nature. It was found that during the cultivation of the *Chlorella vulgaris* ASLI-1 strain on various concentrations of phosphorus-containing wastewater, the highest biomass density was obtained in sample Б, which was taken from a pit at the site of storage of phosphorus-containing sludge, with a waste percentage of 5% and 10%, 10^6 CFU and 10^7 CFU, respectively.

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