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SPECIFIC FEATURES OF NATIVE CHEMOLITHOTROPHIC MICROBIOTA WASTES PRODUCED BY THE BIOENERGY INDUSTRY

I. A. Blayda
T. V. Vasilyeva
L. I. Slyusarenko
V. F. Khytrych

Odesa Mechnikov National University, Ukraine

E-mail: iblayda@ukr.net

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The study's aims were to isolate and establish pure cultures of prevailing microorganisms from the aboriginal consortium in coal fly ash, describe their physiology, biochemistry and practically-useful properties, and compare the efficiency of bioleaching metals from fly ash using pure cultures and the consortium. Through enrichment cultures on standard media we isolated pure cultures of the microorganisms which were then preliminarily identified using standard techniques. This allowed us to isolate from fly coal ash pure cultures of three prevailing strains of mesophilic and moderately thermophilic coal fly ash acidophilic chemolithotrophic bacteriae, belonging to *Acidithiobacillus*, such as *Acidithiobacillus ferrooxidans*, and *Sulfobacillus*. The strains exhibited high oxidative activity in leaching the rare metals Gallium and Germanium, as well as some heavy metals, from fly ash substrate. A comparison of oxidative activity of the isolated strains and the aboriginal consortium under mesophilic conditions led to the conclusion about advantage of consortium, because it had arisen from syntrophy of microbes in the community. This should be taken into account at the developing of bacterial preparations that are optimal for the technogenic substrate.

Key words: fly ash, aboriginal microbial consortia, acidophilic chemolithotrophic bacteriae, leaching activity, Germanium.

Exhaustion of the world's worth of mineral resources and growing volumes of technogenic wastes with complex, multicomponent contents demand alternative technological solutions for recycling. Energy industry wastes, such as fly ash and slag from burning coals, are at the same time dangerous to environment and industrially significant, since they contain rare metals. The most profitable and ecologically sound method of their recycling is bio hydrometallurgical. It uses oxidative activity of microbes of the aboriginal consortium shaped by the wastes' deposition and storage history [1–3]. Microbial biotechnology should be based on research encompassing biological and physicochemical properties of the initial hard substrate and its aboriginal microbial consortium, with subsequent isolation, identification and selection of the most promising highly active strains to create an efficient bacterial preparation. We established that during the process of ash formation, impoundment and storage under the influence of certain technogenic and native factors, a highly efficient and specific consortium is formed, mostly by heterotrophic and acidophilic chemolithotrophic bacteriae. The highest leaching activity was shown when rare metals from initial technogenic substrates were leached by species of *Acidithiobacillus* and *Sulfobacillus* [4–6].

The study's goal was to isolate the prevailing microorganisms from the coal fly ash (FAAC) of industrial wastes into pure cultures, determine their properties, and to compare the efficiency of bioleaching metals from wastes using pure isolates and the aboriginal consortium. It can be considered as a continuation of our earlier work concerning evaluating quantitative and qualitative structure of microbiocenoses of energy industry wastes [7].

Materials and Methods

The object of our research was fly ash obtained by burning a mixture of native fossil coals on DTEK thermal power station in Ladyzhyn (Vinnytsia region, Ukraine). It contained (%): Fe — 5.93; Al — 3.89; Si — 12.10; Ti — 4.16; Ca — 0.20; Cu — $6.82 \cdot 10^{-3}$; Zn — $3.27 \cdot 10^{-2}$; Mn — $5.73 \cdot 10^{-2}$; Pb — $1.09 \cdot 10^{-2}$; Ni — $1.77 \cdot 10^{-2}$; Cd — $5.31 \cdot 10^{-4}$; Sn — $2.07 \cdot 10^{-2}$; Cr — $2.18 \cdot 10^{-2}$; V — $2.15 \cdot 10^{-2}$; Co — $3.05 \cdot 10^{-2}$; Sr — $6.56 \cdot 10^{-2}$; Ba — $6.34 \cdot 1^{-2}$; Zr — $2.37 \cdot 10^{-2}$; Rb — $1.16 \cdot 10^{-2}$; Nb — $1.90 \cdot 10^{-3}$; La — $4.20 \cdot 10^{-3}$; Ce — $7.40 \cdot 10^{-3}$; Ga — $1.02 \cdot 10^{-3}$; Ge — $2.80 \cdot 10^{-3}$; S — 1.24; C (underburning) — 9.98.

To obtain pure cultures of acidophilic chemolithotrophic bacteriae we used enrichment cultures on standard media (Table 1), based on literature as well as our own research which showed that in mineral substrates of both geogenic and technogenic origin, there are mesophilic and thermophilic chemolithotrophic bacteriae and *Sulfobacillus* sp. [3, 8–10]. As energy source, we amended the mineral background with elemental sulfur, its inorganic (sodium thiosulfate $Na_2S_2O_3$) and organic (thio urea $CS(NH_2)_2$) compounds, or bivalent iron ($FeSO_4 \cdot 7H_2O$) [7, 9, 11, 12]. To a 50.0 cm^3 Erlenmeyer flask we put 2.0 g of fly ash and 20.0 cm^3 of a medium, ratio 1:10 (solid:liquid). The enrichment cultures of mesophilic acidophilic chemolithotrophic bacteriae were kept at 35.0±2.0 °C, moderately thermophilic — at 55.0±2.0 °C and *Sulfobacillus* sp. — at 45.0±2.0 °C for 7 days. The microbes' development was evaluated by the change in the medium's pH, appearance of slight suspension and a film on the culture's surface.

The liquid, enriched with bacteriae, was sown on agarized medium of the similar composition, containing sulfur, thiosulfate, thiourea or bivalent iron. The enrichment cultures of *Sulfobacilla* sp. before resowing were kept for ten minutes at 80.0±2.0 °C. The process allowed to purify initial isolates and to obtain microscopically and colonially homogeneous cultures. Pure cultures on the surface of selective agarized media established colonies which could be readily analyzed. Cultures were concluded to be free on the basis of staining and microscopy. Microscopically and morphologically pure cultures were stained after Gram using standard dyes (solutions of gentian violet, Lugol's iodine, and fuchsin), and also determined the range and optimal values of temperature and pH, ability to autotrophic and mixotrophic growth, oxidation of different compounds of sulfur and bivalent iron, reaction to organic matters, and ability to bioleach metals from raw mineral materials.

For the measure of biogeochemical activity of isolated strains and FAAC we took the concentration of metals which went into the culture medium from the solid phase. The degree of metal extraction calculated as the ratio (%) of the amount of metal which entered the solution as a result of the medium's contact with the substrate in the presence of microorganisms, to the amount of the metal in the initial solid substrate (100% stands for all metal being dissolved). As the control, it

Table 1. **Medium composition for acidophilic chemolithotrophic bacteriae**

Minerals	Concentration, g/dm^3	
	9K for *Acidithiobacillus* spp.	Modified 9K* for *Sulfobacillus* spp.
$NH_4(SO_4)_2$	3.0	0.45
KCl	0.1	0.05
K_2HPO_4	0.5	–
KH_2PO_4	–	0.05
$MgSO_4$	0.5	0.5
$Ca(NO_3)_2$	0.01	0.014
Na_2SO_4	–	0.15
Yeast extract	–	0.20
	Energy source	
$Na_2S_2O_3$	5.0	–
SO	2.0	–
$CS(NH_2)_2$	4.8	–
$FeSO_4 \cdot 7H_2O$	44.5	30.0

Note. Here and hereafter 9K* stands for modified 9K medium for *Sulfobacillus* spp.

was used the data obtained in sterile leaching solutions of the media on autoclaved fly ash.

Morphology of cells and the colonies they form were studied with light microscopy (Primo Star PC, Germany) and electron microscopy (PEM100-01, Ukraine). Biomass growth was determined on a spectrophotometer DR 3900 (Germany) at 540 nm wavelength. The concentration of thiosulfate and the presence of intermediate products of its oxidation were measured by a standard iodometry technique [13]. The concentration of metals in solutions was determined by atomic absorption spectroscopy (AAC-1, Germany and C-115PC Selmi, Ukraine) [14]. The significance of the results was evaluated using Student's criterion with $P < 0.05$.

Results and Discussion

As one culture can grow on media differing in both contents and concentrations, primary (initial) isolation of microorganisms using various media can result only in groups of bacteriae, not in pure species [10–12]. In our experiments the development of enrichment culture of mesophilic, moderately thermophilic thionic bacteriae and sulfobacteriae was accompanied by pH increasing (from 1.7 in the beginning to 3.0–4.5) and a change in the culture medium's appearance.

As a result, we isolated from fly ash into pure culture 25 strains of acidophilic chemolithotrophic thionic bacteriae. Three of them were selected for further work, isolated on culture media 9K and 9K* with $FeSO_4 \cdot 7H_2O$ as an energy source. They were numbered according to the conditions of isolation: mesophilic — MesPhLad27, moderately thermophilic — MThPhLad25, sulfobacteriae — SBLad29. The properties of the strains are listed in Table 2, their pictures are given at Fig. 1 and 2.

The properties of strains MesPhLad27 and MThPhLad25 revealed in them representatives of *Acidithiobacillus,* while their ability to oxidize bivalent iron, as long as other energy sources, allowed us to tentatively identify them as *Acidithiobacillus ferrooxidans*. The moderately thermophilic strain SBLad29, isolated and studied on 9K* medium at 45.0±2.0 °C, was similarly classified as *Sulfobacillus* [15]. A definite conclusion about their taxonomy would require a molecular-genetical study.

Next, we compared the ability of the isolated strains of acidophilic chemolithotrophic bacteriae and the aboriginal consortium

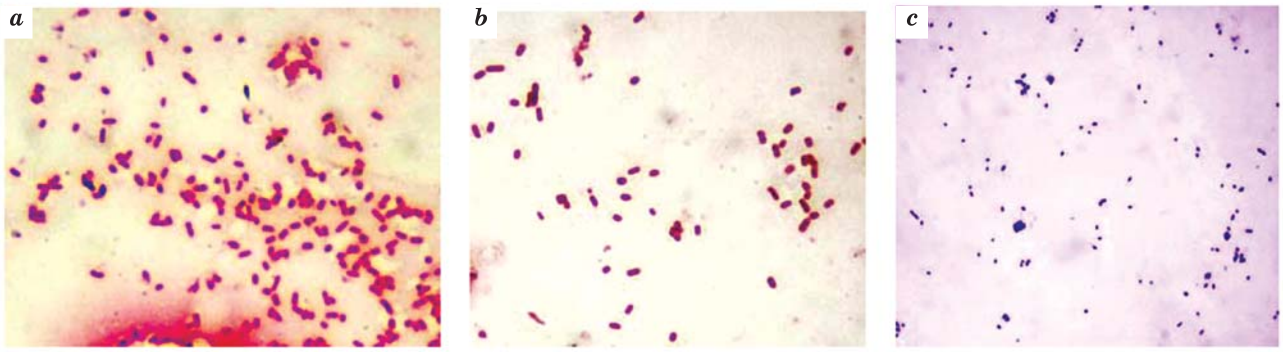

Fig. 1. **Stained microscopic preparations of strains:** MesPhLad27 (*a*); MThPhLad25 (*b*); SBLad29 (*c*). ×1 000

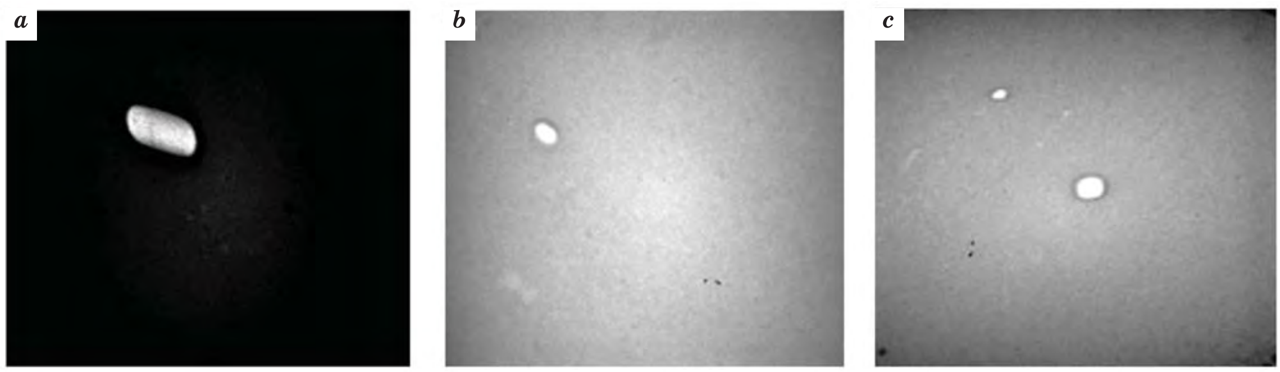

Fig. 2. **Electron microphotographs:** MesPhLad27 (*a*); MThPhLad25 (*b*); SBLad29 (*c*). ×11 000

Table 2. **Morphology of cells and cultures of bacterial strains isolated from fly ash**

Properties		Isolated strains		
		MesPhLad27	MThPhLad25	SBLad29
Morphology, shape of cells		Small thin	Small thin	Small coccobacilli
Gram stainin		Gram-negative	Gram-negative	Gram-negative
Sporulation		Don't sporulate	Don't sporulate	Don't sporulate
Morphology, shape of colonies		Tear-shaped, rounded, convex, grainy, bright-yellow	Tear-shaped, rounded, convex, grainy, bright-yellow	Tear-shaped, rounded, convex, grainy, bright-yellow
PH value	Range	2.0–5.0	2.0–5.0	2.0–5.0
	Optimal	≤ 2.0	≤ 2.0	3.0
Temperature, °C	Range	4.0–37.0	10.0–65.0	10.0–60.0
	Optimal value	35.0±2.0	55.0±2.0	45.0±2.0
Energy source	SO	+	+	+
	$Na_2S_2O_3$	+	+	+
	$CS(NH_2)_2$	+	+	+
	$FeSO_4 \cdot 7H_2O$	+	+	+
Degree of oxidation of $Na_2S_2O_3$,%		47.0	86.0	88.0
Growth under mixotrophic conditions				
In presence of sugars (0.02%)	Glucose	+	+	+
	Sucrose	+	+	+
	Molasses	+	+	+
In presence of yeast extract (0.02%)		+	+	+
Growth on meat peptone agar		–	–	–
Extraction of metals from fly ash		+	+	+

to bioleach metals. The results are shown on Fig. 3. The control for metal extraction by strains MThPhLad25 and MesPhLad27 was sterile medium 9K with bivalent iron as energy substrate at 55.0±2.0 °C (Control 1) and 35.0±2.0 °C (Control 2), respectively, for SBLad29 — sterile 9K* medium with bivalent iron as energy source at 45.0±2.0 °C (Control 3).

The data suggest a relatively high activity of the isolated strains towards extraction of Gallium (67.0–88.2% of extraction), Germanium (75.0–98.8%), Cadmium (53.5–72.1%), copper (87.7–98.8%), nickel (57.0–67.7%), and to a lesser degree — zinc, lead and manganese. Strain MesPhLad27's bioleaching abilities towards the metals were the least among the researched bacteriae.

Table 3 contains data on metal extraction from the same substrate of fly ash by the FAAC. The results suggest that as an association, the mesophilic consortium of acidophilic chemolithotrophic bacteria is more efficient than just the isolated strain MesPhLad27.

This might be a result of common for microorganisms syntrophic relationships, e.g., the substrates might be destroyed by certain bacteriae, without which other bacteriae's action upon them is impossible or substantially slower. Thus, the authors of [8] suppose that *Acidithiobacillus thiooxidans*, which is often present together with *Acidithiobacillus ferrooxidans*, due to a more rapid oxidation of sulfur under a non-direct mechanism of bioleaching create favorable conditions for the growth of iron-oxidizing bacteriae. Therefore, the cycle of substrate oxidation runs significantly faster and more efficient:

$$MeS + Fe_2(SO_4)_3 \rightarrow MeSO_4 + 2FeSO_4 + S^0$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow (\text{bacteriae}) \rightarrow H_2SO_4.$$

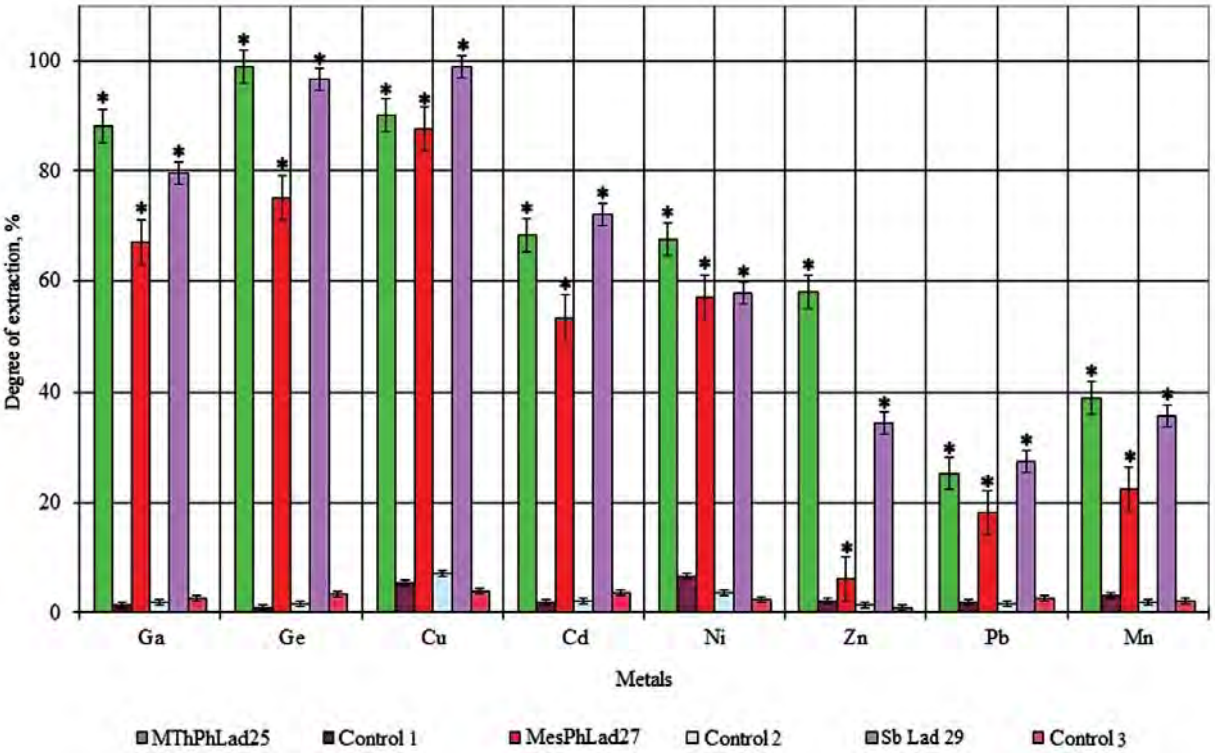

Fig. 3. **Degree of extraction (%) of metals from fly ash by strains, isolated from aboriginal association**
* — $P < 0.05$ compared to the control

Table 3. **Degree of metal extraction from fly ash by the aboriginal consortium of chemolithotrophic bacteriae (%)**

Metals	Mesophilic consortium of acidophilic chemolithotrophic bacteriae	Moderately thermophilic consorrtium of acidophilic chemolithotrophic bacteriae	
	9K medium	9K medium	9K* medium
Ge	99.76	99.98	99.82
Ga	94.92	82.75	95.75
Ni	76.64	84.94	87.63
Cd	99.99	69.95	92.35
Cu	89.54	69.37	16.54
Zn	20.22	20.82	5.92
Mn	37.75	47.85	98.52
Pb	35.67	19.34	22.46

When we compared the bioleaching activity of moderately thermophilic consortium of acidophilic chemolithotrophic bacteriae and of the isolated strains of MThPhLad25 and SBLad29 we didn't observe a general tendency in favor of syntrophic relationships of microorganisms in association.

On the whole, our work on pure cultures of microorganisms from FAAC allowed us to isolate three prevailing strains of mesophilic and moderately thermophilic acidophilic chemolithotrophic bacteriae which we identified as belonging to the genus *Acidithiobacillus*, in particular

Acidithiobacillus ferrooxidans, and *Sulfobacillus*. All isolated strains were characterized by high bioleaching activity towards rare metals Gallium and Germanium, as well as some heavy metals from the studied technogenic substrate. A comparison of activity of the isolated strains and the FAAC under mesophilic conditions provides support for the consortium being a result of syntrophy between microbes in the community.

The results can be used to develop a highly efficient, stable, optimal for a given substrate type bacterial formula, able to leach both rare and heavy toxic metals. However, this would need a study of the properties of isolated strains' growth in autotrophic and mixotrophic conditions. It is also necessary to carry out optimization of medium's composition for the maximal growth rates.

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ОСОБЛИВОСТІ АБОРИГЕННОГО УГРУПОВАННЯ ХЕМОЛІТОТРОФНИХ БАКТЕРІЙ ІЗ ВІДХОДІВ БІОЕНЕРГЕТИКИ

І. А. Блайда
Т. В. Васильєва
Л. І. Слюсаренко
В. Ф. Хитрич

Одеський національний університет імені І. І. Мечникова, Україна

E-mail: iblayda@ukr.net

Метою роботи було виділення домінуючих чистих культур мікроорганізмів з аборигенного угруповання золи-виносу від спалювання вугілля, встановлення їхніх властивостей, порівняння ефективності біовилуговування металів із золи-виносу з використанням чистих культур та аборигенного угруповання. Методом накопичувальних культур із застосуванням стандартних живильних середовищ виділено чисті культури мікроорганізмів, а стандартними мікробіологічними методами встановлено їх передбачувану таксономічну належність. У результаті з аборигенного угруповання мікроорганізмів золи-виносу в чисті культури виділено три домінуючих штами мезофільних і помірно термофільних ацидофільних хемолітотрофних бактерій і встановлено їх належність до представників роду *Acidithiobacillus*, зокрема *Acidithiobacillus ferrooxidans*, а також *Sulfobacillus*. Ці штами відзначалися високою окиснювальною активністю стосовно вилучення рідкісних металів галію і германію, а також деяких важких металів із субстрату золи-виносу. Порівняння окиснювальної активності виділених штамів і аборигенного угруповання в мезофільних умовах свідчить на користь консорціуму як результату синтрофних відносин мікроорганізмів в угрупованні. Це слід враховувати при створенні оптимального для даного техногенного субстрату ефективного бактеріального препарату.

Ключові слова: зола-винос, аборигенне угруповання, ацидофільні хемолітотрофні бактерії, активність вилуговування, германій.

ОСОБЕННОСТИ АБОРИГЕННОГО СООБЩЕСТВА ХЕМОЛИТОТРОФНЫХ БАКТЕРИЙ ИЗ ОТХОДОВ БИОЭНЕРГЕТИКИ

И. А. Блайда
Т. В. Васильева
Л. И. Слюсаренко
В. Ф. Хитрич

Одесский национальный университет имени И. И. Мечникова, Украина

E-mail: iblayda@ukr.net

Целью работы было выделение доминирующих чистых культур микроорганизмов из аборигенного сообщества золы-уноса от сжигания угля, установление их свойств, сравнение эффективности биовыщелачивания металлов из золы-уноса при использовании чистых культур и аборигенного сообщества. Методом накопительных культур с использованием стандартных питательных сред были выделены чистые культуры микроорганизмов, а стандартными микробиологическими методами определена их предполагаемая таксономическая принадлежность. В результате из аборигенного сообщества микроорганизмов золы-уноса в чистые культуры выделены три доминирующих штамма мезофильных и умеренно термофильных ацидофильных хемолитотрофных бактерий и установлена их принадлежность к представителям рода *Acidithiobacillus*, в частности *Acidithiobacillus ferrooxidans*, а также *Sulfobacillus*. Эти штаммы отличались высокой окислительной активностью по отношению к извлечению редких металлов галлия и германия, а также некоторых тяжелых металлов из субстрата золы-уноса. Сравнение окислительной активности выделенных штаммов и аборигенного сообщества в мезофильных условиях свидетельствует в пользу консорциума как результата синтрофных отношений микроорганизмов в сообществе. Это следует учитывать при создании оптимального для данного техногенного субстрата эффективного бактериального препарата.

Ключевые слова: зола-унос, аборигенное сообщество, ацидофильные хемолитотрофные бактерии, активность выщелачивания, германий.