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Screening and Identification of Microorganisms – Potential Producers of Arachidonic Acid

A. V. Goncharova, T. A. Karpenyuk, Y. S. Tsurkan, R. U. Beisembaeva, A. M. Kalbaeva, T. D. Mukasheva, and L. V. Ignatova

***Abstract*—**Microorganisms isolated from water and soil of Kazakhstan to identify potential high-effective producers of the arachidonic acid, exhibiting a wide range of physiological activity and having practical applications were screened. Based on the results of two independent tests (the test on the sensitivity of the growth processes of microorganisms to acetylsalicylic acid - an irreversible inhibitor of PGH-synthase involved in the metabolism of arachidonic acid and its derivatives, the test for inhibition of peroxidase activity of membrane-bounding fraction of PGH - synthase by acetylsalicylic acid) were selected microbial cultures which are potential high-producer of arachidonic acid. They are characterized by a stable strong growth in the laboratory conditions. Identification of microorganism cultures based on morphological, physiological, biochemical and molecular genetic characteristics was performed.

***Keywords*—**Arachidonic acid, aspirin-sensitive culture, bacteria, producers, screening.

I. Introduction

ARACHIDONIC acid (AA) – a polyunsaturated fatty acid (PUFA), is a substrate needed for the synthesis of eicosanoids (prostaglandins, leukotrienes, thromboxanes). It shows a wide range of physiological activities, including controls the activity of various protein kinases, G-proteins, adenylate and guanylate cyclase, it's also involved in the regulation of gene expression at the transcriptional level and in the modulation of calcium-cell responses to thrombin and adenosine triphosphate, regulates the intracellular levels of calcium and potassium induces apoptosis [1], [2]. AA is an active ingredient of drugs and diet components, preventing atherosclerosis, coronary heart disease and other diseases [3], [4].

Fungi *Phycomycetes* of the genus *Mortierella* are highly efficient producers of AA [5], [6]. At present, biotechnological method of obtaining arachidonic acid on the basis of the strain *Mortierella alpina* has developed.

Broaden scope of AA application and its low content in natural sources (porcine liver, adrenal gland, egg yolk) dictates the need for the development of microbial production of AA. Microorganisms have a distinct advantage over the plant and animal raw materials. These advantages include: the rapid growth of cultures in different conditions; the ability to collect and synthesize large amount of lipids (70%), including 50% PUFA from them; the ability to increase the biomass in areas unsuitable for seeding.

Screening of microorganisms isolated from water and soil of Kazakhstan was carried out for identification of the potential high-effective producers of arachidonic acid.

A. V. Goncharova is with the Scientific Research Institute of Biology and Biotechnology Problems; Al-Farabi KazNU, Almaty, Kazakhstan (phone: 727-377-3329; fax: 727-377-3437; e-mail: Alla.Goncharova@kaznu.kz).

T. A. Karpenyuk, Y. S. Tsurkan, R. U. Beisembayeva, A. M. Kalbayeva, T. D. Mukasheva, and L. V. Ignatova are with the al-Farabi Kazakh National University, Almaty, Kazakhstan (phone: 727-377-3329; fax: 727-377-3437; e-mail: Tatyana.Karpenyuk@kaznu.kz, Yana.Tsurkan@kaznu.kz, rbejsembaeva @yandex.ru, Alya.Kalbaeva@kaznu.kz, Togzhan.Mukasheva@kaznu.kz, Lyudmila.Ignatova@kaznu.kz, respectively).

II. Materials and Methods

The objects of study were culture of microorganisms isolated from water and soil of Kazakhstan by limiting dilution in the universal solid medium (MPA) [7], [8]. The purity of isolated cultures was monitored by seeding onto nutrient agar and microscopic examination [9]. Primary screening of microorganisms for the ability to synthesize arachidonic acid carried out by Eroshin V.K method [10]. Bacteria were cultured in medium supplemented with different MPA concentrations of acetylsalicylic acid (ASA) (0.42g/L; 0.84g/L; 1.68g/L). For control we used the medium without adding ASA. The definition of cultural, morphological, physiological and biochemical properties of microorganisms was carried out by common methods [7]-[9]. Preliminary identification of isolated cultures were performed using the "Bergey's Manual of Systematic Bacteriology" [11]-[13].

Identification of cultures by the method of determination of nucleotide sequence of 16S rRNA gene fragments with subsequent determining the nucleotide sequence identity with the sequences deposited in the international database Gene Bank was performed for clarify the species accessory of aspirin-sensitive cultures. For extraction of chromosomal DNA the method of Kate Wilson was used [14].

DNA concentration was measured using a NanoDrop spectrophotometer at 260nm. For the amplification of 16S rRNA gene fragment was performed PCR with universal primers [15] 8f 5 '- AgAgTTTgATCCTggCTCAg-3 and 806R - 5' ggACTACCAgggTATCTAAT. The program of PCR amplification included long denaturation of RNA in 95°C for 7 min; 30 cycles of 95°C - 30 seconds, 55°C-40, 72°C - 1min, final elongation of 7min at 72°C, the PCR program was performed using a thermocycler GeneAmp PCR System 9700 (Applied Biosystems).

Purification of PCR products from the unbound primers performed by the enzymatic method using Exonuclease I

(Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) [16].

Sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions, by followed separation of the fragments on an automatic genetic analyzer 3730xl DNA Analyzer (Applide Biosystems).

The nucleotide sequences of 16S rRNA gene of identified cultures were analyzed using software SeqScape 2.6.0 (Applide Biosystems) that allowed selecting the length of the nucleotide sequences of 730bp, which were identified in the GeneBank by algorithm BLAST (http://www.ncbi.nlm.nih.gov/blast).

Bacterial cells were precipitated by centrifugation (5000 rpm, 15min.) for extraction of membrane-bound fraction of PGH-synthase. The precipitate was responded in a minimum volume of 0.1M Tris-HCl (pH 8.2), transferred to a porcelain mortar, filled with liquid nitrogen and incubated for 2h at 4°C. Damaged cells further triturated, centrifuged (10 000rpm, 10 min) and precipitate was used to determine peroxidase activity of membrane-PGH-synthase. Peroxidase activity was measured by spectrophotometric method and expressed in terms of the amount of oxidized tetraguaiacol [17]. Total protein concentration was determined according to Lowry et al [18]. Experimental results were processed statistically calculating the arithmetic mean and standard deviation [19].

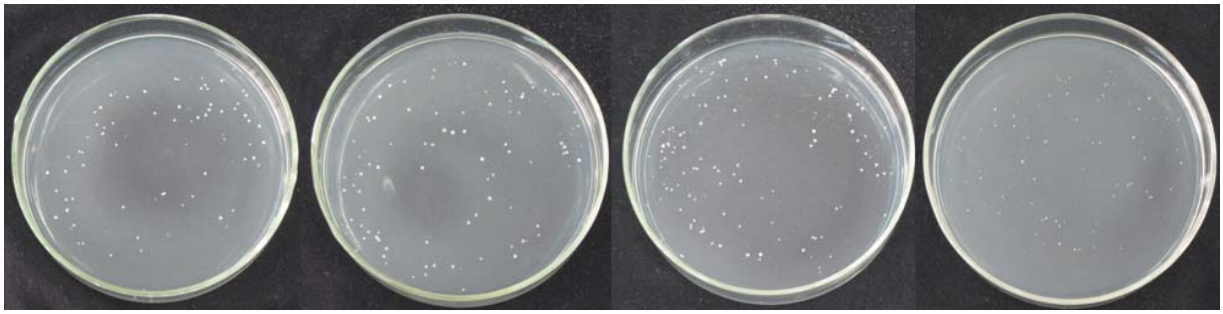
III. Results and Discussion

From the samples of water and soil taken in different regions of Kazakhstan 45 cultures of microorganisms characterizing by consistently strong growth in the laboratory conditions were isolated.

Screening of cultures - potential producers of arachidonic acid was carried out by microbiological method based on the selective effect of ASA on the growth of microorganisms [10]. It is known that aspirin is an irreversible inhibitor of the first enzyme of prostaglandin-synthase bi-enzyme system, prostaglandin H-synthase, the main substrate of which is arachidonic acid [20]. Therefore a preliminary research was performed for 45 selected cultures in the test for sensitivity to aspirin to identify producers of AA. Microorganisms are seeded by Koch's method in medium containing various concentrations of ASA (0.42g/L; 0.84g/L and 1.68g/L), from which selective concentration was 0.84g/L [10] and then analyzed the degree of microorganism growth. The medium without adding ASA used as a control. From 45 cultures of microorganisms taken in experiment, 4 cultures of bacteria were resistant to aspirin and showed stable growth, which coincided with control in all experimental variants. The growth of 24 cultures was completely suppressed by adding to the medium 0.84g/L aspirin, for 12 cultures - at a concentration of ASA in the medium equal to 0.42g/L (Figs. 1-3).

To confirm the ability of cultures sensitive to the presence of ASA in the medium to synthesize AA the peroxidase activity of membrane-bound forms of the enzyme PGH-synthase for which AA is a substrate and ASA acts as an irreversible inhibitor was determined. Studies were performed on 12 cultures, the growth of which was inhibited by the addition of ASA in the medium at a concentration of 0.42 g/L.

It was found that all studied cultures showed peroxidase activity within the limits of a $14.37 \pm 0.2 – 68.44 \pm 0.42$μM of tetraguaiacol at 1mg of protein per 30min, that was inhibited by the addition in the incubation medium ASA (Table I).



(a) (b) (c) (d)

Fig. 1 Effect of aspirin on the growth and development of the aspirin sensitive culture (a) control (b) medium supplemented with ASA 0.42 (c) 0.84 (d) 1.68g/L

(a) (b) (c) (d)

Fig. 2 Effect of aspirin on the growth and development of the aspirin sensitive culture (a) control (b) medium supplemented with ASA 0.42 (c) 0.84 (d) 1.68g/L

(a) (b) (c) (d)

Fig. 3 Effect of aspirin on the growth and development of the culture aspirin sensitive culture (a) control (b) medium supplemented with ASA 0.42 (c) 0.84 (d) 1.68g/L

TABLE I
INVESTIGATION OF PEROXIDASE ACTIVITY OF PGH-SYNTHASE FOR MICROORGANISM CULTURES

№ culture	Peroxidase activity (μM of tetraguaiacol at 1 mg of protein per 30 min)
1	17.48±0.24
2	54.32±0.39
3	14.37±0.2
4	17.25±0.21
5	34.68±033
6	14.84±0.25
7	68.44±0.42
8	15.4±0.21
9	18.21±0.28
10	2005±0.3
11	15.86±0.22
12	29.26±0.32

Thus, based on the results of two independent tests we have selected AA-producing microorganism cultures.

To determine the genus affinity of selected cultures their morphological and cultural, physiological and biochemical characteristics were investigated. To clarify the bacteria species affinity the molecular-genetic identification of the aspirin sensitive cultures was carried out.

It was established that 5 aspirin sensitive cultures belong to the genus of *Bacillus*, 3 – to *Pseudomonas*, 4 – to Flavobacterium, *Enterobacter*, *Ochrobactrum*, *Kocuria* (Table II).

TABLE II
THE RESULTS OF BACTERIAL CULTURES IDENTIFICATION

№ cultures	Results of identification
1	*Pseudomonas peli*
2	*Pseudomonas mandelii*
3	*Pseudomonas migulae*
4	*Bacillus safensis*
5	*Ochrobactrum thiophenivorans*
6	*Bacillus pumilus*
7	*Flavobacterium saliperosum*
8	*Enterobacter asburiae*
9	*Bacillus thuringiensis*
10	*Bacillus endophyticus*
11	*Bacillus simplex*
12	*Kocuria erythromyx*

Obtained data testify that selected cultures are promising for further study as sources of arachidonic acid.

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