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IMPACT OF WATER EXTRACTS OF SPIRULINA (WES) ON BACTERIA, YEASTS AND MOLDS

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ABSTRACT

Background. Due to its chemical composition, Spirulina is widely used as a dietary supplement that exerts positive effects on the human body. It also has the ability to inhibit the growth of certain microorganisms, both pathogens that pose a health hazard, as well as those that cause food spoilage in all branches of food industry. The main aim of this study was to determine the impact of water extracts of *Spirulina* (WES) on the growth of various microorganism both useful and harmful for humans and the economy.
Material and methods. The impact of different WES concentrations (0.1, 1.0, 2.5, or 5.0%) on the growth of various bacteria, yeasts and molds was determined by diffusion method on solid medium.
Results. It was demonstrated that WES have a diversified impact on microorganisms, depending on the species. The inhibitory activity was shown against *Bacillus subtilis*, *Micrococcus luteus*, *Rhodotorula*, and *Penicillium*. WES had strong stimulating effect on *Alicyclobacillus acidoterrestris* and *Geotrichum*. Moreover, higher concentrations of WES stimulated also the development of mycelium and production of conidiophores by *Cladosporium* and *Aspergillus niger*.
Conclusions. Inhibitory impact of WES on microorganisms that cause food spoilage may be used in food production. However, the obtained results indicate the need for further studies, particularly in order to evaluate the effect of the WES on microflora in the food matrices.

Key words: *Arthrospira*, bacteria, food spoilage, food supplementation, growth inhibition and stimulation, molds, yeasts

INTRODUCTION

Spirulina (Arthrospira) platensis is a photosynthetic, multicellular, and filamentous blue-green microalgae, which naturally grows in warm climates. Because it can be used as a food, animal feed, dietary supplements or functional foods *Spirulina* became the object of intensive research [Belay et al. 1996, Belay 2008]. Today, many companies around the world produce preparations of the cyanobacteria biomass from the genus *Arthrospira*, mainly *Arthrospira platensis* and *Arthrospira maxima*, which are sold under the trade name "Spirulina". The genus names *Arthrospira* and *Spirulina* are often used interchangeably with the understanding that all the edible forms that are under commercial cultivation and sold as Spirulina actually belong to the genus *Arthrospira* [Belay 2008].

The chemical composition and nutritional value of Spirulina has been already recognised quite well. It is a very rich source of easy digested and wholly utilized protein (60 to 70% on dry weight basis). *S. platensis* contains also significant amounts of carbohydrates (~16.5%), polyunsaturated fatty acids (particularly γ-linolenic acid), antioxidants, carotenoids, chlorophyll,

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phycocyanin, and xanthophylls (zeaxanthin, cryptoxanthin, lutein, lycopene). Among other nutrients vitamin A, tocopherols, B group vitamins, biotin, folic acid as well as minerals (mainly significant amounts of calcium, iron, magnesium, manganese, potassium, zinc, and selenium) should be mentioned [Ciferri 1983, Mühling et al. 2005, Becker 2007, Dartsch 2008, Wu and Ho 2008, Gouveia et al. 2008, Chu et al. 2010].

Spirulina platensis, thanks to its chemical composition, is widely used primarily as a dietary supplement that exerts positive effects on the human body. Among other things, it reduces the levels of free fatty acids and triglycerides in the blood [Mühling et al. 2005], is effective to treat different diseases like tumors, arthritis, diabetes, hepatotoxicity, cardiovascular diseases, hyperglycemia, hyperlipidemia, allergies and anemia [Mani et al. 2008]. Spirulina has anti-inflammatory, antibacterial, antifungal, and antiviral properties, can stimulate and regulate the work of the immune system, helps in the treatment of radiations sickness, immune deficiencies and intoxications [Hernández-Corona et al. 2002, Kaushik and Chauhan 2008, Wu and Ho 2008, Duda-Chodak et al. 2010]. In addition to the above-mentioned properties of *Spirulina*, preparations contribute to the growth of intestinal bacteria, mainly *Lactobacillus*, lower blood glucose levels, and protect the kidneys from damage caused by heavy metals and drugs [Belay et al. 1996, Kutala et al. 2008].

Cyanobacteria of the genus *Arthrospira* also have the ability to inhibit the growth of certain microorganisms, both pathogens that pose a health hazard, as well as those that cause food spoilage in all branches of food industry [Kaushik and Chauhan 2008]. Thanks to this *Spirulina* may be an alternative to the widely used in food technology chemical preservatives.

The main objective of this study was to determine the effect of water extracts of *Spirulina* (WES) on the growth of various bacteria, yeasts and molds. On the basis of the obtained results further research for verifying the interactions between WES and the microflora in different food matrices could be design.

MATERIAL AND METHODS

Material

The commercially available preparation Bio-Spirulina (Anagram Company, Taiwan) purchased in a shop with organic food was used for the study. Pure cultures of bacteria: *Micrococcus luteus* (DSM 20030), *Escherichia coli* (DSMZ 4261), *Bacillus subtilis* (DSM 10) and *Alicyclobacillus acidoterrestris* (DSM 2498) were purchased in The Deutsche Sammlung von Mikroorganismen Und Zellkulturen (DSMZ) – German Collection of Microorganisms and Cell Cultures. All yeasts (*Rhodotorula* sp., *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombe*) and molds (*Penicillium* sp., *Aspergillus niger*, *Rhizopus oryzae*, *Fusarium* sp., *Geotrichum* sp.) were provided by The Collection of Pure Cultures of the Department of Fermentation Technology and Technical Microbiology (University of Agriculture, Krakow, Poland).

Preparation of water extracts of Spirulina (WES)

The tablets of Bio-Spirulina were triturated in a mortar to a powder. Then 40 g was weighed and poured with 1 l of deionized water. The mixture was boiled for 2 h, under cover, over low heat. The extract was transferred to a centrifuge vials and centrifuged (5000 rpm/min, 10 min). Supernatant was collected, filtered through a filter paper. Such prepared solution was lyophilized. The resulting lyophilized cyanobacteria were dissolved in distilled water to prepare aqueous extracts of the following final concentrations: 0.1%, 1%, 2.5% and 5%. All extracts have been sterilized by filtration (0.45 µm) and kept in the refrigerator before experiments.

Assessment of the WES impact on microorganisms' growth

The impact of water extracts of Spirulina on the growth of microorganisms was determined by diffusion method. For this purpose, the Petri dish was poured with suitable medium (Table 1), which after solidification was inoculated with an appropriate strain of organism tested (200 µl of 24-h liquid bacterial or yeasts culture, 0.5 McFarland inoculum or 50 µl of molds culture). Microorganisms were spread over the surface of the medium and left for 20 minutes to absorb. Then 4 wells (7 mm in diameter) were made in the medium, and 50 µl of water extract of Spirulina (0.1, 1.0, 2.5, or 5.0%) was added to each one. The controls were antibiotics solutions for fungi or ready to use discs soaked with appropriate antibiotics for bacteria (Antimicrobial Susceptibility Discs, Biolab Inc., Budapest, Hungary; Table 1). The zone of microbial growth inhibition around the wells was measured after 24 h of incubation at proper temperature. The results were expressed as distance (mm) from the edge of the well to the point at which microorganisms begin to grow again.

RESULTS

The present study demonstrated that the influence of water extracts of Spirulina depended on the microorganism species, and was from the inhibitory to highly stimulating impact.

The size of inhibition zone, calculated as the length (mm) from the edge of the wells to the point where microbes begin to grow again, is presented in Table 2. No inhibition was determined by the symbol ø. By contrast, when the extract did not inhibit, but stimulated the growth of a microorganism, the + sign before the number indicating the size area of high growth was used. For example, "+3" indicates the stimulation of growth in length of 3 mm around the well edge.

Bacteria

Analysis of the bactericidal properties of water extracts of Spirulina (WES) has demonstrated the ability to growth inhibition of *Bacillus subtilis* and, to a lesser extent, of *Micrococcus luteus* (Table 2 A). In the case of *Bacillus subtilis*, higher concentrations of extract (2.5% and 5%) showed inhibitory activity, and 5% WES was more potent toward *Bacillus* than penicillin (10 μg), comparable with chloramphenicol (30 μg) and approximately twice weaker than gentamicin (120 μg). However, in the case of *M. luteus* only 0.1% WES had inhibitory impact, and its potential was weaker than that of the antibiotics used.

Extracts of Spirulina had no impact on *E. coli*, but all analysed concentrations of WES had stimulated *Alicyclobacillus acidoterrestris.* The stimulation zone around well with 5% WES had almost 11 mm. It should be highlighted that *A. acidoterrestris* showed particular sensitivity to all antibiotics applied, with inhibition zone diameter ranging from 7 to 16 mm. In contrary, *Bacillus subtilis* and *Escherichia coli* were completely resistant to ampicillin.

Yeasts

WES exhibited low inhibitory activity against yeasts used in experiments. There were no inhibition zones in samples with *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Table 2 B). Brewer's yeast *Saccharomyces carlsbergensis* showed little sensitivity to 0.1% of WES. 2.5 and 5% WES slightly inhibited the growth of *Rhodotorula* yeast, and this effect was significantly weaker than the impact of antibiotics used, especially of NST1 (Fig. 1 A).

Molds

Water extracts of Spirulina had no influence on analysed in the study *Rhizopus oryzae* and *Fusarium* sp. (Table 2 C). In the *Aspergillus niger* cultures, no inhibition zone was observed, but with increasing concentration of WES the stimulated formation of asexual fruiting structure with conidia and conidiophores could be observed in the vicinity of the wells

Table 1. Basic parameters of experiments – incubation temperature, medium and control samples applied for particular microorganisms

Microorganism	Medium	Incubation temperature	Controls
Bacillus subtilis, *Micrococcus luteus*, *Escherichia coli*	Nutrient agar (Biocorp, Poland)	32°C	P10 – penicillin 10 μg CN120 – gentamicin 120 μg C30 – chloramphenicol 30 μg AM10 – ampicillin 10 μg
Alicyclobacillus acidoterrestris	BAT agar (Biocorp, Poland)	45°C	
Fungi	Sabouraud Dextrose Agar (Biocorp, Poland)	28°C	NST1.0 – Nystatin 1 mg/ml NST0.1 – Nystatin 0.1 mg/ml AmfB – Amphotericin B 0.4 mg/ml

Table 2. The impact of different concentrations of water extracts of Spirulina (WES) and antibiotics on the growth of bacteria (A), yeasts (B) and molds (C)

A

Microorganism	WES				P10	C30	CN120	AM10
	0.1%	1.0%	2.5%	5.0%				
B. subtilis	ø[a]	ø[a]	4.3 ±0.5[b]	6.3 ±0.5[c]	10	5	10	ø
E. coli	ø	ø	ø	ø	1	8	12	ø
M. luteus	1.0 ±0.0	ø	ø	ø	3	5	11	4
A. acidoterrestris	+1.3 ±0.5[a]	+5.3 ±1.4[b]	+5.3 ±1.8[b]	+10.7 ±1.0[c]	11	14	16	7

B

Microorganism	WES				NST1.0	NST0.1	AmfB
	0.1%	1.0%	2.5%	5.0%			
Saccharomyces cerevisiae	ø	ø	ø	ø	6	1.5	2.5
Saccharomyces carlsbergensis	<1.0[a]	ø[b]	ø[b]	ø[b]	6.5	1.5	3.5
Schizosaccharomy-ces pombe	ø	ø	ø	ø	1	ø	2.5
Rhodotorula sp.	ø[a]	ø[a]	<1.0[b]	1.1 ±0.3[b]	8	1	4.5

C

Microorganism	WES				NST1.0	NST0.1	AmfB
	0.1%	1.0%	2.5%	5.0%			
Rhizopus oryzae	ø	ø	ø	ø	1	ø	ø
Fusarium sp.	ø	ø	ø	ø	12	6	ø
Aspergillus niger	ø	ø	ø*	ø*	7.5	3.5	7.5
Penicillium sp.	3.0 ±0.9	ø	ø	ø	14	4	6
Cladosporium sp.	3.0 ±0.9[a]	ø[b]	ø[b]	+3.5 ±0.6[c]	6	1	1
Geotrichum sp.	ø[a]	+3.0 ±0.9[b]	+5.4 ±1.1[c]	+8.5 ±0.6[d]	4	ø	2

Values represent the length (mm) of inhibition or stimulation (+) zone measured as a distance between the edge of well and place where normal growth of bacteria can be observed.
ø – no inhibition.
*Increased mycelium maturation and stimulation of conidia formation by 5% WES.
a, b, c – the same letters mean the lack of significant differences ($p < 0.05$) between impact of different WES concentrations on particular microorganism.

(Fig. 1 B). Water extracts of Spirulina at the concentration of 0.1% showed inhibitory activity against mold of the genus *Penicillium*, but to a lesser extent than the antibiotics used in the experiment. Interesting results were obtained for *Cladosporium* mold. 0.1% WES inhibited the growth of this mold, and the impact was

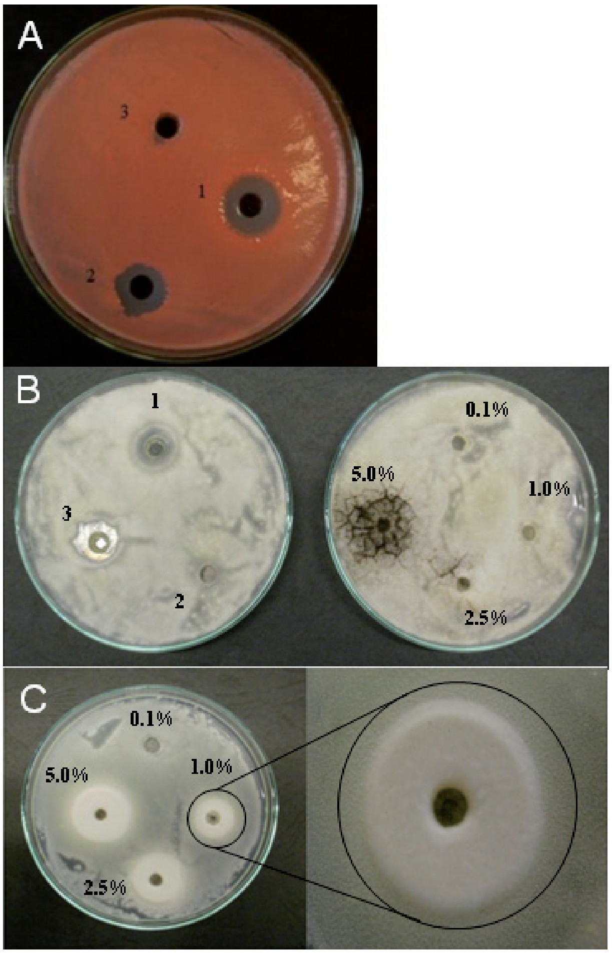

Fig. 1. Impact of antibiotics and water extracts of Spirulina (0.1%, 1.0%, 2.5% and 5.0% WES) on the molds growth: A – inhibitory effect of antibiotics on *Rhodotorula* sp., B – inhibition of *Aspergillus niger* growth by antibiotics and its stimulation by 2.5% and 5.0% WES, C – stimulations of *Geotrichum* sp. growth by 1.0-5.0% WES; 1 – nystatin 1.0 mg/ml, 2 – amphotericin B 0.4 mg/ml, 3 – nystatin 0.1 mg/ml

3-fold stronger than amphotericin B and 0.1 mg/ml nystatin. However, the higher concentration (5% WES) stimulated the growth and the mycelium maturation of *Cladosporium*, which manifested as significantly more developed green mycelium with numerous conidiophores. Water extracts of Spirulina stimulated also the growth of molds of the genus *Geotrichum*, and the strength of the stimulation was directly proportional to the WES concentration (Fig. 1 C).

DISCUSSION

The activity of unfavourable microflora causes the degradation of numerous components present in food. This results in reduced sensory and nutritional value of foodstuff, and contributes to economic losses. The possibility of increasing the microbiological stability of food with its simultaneous enrichment in bioactive ingredients of healthy properties is the dream of every producer. This combination would enable the reduction of the amount of artificial preservatives added. Moreover, it could increase the nutritional value of the product and its stability, and thus the shelf life of the product could be longer. The reduction of losses caused by food spoilage significantly would increase the company's competitiveness in the market.

For example, *B. subtilis* causes ropiness of bread, while fruits and vegetables are particularly susceptible to spoilage due to growth of *Penicillium*, *Aspergillus*, *Alternaria*, *Botrytis*, *Rhizopus*. On the meat surface species of the bacteria genera *Pseudomonas*, *Bacillus*, *Micrococcus*, *Alcaligenes*, *Proteus*, as well as molds *Rhizopus* and *Mucor* can be found [Sivasankar 2002, Bibek 2005]. No activity of WES against baker's yeast *Saccharomyces cerevisiae* together with the inhibition of *Bacillus subtilis* growth, both proved in this study, suggest that Spirulina extracts probably can be used as a supplement for bread that will enrich it in bioactive components and simultaneously protect from spoilage. Further studies should be conducted, that will evaluate the most optimal dose of WES in the bread taking into account its sensory and nutritional value, as well as its microbiological stability. The Spirulina addition to meat products could also contribute to enhancing their nutritional value and simultaneously slow down the multiplication of undesirable microflora of the genus *Micrococcus* and *Bacillus*.

In the present study, no inhibitory effect of Spirulina on *Fusarium*, *Rhizopus oryzae* and *Aspergillus niger* was shown. Therefore the WES addition to fruit and vegetable products probably will not increase their stability and resistance to deterioration caused by these microorganisms. What's more, too high WES concentration can stimulate the mycelium maturation of *Aspergillus niger*, *Cladosporium*, and *Geotrichum*. These results suggest that, if Spirulina would be used as a food supplement, the concentration used should be very carefully determined, to avoid growth stimulation of these fungi by too high cyanobacteria dose. Especially as the excessive growth of the mold *Geotrichum candidum* leads to discoloration of the surface of butter and changes in taste [Bibek 2005, Ledenbach and Marshall 2009].

A particularly big problem in fruit and vegetable industry is *Alicyclobacillus acidoterrestris*. This acidophilic bacterium can survive standard pasteurization conditions and then grow during storage causing deterioration of fruit juices manifesting by unpleasant pharmaceutical odor and turbidity [Oteiza et al. 2011]. The results of this study had show the significant stimulation of *A. acidoterrestris* growth by water extracts of Spirulina. In the next research it will be checked whether the observed effect is the same in real conditions of food matrix, i.e. in the apple juice inoculated with *Alicyclobacillus acidoterrestris*. If these experiments confirm the stimulatory effect of WES to *A. acidoterrestris* in food matrix it will mean that the Spirulina cannot be used to the enrichment of fruit juices. At the present moment, the food supplementation with various nutrients or plant raw materials having pro-health properties, such as Spirulina, has growing popularity. Any research that shows the negative side effects of such supplementation is particularly important and should be distributed not only in the scientific world but also among food producers.

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WPŁYW WODNYCH EKSTRAKTÓW SPIRULINY (WES) NA BAKTERIE, DROŻDŻE I PLEŚNIE

STRESZCZENIE

Wstęp. Ze względu na skład chemiczny, Spirulina jest stosowana powszechnie jako suplement diety, który wywiera korzystne działanie na organizm człowieka. Ponadto Spirulina ma zdolność hamowania wzrostu niektórych mikroorganizmów, zarówno patogenów będących zagrożeniem dla zdrowia, jak i powodujących psucie się produktów wytwarzanych we wszystkich gałęziach przemysłu spożywczego. Głównym celem prezentowanej pracy było określenie wpływu wodnych ekstraktów Spiruliny (WES) na wzrost różnych mikroorganizmów, zarówno użytecznych, jak i szkodliwych dla zdrowia człowieka bądź z ekonomicznego punktu widzenia.
Materiał i metody. Wpływ różnych stężeń WES (0,1; 1,0; 2,5 lub 5,0%) na wzrost wybranych gatunków bakterii, grzybów drożdżowych i pleśniowych oznaczano metodą dyfuzyjną na podłożach stałych.
Wyniki. Wykazano, że WES mają zróżnicowane działanie na mikroorganizmy w zależności od badanego gatunku. Działanie hamujące obserwowano wobec *Bacillus subtilis*, *Micrococcus luteus*, *Rhodotorula* oraz *Penicillium*. Natomiast silnie stymulujący wpływ wykazano wobec *Alicyclobacillus acidoterrestris* oraz pleśni *Geotrichum*. Ponadto większe stężenia WES stymulowały rozwój grzybni i wytwarzanie konidioforów przez *Cladosporium* oraz *Aspergillus niger*.
Wnioski. Hamujący wpływ WES na drobnoustroje powodujące psucie się żywności może być wykorzystany w produkcji żywności. Jednakże uzyskane wyniki wskazują na potrzebę dalszych badań, szczególnie w celu stwierdzenia wpływu WES na mikroflorę w matrycy żywności.

Słowa kluczowe: *Arthrospira*, bakterie, psucie żywności, suplementacja żywności, zahamowanie i stymulacja wzrostu, pleśnie, drożdże

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