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Sunflower Protein Enzymatic Hydrolysates as a Medium for Vitamin B2 and B12 Biosynthesis

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Lowering the costs of industrial fermentation processes is a current challenge of modern biotechnology. In this sense, has been studied the possibility to replace the conventional medium for vitamin B2 and B12 biosynthesis with new effective medium from renewable plant-based sources. Riboflavin (vitamin B2) and cobalamin (vitamin B12) are essential compounds in humans diets and their demand is increasing globally. Current manufacturing process of these vitamins is unsustainable because of using costly unrenewable resources and generating hazardous wastes. Meat peptone is conventionally used as a nitrogen source during the fermentation process, however it is relatively expensive. Sunflower protein is a secondary product, which can be obtained from sunflower meal after oil extraction, and rich on its nutritional value. The objective of this study was to select and evaluate fermentative potential of sunflower protein and its enzymatic hydrolysates as a nitrogen source for riboflavin and cobalamin biosynthesis by evaluating the kinetic parameters of strains growth, specific substrate consumption and formation of metabolites. The fermentation of strains was carried out using conventional mineral medium with meat peptone in 250 mL Erlenmeyer flasks, and the results obtained were compared with fermentation where sunflower protein and it's enzymatic hydrolysates were used as a nitrogen source instead of meat peptone. It was observed that when compared with meat peptone, some strains show positive effect in growth when nitrogen source was substituted with sunflower protein enzymatic hydrolysates. This allows affirming that sunflower protein and its enzymatic hydrolysates are promising in the use as a nitrogen source for riboflavin and cobalamin biosynthesis. The characteristics and parameters of studied fermentation processes reveals their potential for residue products applications for obtaining products with high added value.

1. Introduction

Sunflower is being one of the most popular oil seed culture, and the Russian Federation estimated to have 10.5 million tons of sunflower in 2018, being the second worldwide supplier of sunflower seed after Ukraine according to Nuseed Europe (2019). Sunflower seed is mainly used for oil production, while the secondary product of oil extraction - sunflower meal can be used for sunflower protein isolate production with crude protein content up to 86.0 % (Baurin et al., 2015). Sunflower protein isolate is characterized by high digestibility (up to 90 %), good emulsifying, fat-holding, foaming properties, while lipophilic properties are manifested due to the presence of non-polar protein chains. High crude protein content and product parameters as well as low cost of sunflower protein isolate make it a promising source ammonia nitrogen. High costs of conventional microbiological nitrogenous sources such as meat peptone restrict their use in industrial scale for animal fodder production by means of microorganism fermentation.
Vitamin B12 is a fascinating molecule, comprising of two coenzymes - methyl cobalamin and adenosylcobalamin, commercially known as cyanocobalamin, which plays an important role in fats and proteins metabolism, as well as in DNA and hemoglobin synthesis and regulation. To meet the needs for vitamin B12, humans may obtain it with their nutrition by consuming meat, eggs, fish and dairy products, while the conventional fodder for livestock, such as plant meals and silage are poor in vitamin B12 (Watanabe et al.,

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2018). Production of cyanocobalamin by means of chemical synthesis requires more than 60 steps and the final product is polluted with impurities that are hard to separate (Martens J. H. et al., 2002). Microbiological fermentation of cyanocobalamin is an alternative way to obtain vitamin B12 naturally. Several researches were carried out in order to enhance the vitamin B12 fermentation process using aerobic microorganisms (such as *Pseudomonas denitrificans* (Fang H. et al, 2018)) and anaerobic microorganisms *Lactobacillus reuteri* (Taranto M. P. et al., 2003), *Salmonella typhimurium*, *Propionibacterium shermanii* and *Bacillus megaterium* (Kang Z. et al., 2012). Several studies were carried out in order to describe the vitamin B12 biosynthesis by genetically modified *Bacillus megaterium* – gram-positive bacteria, strict aerobe, which was one of the first discovered to be able to produce vitamin B12 even at very low O_2 concentrations (Biedendieck R. et al., 2010). Process optimization allowed to increase vitamin B12 concentration on the fermentation medium up to 205 mg/l, after genetically modified *Bacillus megaterium* DSM319 fermentation on synthetic medium based on meat peptone (Mohammed Y. et al., 2014), which is not suitable for industrial fodder, enriched with vitamin B12 and riboflavin, production because of high medium costs. Riboflavin (or vitamin B2) is water-soluble compound, composed of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which take part in a great variety of biochemical reactions. Riboflavin can be synthesized by plants and microorganisms, it was firstly isolated from egg whites in 1930s (Kuhn et al. 1933). Until 2002 chemical synthesis from D-glucose and D-ribose (Schwechheimer S. K. et al., 2016) was the only way to obtain riboflavin, while nowadays the most of the riboflavin production has been replaced via genetically modified *Bacillus subtilis* fermentation (Barbau-Piednoir E. et al, 2015). However, several studies report the increment in riboflavin production during *B.megaterium* ATCC 13639 fermentation when corn meal has been assumed as a source of nitrogen (Chung H. J., 1986). Enzymatic hydrolysates are being widely used as medium for microbial fermentation in order to obtain wide range of industrial products such as arabitol (Loman A. A. et al., 2018), xylitol (Mardawati E. et al., 2018), probiotics (Karetkin B. A. et al., 2018). Our approach was to develop the fermentation process of *B. megaterium* for fodder, enriched with vitamin B12 and riboflavin, production by using a cheap alternative nitrogen source to conventional meat peptone such as sunflower protein enzymatic hydrolysates.

2. Materials and Methods

2.1 Enzymatic hydrolysis

Unpurified sunflower protein isolate was kindly provided by LLC “Sunprotein” (Altai Region, Russian Federation) with following characteristics (%): crude protein 82,0; carbohydrates 5,5; moisture 5,5.
Five enzyme mixtures Fermgen, Proteinase T, Protex 40E, Protex 51 FP, Protex 7L were kindly provided by Genencor (Danisco International Moscow, Russia). Only this enzyme mixtures of this manufacturer were tested due to the availability. All the proteases were alkaline (except Fermgen) and food grade with optimum pH range from 4.0 to 12.0 and it corresponded with solubility of sunflower protein isolate. Enzymes characteristic and incubation conditions are presented in Table 1. Enzymatic hydrolysis was performed in 250-mL Erlenmeyer flask at pH and temperature value, presented in Table 1, depending on the enzyme used. 10 g of sunflower protein isolate were mixed with 100 mL of distilled water, the pH was adjusted with 2 M NaOH or 2 M HCl. 0,1 mL of enzymatic mixture was added to PBS buffer with pH 7.2 and the obtained mixture was then added to the protein suspension. The hydrolysis was carried out in the water bath for 24 hours using magnetic stirrer. The obtained hydrolysates were used as a medium for bacteria fermentation.

Table 1. Enzymes characteristic and incubation conditions

Enzyme	Type	Optimum pH*	Activity as given by the manufacturer	Micro-organism	Incubation	
					pH	Temperature, °C
Fermgen	Acid fungal protease	3.5-5.0	1000 SAPU/g	*Trichoderma reesei*	4.0	65
Proteinase T	Thermostable neutral proteinase	6.5-9.0	65.5 μmol/min/mg	*Geobacillus stearo-thermophilus*	7.0	37
Protex 40E	Bacterial alkaline protease	7.0-12.0	38000 GSU/kg	*Bacillus subtilis*	7.0	60
Protex 51FP	Endo/exo-peptidase	6.0-9.0	400000 HU/g	*Aspergillus oryzae*	7.0	50
Protex 7L	Bacterial alkaline endopeptidase	6.0-8.0	1600 AU/g	*Bacillus amylo-liquefaciens*	7.0	50
Pancreatin	Commercial mixtures of amylase, lipase, and protease	5.0-7.5	25 USP protease activity/mg	ofBos Taurus (Fam. Bovidae)	7.0	37

* range as given by the manufacturer

GSU/g (Genecor Subtilisin Units) is measured against an internal Genecor standard using a peptide substrate.
HU/g (Hemoglobin Units) is the activity which will liberate 0.0447 mg of non-protein nitrogen in 30 minutes.
DU/g is based on the ability of a protease to cleave p-nitroanilide from synthetic peptide substrate. The absorbance 405 nm is directly related to protease activity via the use of enzyme standards.
AU/g is based on hydrolysis of azo-casein substrate at pH 7.5 for 5 minutes at 30 ◦C

2.2 Microorganism and Media

Bacillus megaterium (VKPM B-3750) strain was purchased from VKPM Russian National Collection of Industrial Microorganisms. Standard medium containing 10 g/L meat peptone and 5 g/L NaCl was used as a control medium. Experimental fermentation medium was composed of the following: 100 mL of sunflower protein isolate enzymatic hydrolysate; 10 g/L NaCl. The control fermentation medium was composed of the following: 10 g/L of sunflower protein isolate; 10 g/L NaCl. The initial pH was adjusted to 6.6-6.8 with 2 M HCl prior to sterilization.

2.3 Fermentation in Shake Flasks

Bacillus megaterium was grown on agar slant (18×180 mm) at 37 °C for 24 h, and the fresh cell was washed with 10 mL of sterilized water. One mL of the suspended cell was then inoculated into a 250-mL Erlenmeyer flask containing 100 mL of seed medium, and the cultivation was performed at 37 °C on a rotary shaker at 180 rpm. When the optical density value (determination at 700 nm) of the seed biomass reached 9–10, the seed culture was then transferred into a 250-mL Erlenmeyer flask containing 100 mL of fermentation medium with 10 % inoculum and incubated at 37 °C on a rotary shaker at 180 rpm for 24 h.
The amount of the colony forming units (CFU) after the fermentation was calculated according to the micromethod analysis (Baron F. et al., 2006).

2.4 Analytical methods

The fermentation process was analyzed according to the ammonia nitrogen reduction in the fermentation medium by Sorensen's formol method (Brown J.H., 1923). The concentration of total sugar in the broth was determined by the dinitrosalicylic acid reagent (DNS) method (Miller G. L., 1959). The data given represented the average values obtained from triplicate experiments.

3. Results and discussion

3.1 Effect of meat peptone substitution with unpurified sunflower protein isolate

In order to investigate the effect of nitrogen source substitution on cell growth two fermentations were performed using the described conditions. During the first fermentation 10 g/L of meat peptone was used as nitrogen source. The results obtained were compared with the bacteria fermentation on medium where 10 g/L of sunflower protein isolate was used as nitrogen source instead of meat peptone. After 24 h of shake-flask fermentation the CFU/mL was determined using micromethod and the ln(CFU/mL) was calculated fir both fermentations. Ammonia nitrogen consumption was determined and the results obtained are presented on Figure 1.
The substitution of meat peptone with unpurified sunflower protein isolate did not inhibit the cell growth, maximum cell concentration reached $3.3 \cdot 10^9$ on 10th h of shake flask fermentation on medium with meat peptone, and $4.5 \cdot 10^8$ on 10th h of shake flask fermentation on medium with sunflower protein isolate. The pH increased from 6.6 to 8.4 and to 8.21 during 24 h fermentation on medium, containing meat peptone and sunflower protein isolate respectively. However, the ammonia nitrogen concentration was 51 % lower in medium based on sunflower protein isolate, comparing to medium based on meat peptone (364 mg/L and 714 mg/L, respectively). The lag phase took 6 h while using medium with sunflower protein as nitrogen source (comparing to 2 h while using meat peptone), which was caused by low content of free low molecular weight compounds available in the medium based on sunflower protein for fast accumulation by bacteria.
The results obtained confirmed previous suggestions that the expensive compound used in medium preparation for *Bacillus megaterium* fermentation can be substituted with less expensive sunflower protein isolate, obtained during secondary plant raw material (sunflower meal) bioconversion.
In order to increase bioavailability of sunflower protein, high molecular weight protein complexes can be broken down into available nutrients for bacteria growth by applying industrial proteases.

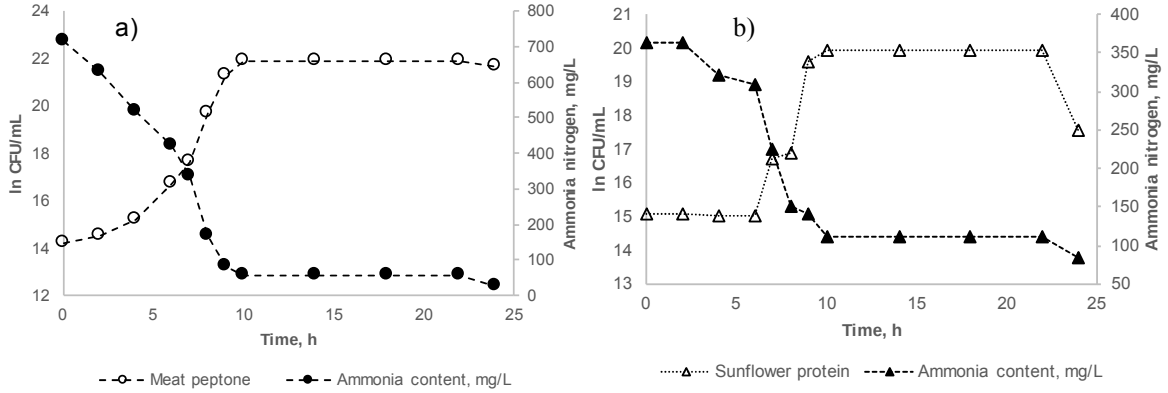

Figure 1: Time courses of ln(CFU/ml) growth and ammonia nitrogen (mg/L) consumption during Bacillus megaterium shake flasks fermentation on medium, containing meat peptone(a) and unpurified sunflower protein isolate (b)

3.2 Effects of sunflower protein isolate enzymatic hydrolysis on *Bacillus megaterium* shake flasks fermentation

As a result of sunflower protein enzymatic hydrolysis by commercial enzymes Protex 40E, Proteinase T, Protex 51FP, Protex 7L, Fermgen and Pancreatin, six hydrolysates were obtained.

The amino acid composition of the obtained sunflower protein enzymatic hydrolysates significantly differs from the control sample containing the protein solution in sodium phosphate buffer (Table 2). The amino acid profile of the hydrolysate, obtained using Pancreatin has the highest concentration of amino acids due to the complex effect of the enzyme, containing lipase, alpha-amylase, trypsin, chymotrypsin. Also, the amino acid content in the hydrolysate, obtained using Protex 7L increased significantly compared to the hydrolysate, obtained with Protex 51FP.

Table 2. Amino acid analysis of of sunflower protein enzymatic hydrolysates

Amino acid	Sunflower protein	Protex 40E	Proteinase T	Protex 51FP	Protex 7L	Fermgen	Pancreatin
Ala	< 12,5	< 12,5	22	< 12,5	122	28	221
Arg	< 25	< 25	87	< 25	16	< 25	92
Asn	< 25	< 25	16	< 25	25	9	106
Val	< 25	< 25	71	< 25	156	32	348
His	< 25	10	40	< 25	74	15	241
Gly	< 12,5	< 12,5	14	< 12,5	33	7	79
Gln	< 25	< 25	8	< 25	105	16	633
Lys	< 12,5	< 12,5	28	< 12,5	76	25	104
Leu + Ile	< 12,5	< 12,5	186	< 12,5	505	73	1100
Trp	<10,0	<10,0	<10,0	<10,0	<10,0	<10,0	<10,0
Met	27	45	96	< 12,5	175	9	350
Pro	< 12,5	< 12,5	10	< 12,5	22	10	20
Ser	< 12,5	< 12,5	27	< 12,5	38	9	84
Tyr	< 12,5	38	89	< 25	54	33	172
Thr	< 25	< 25	22	< 25	31	9	148
Phe	< 12,5	< 12,5	282	< 12,5	420	75	1220
Cys	< 5,0	< 5,0	< 5,0	< 5,0	< 5,0	< 5,0	< 5,0

Enzymatic treatment of the protein leads to the formation of low molecular weight hydrolysis products, the fractional composition of which was studied. Proteins with a molecular weight of 40-60 kDa prevail in the hydrolysates, which corresponds to 11S globulins of sunflower seed (Figure 2). The most complete hydrolysis of sunflower protein occurred during treatment with Protex 7L (3).

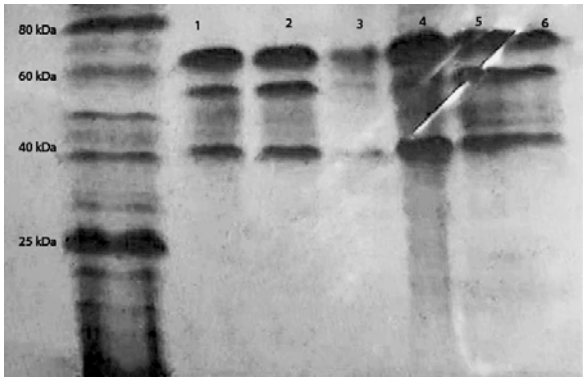

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of sunflower protein enzymatic hydrolysates obtained with (1) Pancreatin, (2) Fermgen, (3) Protex 7L, (4) Protex 51 FP, (5) Proteinase T, (6) Protex 40 E. Molecular weight markers (kDa) are indicated on the left.

In order to investigate the effect of using sunflower protein isolate enzymatic hydrolysates on cell growth, enzymatic hydrolysis was carried out prior to *Bacillus megaterium* fermentation according to conditions presented in Table 1. The obtained medium was inoculated with *B. megaterium*. After 24 h of shake-flask fermentation the ln(CFU/mL) was calculated and ammonia nitrogen consumption (mg/L) were determined and the results presented on Figure 3.

The substitution of meat peptone with sunflower meal enzymatic hydrolysates did not significantly differ. Maximum cell concentration reached $5.1 \cdot 10^9$ CFU/mL on 10th h of shake flask fermentation on medium, based on enzymatic hydrolysates, obtained with Fermgen and $4.5 \cdot 10^8$ on 10th h of shake flask fermentation on medium with sunflower protein isolate. The pH increased from 6.6 to 8.4 and to 8.21 during 24 h fermentation on medium, containing meat peptone and sunflower protein isolate respectively. However, the ammonia nitrogen concentration was 51 % lower in medium based on sunflower protein isolate, comparing to medium based on meat peptone (364 mg/L and 714 mg/L, respectively). The lag phase took 2-4 h while using medium with sunflower protein as nitrogen source (comparing to 2 h while using meat peptone and 6 h while using sunflower protein), which was caused by low content of free low molecular weight compounds available in the medium based on sunflower protein for fast accumulation by bacteria. High molecular weight protein complexes can be broken down into available nutrients for bacteria growth by applying industrial proteases.

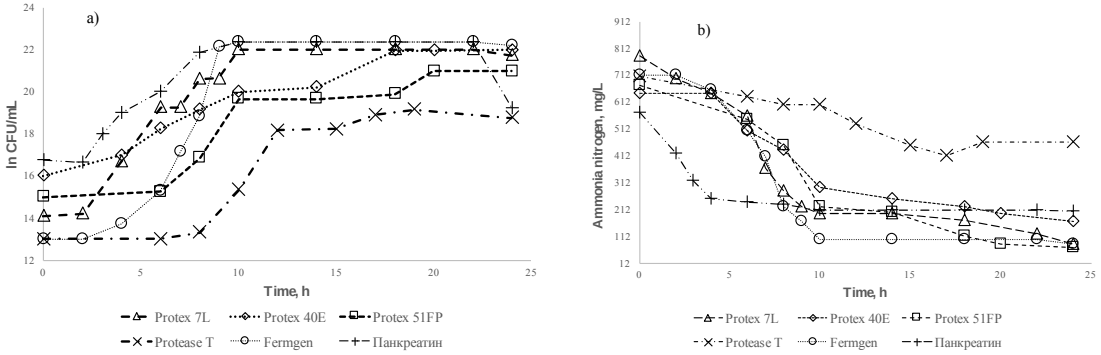

Figure 3: Time courses of cell growth ln(CFU/mL) (a) and ammonia nitrogen (mg/L) consumption (b) during Bacillus megaterium fermentation on unpurified sunflower protein enzymatic hydrolysates

4. Conclusions

The present study investigated the possibility to substitute meat peptone with sunflower protein isolate as a nitrogen source for *Bacillus megaterium* B-3750 fermentation. Compared to the fermentation on medium, containing meat peptone, it was observed that using sunflower protein isolate did not significantly influenced the fermentation process, which leads to its promising application for lowering the vitamin B12 and riboflavin industrial production costs.

In order to increase bioavailability of sunflower protein, enzymatic hydrolysis with industrial enzymatic compositions was suggested as a preliminary stage. Five industrial proteases (Fermgen, Proteinase T, Protex 40E, Protex 51 FP, Protex 7L) were tested for fermentation media preparation. The aim of this study was testing the possibility of a potential fermentation process on sunflower protein isolate enzymatic hydrolysates. Sunflower protein isolate enzymatic hydrolysates not only accelerated the bacteria growth, and should be promising in further enhanced vitamin B12 and B2 biosynthesis. *B. megaterium* fermentation on medium composed of sunflower protein isolate treated with acidic proteinase Fermgen as well as alkaline endopeptidase Protex 7L demonstrated the highest bacteria growth, which means they could be good candidate for industrial production of fodder enriched with vitamin B12 and riboflavin.

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