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FACULTY OF TECHNOLOGY AND METALLURGY

ABDALLA ALI SALIM

PRODUCTION OF HYDROLYTIC ENZYMES BY FERMENTATION ON AGRICULTURAL BY-PRODUCTS USING *BACILLUS* SP.

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ABDALLA ALI SALIM

PROIZVODNJA HIDROLITIČKIH ENZIMA FERMENTACIJOM NA POLJOPRIVREDNOM OTPADU POMOĆU RAZLIČITIH VRSTA IZ RODA *Bacillus*

Doktorska disertacija

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SUPERVISOR:

_____

Dr Zorica Knežević-Jugović, full professor

Faculty of Technology and Metallurgy, University of Belgrade

COMMISSION:

_____

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Faculty of Technology and Metallurgy, University of Belgrade

_____

Dr Nataša Šekuljica, scientific associate

Innovation Center, Faculty of Technology and Metallurgy

DATE OF DEFENSE:

Greetings

Production of hydrolytic enzymes by fermentation on agricultural by-products using *Bacillus sp.*

ABSTRACT

During the production and processing of food, large quantities of waste are generated which need to be properly stored and, at best, found new use and utilized. An extremely large problem worldwide is certainly the large amount of agroindustrial waste that is generated on a daily and especially annual basis. If agroindustrial waste is directly stored in the environment, it causes a greenhouse effect and is a large-scale environmental problem. However, agroindustrial waste has recently been regarded as an extremely valuable raw material that can be converted into an added-value raw material by applying different biotechnological approaches. Accordingly, this doctoral dissertation deals with the global problem of utilization and quality improvement of various agro-industrial wastes through the application of biotechnology.

Modification and improvement of quality is achieved by growing the production microorganism on a solid surface, that is, agroindustrial waste. This is a rather new method which involves the use of cheap materials but they usually contain large amount of protein, sugar and mineral as well as bioactive compounds such as polyphenols, carotenoids and dietary fibers. Accordingly, the first part of this doctoral dissertation is the selection of the microorganism producer. During the selection, a rich collection of various commercial and natural bacterial strains was utilized, which is available at the Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy.

Various strains have been tested for the ability to produce α-amylase, cellulase, pectinase and protease using selective medium on agar plates. In this way, one of the *Bacillus* strains was found to be particularly prominent in the production of targeted enzymes. Due to the fact that this strain is a natural isolate, identification by sequencing 16SrRNA encoding gene and subsequent analysis of the obtained sequence using the BLAST (Basic Logical Alignment Search Tool) was

performed. Further, the obtained and isolated strain (*Bacillus* sp. TMF-1) was used in the solid-state fermentation process with the main objective to examine the possibility of the selected microorganism to produce desired enzymes under the different substrate:water ratios by using different types of agroindustrial waste materials such as wheat bran, soybean meal, olive oil cake, sunflower meal for growth. Following the cultivation of microorganism producer and production of targeted enzymes, it has been found that agro-industrial waste can be transferred to an even better growing medium if a certain treatment of the raw material is carried out. Accordingly, the aforementioned agroindustrial wastes have been chemically treated by the use of acids and bases, the use of ultrasound as well as microwave technology and used for enzymes production by *Bacillus* sp. TMF-1.

B. subtillis TMF-1 has proven to be an outstanding producer of the hydrolytic enzymes of α-amylase, cellulase and protease on soybean meal. However, in addition to producing targeted enzymes in high yield, it is extremely important and efficient to extract the enzymes from the fermented medium. Accordingly, in the next part of this doctoral dissertation, the first step in the downstream process will be examined, that is the selection of an appropriate technique for extracting the enzymes produced. Herein, the conventional extraction techniques as well as innovative technologies such as ultrasound technology are examined in terms of eco-friendly enzyme recovery in high yield. The influence of the parameters of a conventional extraction technique such as pH, the solid: liquid ratio, mixing intensity and extraction time on enzyme yield is examined in detail. Furthermore, ultrasound-assisted extraction is optimized in detail in terms of extraction time, ultrasound frequency and specific energy. The data regarding the protease/α–amylase/cellulase ultrasound-assisted recovery from soybean meal fermented with *B. subtillis* TMF-1 are compared with the enzyme recovery data obtained using the conventional extraction technique.

Agroindustrial waste also contains numerous elements that must be removed or their levels must be reduced in order to be suitable for human and animal nutrition after fermentation. These elements are called anti-nutritional factors and are the most important protease inhibitors, i.e. trypsin inhibitors as

well as phytic acid. The third part of this doctoral dissertation deals with the monitoring of the profiles of the mentioned anti-nutritional factors. The content of anti-nutritional factors is monitored during the 8 days of *B. subtilis* TMF-1 cultivation on soybean meal, at different soybean meal: water ratios.

Soybean meal contains bioactive compounds which have a myriad of positive effects on human health, such as a positive effect on cardiovascular diseases but also degenerative diseases such as cancer and diabetes. Accordingly, there is an increased interest in application of solid state fermentation which has proven to be a technique for the significantly higher yields production of bioactive components in soybean meal. In the fourth part of this doctoral dissertation, the profile of phenolic compounds in soybean meal after fermentation of *B.subtilis* at different initial moisture contents is followed. Also, the content of phenolic compounds is directly related to the antioxidant capacity of soybean meal, so the biological activity profile of soybean meal after fermentation is also shown.

Key words: hydrolytic enzymes production; soli-state ferementation; agroindustrial waste; antinutritional factors; biological activity.

Academic Expertise: Engineering technology

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Proizvodnja hidrolitičkih enzima fermentacijom na poljoprivrednom otpadu pomoću različitih vrsta iz roda *Bacillus*

IZVOD

U toku proizvodnje i obrade hrane stvaraju se velike količine otpada koje je neophodno skladištiti na pravi način i u najboljem slučaju ponovo iskoristiti. Izuzetno veliki problem širom sveta je svakako stvaranje velikih količina agro-industrijskog otpada na dnevnom a naročito godišnjem nivou. Ukoliko se agro-industrijski otpad direktno skladišti u okolnu životnu sredinu nastaje efekat staklene bašte i to je ekološki problem velikih razmera. Međutim, agro-industrijski otpad je skoro klasifikovan kao izuzetno vredna sirovina koja se može prevesti u sirovinu sa dodatnom vrednošću primenom različitih biotehnoloških pristupa. Shodno tome, ova doktorska disertacija se bavi globalnim problemom iskorišćenja i unapređenja kvaliteta različitog agroindustrijskog otpada primenom biotehnologije.

Modifikacija i unapređenje kvaliteta je postignuta gajenjem proizvodnog mikroorganizma na čvrstoj podlozi, tj. agro-industrijskom otpadu. Ovo je prilično nova metoda koja podrazumeva upotrebu jeftinih materijala koji sadrže velike količine proteina, šećera i minerala kao i bioaktivnih jedinjenja kao što su polifenoli, karotenoidi i dijetetska vlakna. Prvi deo ove doktorske disertacije je selekcija mikroorganizma producenta. Tokom ove selekcije upotrebljena je bogata kolekcija različitih prirodnih i komercijalnih sojeva bakterija dostupnih na Katedri za Biohemijsko inženjerstvo i biotehnologiju, Tehnološko-metalurškog fakulteta. Različiti sojevi su ispitani na sposobnost proizvodnje α-amilaza, celulaza, pektinaza i proteaza korišćenjem selektivnog medijuma na agar pločama. Na ovaj način utvrđeno je da jedan od *Bacillus* sojeva ima izuzetan potencijal za proizvodnju ciljanih enzima. Shodno činjenici da je ovaj soj prirodnog porekla, izvršena je identifikacija sekvencionisanjem gena za kodiranje 16SrRNA i naknadnom analizom dobijene sekvence korišćenjem osnovnog alata za pretraživanje logičkog poravnanja (na *engl.''* Basic Logical Alignment Search Tool'', BLAST). Zatim,

dobijeni i izolovani soj (*Bacillus* sp. TMF-1) je korišćen za fermentaciju na čvrstoj podlozi sa osnovnim ciljem da se ispita mogućnost odabranog mikroorganizma da proizvede željene enzime pri različitom odnosu supstrat:voda korišćenjem za rast različitog agro-industrijskog otpadnog materijala kao što su: pšenične mekinje, sojina sačma, pogača nakon ceđenja maslinovog ulja, suncokretova sačma. Praćenjem gajenja mikroorganizma producenta i proizvodnje ciljanih enzima, utvrđeno je da se agroindustrijski otpad može prevesti u još bolji medijum za rast ukoliko se sirovine podvrgnu određenim pretretmanima. Prema tome, pomenuti agro-industrijski otpadi su pretretirani hemijski korišćenjem kiselina i baza ali i primenom tehnologije ultrazvuka i mikrotalasa i korišćeni za proizvodnju enzima *Bacillus* sp. TMF-1-om.

B. subtillis TMF-1 se pokazao kao izuzetan producent hidrolitičkih enzima α-amilaza, celulaza i proteaza na sojinoj sačmi. Međutim, pored produkcije ciljanih enzima u visokom prinosu, izuzetno je važno i efikasno ekstrahovati enzime iz profermentisane podloge. Dakle, u sledećem delu ove doktorske disertacije, ispitan je prvi korak u izdvajanju koji podrazumeva selekciju pogodne tehnike ekstrakcije proizvedenih enzima. Ovde su ispitane konvencionalne tehnike ekstrakcije i inovativne tehnologije kao što je tehnologija ultrazvuka u pogledu ekološke prihvatljivosti i visokog prinosa. Detaljno je ispitan uticaj parametara konvencionalnih tehnika ekstrakcije kao što su pH, odnos čvrst:tečno, vreme i intenzitet mešanja. Dalje, ekstrakcija primenom tehnologije ultrazvuka je optimizovana u pogledu vremena ekstrakcije, frekvencije ultrazvuka i specifične energije. Podaci o ekstrakciji enzima, proteaza/α-amilaza/celulaza iz sojine sačme fermentisane *B. subtillis* TMF-1-om konvencionalnom tehnikom su upoređeni sa podacima ekstrakcije potpomognute tehnologijom ultrazvuka.

Agroindustrijski otpad sadrži brojne elemente koji moraju da se uklone ili da se smanji njihova količina kako bi se mogla sirovina koristiti u ishrani životinja i ljudi. Ovi elementi se nazivaju antinutritivnim faktorima i među njima najbitniji su inhibitori proteaza, tj. tripsin inhibitor i fitinska kiselina. Treći deo ove doktorske disertacije je posvećen praćenju profila ovih antinutritivnih faktora. Sadžaj

antinutritivnih faktora praćen je tokom 8 dana fermentacije sojine sačme *B. subtillis* TMF-1-om pri različitom početnom sadržaju odnosu sojina sačma:voda.

Sojina sačma sadrži bioaktivne komponente koje ispoljavaju bezbroj pozitivnih efekata na zdravlje ljudi, kao što su pozitivni efekti na kardiovaskularna oboljenja ali i različita degenerativna oboljenja kao što su kancer i dijabetes. Shodno tome, teži se povećanju sadržaja ovih jedinjenja u sojinoj sačmi, pa se postoji povećan interes za fermentaciju na čvrstoj podlozi za koju je dolazano da je tehnika kojom je moguće značajno povećati sadržaj ovih bioaktivnih jedinjenja. U četvrtom delu ove doktorske disertacije, praćen je profil fenolnih jedninjenja tokom fermentacije sojine sačme *B. subtillis* TMF-1-om pri različitom početnom sadržaju odnosu sojina sačma:voda. Takođe, sadržaj fenolnih jedinjenja je direktno povezan sa antioksidativnim kapacitetom sojine sačme, pa je prikazan i profil biološke aktivnosti sojine sačme nakon fermentacije.

Ključne reči: proizvodnja hidrolitičkih enzima; fermentacija na čvrstoj podlozi; agroindustrijski otpad; antinutritivni faktori; biološka aktivnost.

Naučna oblast: Tehnološko inženjerstvo

Uža naučna oblast: Biohemijsko inženjerstvo i biotehnologija

UDK broj:

Contents

1 INTRODUCTION

The world is facing the increasing problem of generating large quantities of waste. Annually in Europe alone, food waste amounts to 89 million tons of food per year where 39% of that number is the food waste that occurs during manufacturing processes. The number is much higher when total agricultural residual production (crop residues: straw, stem, stalk, leaves, husk, shell, spent grains) is taken into account: 367 million tons per year (Ravindran *et al.*, 2018).

If not treated, that kind of waste has an important negative environmental impact. Since it has a high content of organic material, disposal of such waste can cause pollution if not managed properly. The direct disposal of agro-industrial by-products as a waste in the environment represents a major cause for environmental pollution. In addition, it contributes to the green house effect by increasing a number of greenhouse gases. Therefore it can have a harmful effect on human and animal health (Sadh *et al.*, 2018). Management of such waste is a major concern of modern science. It is fair to say that only a small amount of this large quantity is being valorized. One of the most logical ways of utilizing, at least, a part of enormous amount of agro industrial waste is the production of animal feed. Agro-industrial wastes differ in composition but usually contain large amounts of protein, sugars and minerals. They are rich in many bioactive compounds (polyphenols, carotenoids) as well as dietary fibers.

However, the usage of agricultural waste as an animal feedstock has its limitation like poor quality and poor nutrient content. Also the drawback is the presence of antinutritional factors. By definition the antinutritional factors are substances that, when present in animal feed or water, either by themselves or through their metabolic products reduce the availability of one or more nutrients (Yakout, 2016). In fact, many byproducts of agro-industrial waste can be used as animal feedstock without any modification, but a large part must be used only after fermentation of the agro-residues. There are several approaches to this problem, where solid state fermentation (SSF) is the most promising one.

Apart for the animal feedstock, as noted before, the agro-industrial waste is rich in sugars, minerals and proteins and as such can be used as solid support, carbon and nutrient source in solid state fermentation processes for the production a large amount of value-added compounds such as organic acids, flavor and aroma compounds, enzymes and bioactive compounds (Sadh *et al.*, 2018). This work is focused on developing an environment-friendly bioprocess based on the use of agro-industrial residue as substrates for enzyme production. The goal is to develop a process that will have a comprehensive benefit concerning both the environmental issue and valorization of agricultural waste. Although, up to date, some progress has been made in the conversion of various lignocellulosic and other substrates into valuable added products, there are still many unresolved problems that prevent industrial development. The dissertation deals with the most sensitive and unresolved issues in the fields of enzyme production. Particularly important is the selection of the production microorganism for the biosynthesis of important hydrolytic enzymes such as cellulase, mannanase, protease and amylase. In terms of the applicability of production microorganism in an industrial process, it should be able to grow and produce enzymes on cheap raw materials as substrates and produce the enzyme with desired temperature/pH profile, specificity towards substrates, thermal stability and stability in the presence of specific agents.

In recent years, interest in enzyme production has been growing through low-cost methods such as the cultivation of microorganisms on solid substrates. One of the main reasons for this expansion is the possibility of using agricultural waste as substrates for microorganisms` growth. Agricultural and industrial waste is a major polluter of the environment. Conversion of these wastes into useful product can greatly alleviate the problems that such waste causes. On the other hand, the same waste represents a rich source of fermentable sugar and protein. There is a need for the revalorization of these wastes in order to obtain valuable microbiological metabolites. In addition to the ecological advantages of such processes, the production of industrially useful enzymes, as a valuable metabolite, significantly reduces the cost of enzyme production, thereby increasing the

possibility of their wider industrial application, in another words, it makes industrial enzymatic processes competitive with chemical ones. Solid state fermentation of microorganisms on solid substrates is a simple technology in terms of process equipment and potentially can achieve higher yields in comparison to the fermentation in a liquid medium. Furthermore, screening a large number of microorganisms for enzymes with improved performances combined with genetic engineering, and SSF optimization approaches can lead to more productive and cost-efficient enzyme production.

2 SOLID STATE FERMENTATION

There are two different types of microorganism cultivation: solid state fermentation (SSF) and submerged fermentation (SmF). The classification is mainly based on the type of substrate used during fermentation; SSF utilizes solid substrates (such as bran, bagasse and paper pulp) where SmF utilizes free flowing liquid substrates such as molasses and broths (Subramaniyam and Vimala, 2012). Although SmF is the most common method for commercial production of different types of products, the use of solid state fermentation is considered a promising way of producing enzymes with higher yield and higher activity obtained (Hansena *et al.* 2015).

Cultivation of microorganisms on solid support for enzyme production has a very long tradition. First industrial enzymes have been produced on this way in the countries of Far East, and later this technology has been developed in the Western countries. Nowadays, it has a huge commercial importance in the production of amylases, pectinases, lipases and other hydrolases, especially when the raw fermented product can be used directly without the need for subsequent steps of extraction and purification. Solid state fermentation is, by definition, the cultivation of microorganisms under controlled conditions on solid substrates in the absence, or in the presence of a small amount of free water. Consequently, this type of fermentation is mainly used in cultivation of fungi and yeast, which are able to grow in a low water activity environment. However, there are also some species of bacteria (*Bacillus subtilis*, *Bacillus thurigiensis* and *Lactobacillus* sp.) that have been reported to successfully produce enzymes in solid state conditions (Soccol *et al.*, 2017).

The SSF is still undervalued in comparison to SmF but it is gaining more attention from scientific community and large companies in industry, primarily for some obvious advantages such as small investments, lower capital, operating costs and biotechnological advantages: higher fermentation capacity, higher end-

product stability, lower catabolic repression and cost-effective technology (Costa *et al.*, 2018). In Table 2.1 as stated before, big gain from this kind of fermentation is that it can be of great importance in cases where raw fermentation product can be directly used as an enzyme preparation, even without biomass separation. Recent studies have shown that in the case of enzyme production, the yield is higher for SSF than for SmF when comparing the same strain (Hansena *et al.*, 2015).

Some authors emphasize two main reasons for SSF uprise. The first is the phenomenon that is attributed to SSF, is the resistance of microorganisms to catabolic repression in the presence of abundant substrates. And secondly is, as stated before, the possibility of using agro-industrial waste as substrate for the metabolite production (enzymes, antibiotics, organic acids, biosurfactants, aroma compounds) at low production cost, thereby this reducing the environmental problems (such as soil pollution) that occur with agro-industrial waste (Lizardi-Jiménez and Hernández-Martínez, 2017).

Table 2.1. Advantages and disadvantages of solid state fermentation (SSF) compared to submerged fermentation (SmF)

Advantages	Disadvantages
High productivity, high yield compared to the correspondent submerged cultures/	Applicable for small numbers of micro-organisms due to low water content; Solid supports make the biomass determination very challenging.
The low content of water allowing working in aseptic conditions in some cases.	Only microorganisms which are capable to grow at low moisture levels can be used.
Facilitated separation and purification	

of enzymes; the dried fermented substrate is most often used directly as enzyme source.	Minimum process control (difficult monitoring of process parameters); Agitation may be very difficult.
Culture media are often quite simple, providing all the nutrients necessary for growth. simulate the living conditions of many higher filamentous fungi	Possibility of contamination by undesirable fungi and yeast.
Simple reactor design with few spatial requirements can be used.	Many important basic scientific and engineering aspects have not yet been elucidated. Information about the reactor design and on a large scale is scarce; Aeration can be demanding because of high solid concentration.
Reduced energy and investment costs; Fewer requirements of solvents are necessary for the enzyme extraction due to its high concentration.	The removal of carbon dioxide and metabolic heat generated during fermentation may be very difficult; Mass transfer limitation.
The low moisture content may favor the production of unstable enzymes that may not be produced in SmF.	Substrates often require pre-treatment (reduction of size by grinding, physical, chemical or enzymatic hydrolysis, steam treatment).

There are several factors of solid state fermentation that have to be considered when applying this type of fermentation. Some of these factors crucial for process development are selection of microorganism and substrate, chemical composition and physical state of substrate, physical and/or chemical pre-treatment of the substrate, particle-size of substrate, supplementation of growth medium, moisture content and water activity, inoculums density, temperature, pH, oxygen intake, carbon dioxide removal and separation of biomass. The main problem that has to be addressed with this kind of fermentation is the accessibility of substrate nutrients, thus some substrate pretreatment is necessary, and inadequate heat/mass transfer making it difficult to further process scale-up. Thus, despite the advantages, the solid state fermentation has found limited applications in processes using unicellular organisms.

Basically, the two most important factors that have to be considered during the development of a SSF are the choice of microorganism and the choice of substrate. One of the reasons why traditional SSF came about is the need to dispose agricultural and farm waste materials. Therefore, we will give a short summary of possible waste that can be used as substrates for the SSF.

2.1 Substrate for SSF

2.1.1 Soybean and soybean meal

The soybean (*Glycine max*) or soya bean is a species of leguminous plant related to peas and cloves originate in Asia. It has been grown in China, Korea and Japan more than three thousand years ago. Grains grow in beads containing 3-5 grains and they are round or oval. The main reason for soybean cultivation around the world is the suitable chemical composition of the grain. Soybeans have around 8% seed hull, 90% cotyledons and 2% germ. Based on dry matter, dehulled soybean contain 20% oil, 40% protein, 35% carbohydrates and 5% minerals. After the oil extraction, the remaining product called the soybean meal has around 48% protein, 35-40% carbohydrates, 7-10% water, 5-6% mineral and less than 1% fat (Choct *et al.*, 2010). Generally, soybean meal is characterized by a high protein content (43-53%) and low crude fibre content (<3%). Due to its excellent amino

acid composition and high level of digestibility, the soy products represent an important source of protein for animal feed. About 80% of crude proteins in soybean meal are storage protein. Soybean meal contains high amounts of lysine, tryptophane, threonine and isoleucine, but low amounts of cystine and methionine. Soybean meal is commercially divided into two categories: “high protein” soybean meal with 47-49% protein and 3% crude fiber (hulled has been removed) and “conventional” soybean meal with 43-44% protein (the hulls are added back after the extraction).

Polysaccharides present in soybean meal are predominantly non-starch polysaccharides and free sugars, such as, mono-di and oligosaccharides, whereas starch is present in less than 1%. Non-starch polysaccharides can be classified into three main groups: cellulose, non-cellulosic polymers and pectic polysaccharides (Choct *et al.*, 2010). Some carbohydrates, such as stachyose and raffinose, actually present anti-nutritive soybean ingredients because monogastric animals cannot hydrolyze the α-galactoside and β-fructoside bonds that the soya contains due to the lack of a specific endogenous α-galactosidase. Soya is rich in minerals (mostly calcium and iron), vitamin B complex, beta carotene and biologically active components (isoflavonoids, lecithin, saponin, phytosterols, and oligosaccharides). Numerous soybean products are intended for human and animal nutrition, with special emphasis on the production of functional foods. Soybean meal, is a by-product of the extraction of soybean oil. There are several extraction methods, but the most commonly used is solvent extraction (over 99%). In that process the soybeans are cracked, dehulled (commonly), heated.

The oil is extracted and the hull is mixed and grinded into the meal. The process is performed at high temperature, in order to inactivate the antinutritive factors that are otherwise present in the raw soybean. However, the temperature should not be too high, so that the basic nutrients are intact. It has been shown that excessive temperatures or long duration on lower temperatures, may result in disturbance of digestibility of amino acids due to the Mayellard reaction, which can be notices by the changing of the color of soybean to dark brown (Del Valle, 1981; Caprita and Caprita, 2009).

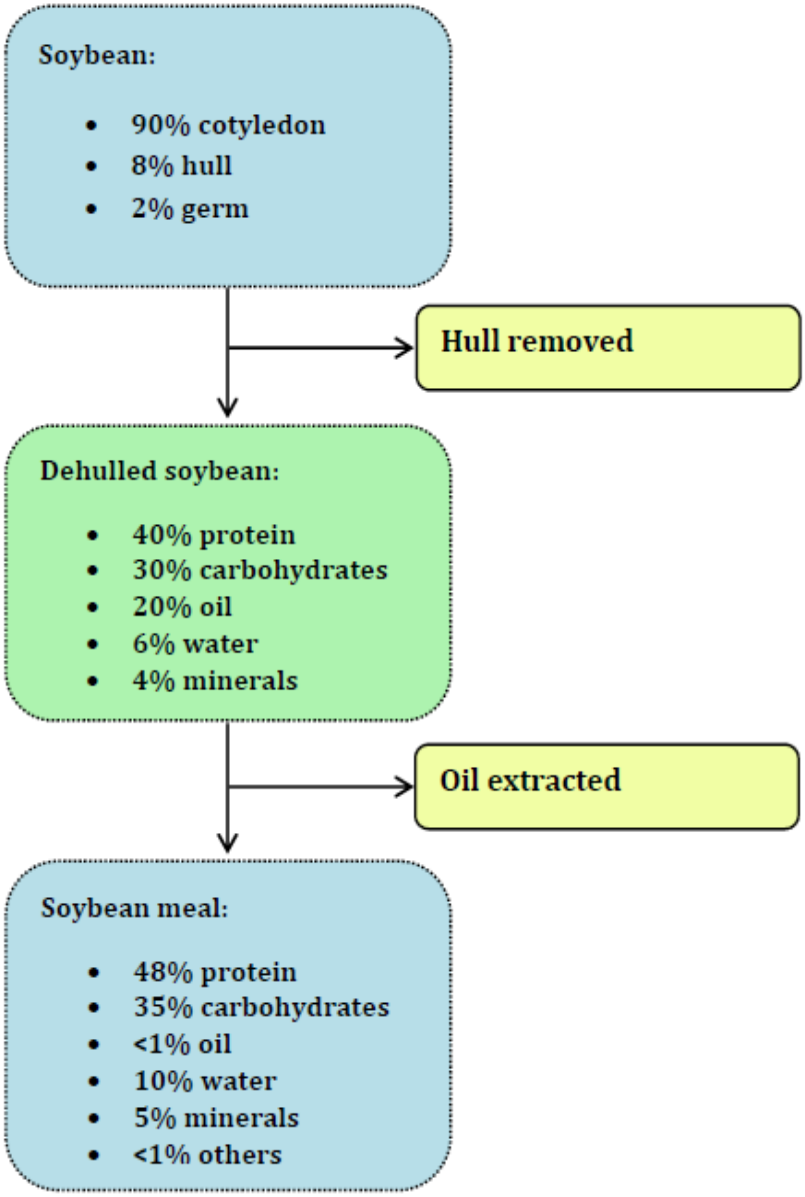

Figure 2.1. Nutrient composition of soybean and soybean meal (Choct *et al.*, 2010).

It is important to say that based on the genotype, origin and climate conditions there can be significant changes in the chemical composition and protein quality in soybean meal. Also, differences arise based on processing and storage conditions. This may translate into differences in amino acid digestibility, especially in broilers. For example, research has shown that soybean meal with

higher levels of crude protein and lower levels of trypsin inhibitor activity showed higher amino acid digestibility (Pettersson and Pontoppidan, 2013).

Soybean meal is the most important source of high quality protein for animal nutrition. It represents nearly two-thirds of the total world protein market. The largest producers of soybean, nowadays, are USA, Brazil, China and Argentina.

2.1.2 Sunflower meal

Sunflower meal is a byproduct of the extraction of oil from sunflower seeds. Extraction of the oil can be either mechanical and/or by solvent. The dominant way of oil extraction is the solvent extraction but with the growth of organic farming, mechanical extraction is gaining more attention. Figure 2.2 shows the sunflower meal with hull and dehulled (Heuzé *et al.*, 2019).

Figure 2.2. Sunflower meal, a) with hull; b) dehulled.

Sunflower meal is used as livestock feeds, predominantly for ruminants. It is one of the most important oil meals that is used in farming along with rapeseed meal, soybean meal and cotton seed meal. Sunflower meal has a relatively high protein percentage: hulled meal is around 28%, and 42% for the dehulled seed meal. Commonly produced sunflower meal contains 30-34% of protein, 20-25% cellulose and 8-10% lignin. In comparison to soybean meal, the sunflower meal contains lower energy and lysine but higher fiber and methionine content (Sánchez-Muniz and Cuesta, 2003). The lower lysine concentration can lead to insufficient utilization of rich amino acid potential that sunflower has (Sredanovic

et al., 2006). Therefore, the sunflower meal is usually combined with soybean meal for livestock feed (Gulya, 2004). The fiber content is depended on the presence of the hull: dehulled meal has 20-26% of crude fibre, and the non-dehulled meal has 27-31% of crude fiber. It is desirable to have less fiber content because the high fiber content can reduce nutrient digestibility (Heuzé *et al*., 2019).

One big difference between the soybean meal and sunflower meal is the absence of any antinutritional factors in sunflower meal. Therefore, there is no need for subsequent heating or additional processing. There is a wide range of product that is available on the market based on the quality of the meal, weather it is high quality flour or a semi processed meal with straws and hulls. The meal itself is sold on the basis of protein content as the most important parameter.

Like with other plants, the quality of the sunflower meal depends on the plant and climate characteristic as well as on processing and storage conditions. For example, if the dehulling process isn't fully completed the extraction will be less successful and the quality of both oil and meal will be reduced.

Table 2.2. Composition of sunflower meal and soybean meal

Item	Sunflower meal	Soybean meal
Crude Protein	32%	47%
Fat	1%	1.50%
Fiber	21%	3.10%
Ash	6%	6.40%
Lysine	1.14%	2.99%
Arginine	2.46%	3.40%
Cystine	0.55%	0.73%
Valine	1.75%	2.26%

Isoleucine	1.38%	2.10%
Methionine	0.68%	0.68%
Threonine	1.13%	1.85%
Tryptophan	0.65%	0.65%

2.1.3 Wheat bran

Wheat kernel is consisted from three layers: germ, endosperm and wheat bran. Wheat bran is a byproduct from the milling process when it is stripped away from the wheat cornel. It has food and nonfood application. Wheat bran is rich in fiber, minerals, vitamin B, vitamin E, fat, thiamine and antioxidants such as phenolic compounds (ferulic and p-coumaric acid) (Stevenson *et al.*, 2012). Wheat bran consists of non-starch polysaccharides (35%), starch (15%), protein (15%) and lignin (5%). The structure of the non-starch polysaccharides is such that almost 70% are arabinoxylans, 19% is cellulose and 6% β-(1,3)/β-(1,4)-glucan (Merali *et al.*, 2015). The content of dietary fiber in wheat bran is 35-50%, but only 2-3% is the soluble dietary fiber. That low percentage of soluble dietary fiber is undesirable when wheat bran is used as high-quality dietary fiber supplement. High percentage of nonsoluble fiber has a negative correlation with animal feed intake, and therefore wheat bran has a limited usage in animal feed. One of the solutions is to apply solid state fermentation on that kind of substrate in order to eliminate apparent disadvantages and increase nutrient digestibility. Wheat bran also has the potential to be used as a low-cost stock for the production of different value-added compounds such as enzymes, amino acids, organic acids, flavors and other compounds that have usage in the food industry.

2.1.4 Maize bran

Maize bran or corn bran is a byproduct from maize industry (corn industry). The maize processing industry implies several different processes such as bioethanol production, starch production and various maize-based food productions. The

maize bran is usually a mixture of bran fraction and other maize products depending on the industry (Heuzé *et al*., 2019). For example, maize bran form starch extraction processes is usually mixed with steep liquor. Maize bran obtained from ethanol production is usually the mixture of the bran fractions and distillers soluble (Kalscheur *et al.*, 2012). And most commonly, the maize bran obtained from maize grits production is usually maize bran combined with broken kernels, germ residue and inseparable fractions of germ. So the maize bran is loosely defined product with highly variable composition. Basically, it is a product that is high on energy but with low protein content, based on dry matter there is 9-15% of protein with fiber content 5-20% and usually less than 10% of oil. Potential constraint is the possible mycotoxin contamination. Maize bran is predominantly used as an energy source for ruminants. Due to its low protein content it is usually mixed with other food ingredients and supplementary food components. The quality of the maize bran largely depends on the plant and climate characteristic, storage conditions and the processing industry.

2.2 Application of solid state fermentation

2.2.1 Organic acid

Solid state fermentation is used for the production of variety of valuable compounds. The longest application has the production of enzymes and it is considered a classical applications. Recent developed products are bioactive compounds, flavor enhancers, antioxidants and organic acids (citric acid, lactic acid). Organic acid are commonly used in food industry primarily as preservative agents, but the range of their application is very wide. Due to their versatility solubility, hygroscopic and ability of buffering and chelation, they are widely used in food industry. Acids are added to food (animal feed or human consumption) in order to control microbiological activity, to improve digestibility of proteins and amino acids, reduce ammonia and biogenic amines and increase the activity of digestive enzymes. The SSF for the production of citric and lactic acid has been employed for years but recently other acids have been produced such as oxalic, gluconic and gallic acid (Mussatto *et al.*, 2012). Acids have been used in animal feed

instead of antibiotics and prove to be a good alternative. In order to prevent the infection of animals by microorganisms it is necessary to control their activity, to prevent contamination and recontamination. Decontamination can be achieved by thermal processes, application of high temperature and water vapor (different types of conditioners), as well as hydrothermal and mechanical processes (extrusion, expansion). Aside from these thermal processes, decontamination can be achieved by adding organic acid. Adding organic acids also prevents recontamination during storage and transport.One of the most important acids produced on the industrial level is citric acid. The natural production of citric acid, from fruit, is unprofitable and too expensive, and since the end of the 19th century began with successful experimentation with various types of molds. In 1893, chemists discovered that *Penicilium* strain can produce significant amounts of citric acid and vitamin C, but mass production of it began only after the discovery that *A. niger* created 70% more citric acid in comparison to *Penicilium* strain (Barrington *et al.*, 2009). Several microorganisms have been tested for citric acid synthesis by SSF such as bacteria (*Arrthrobacter, Bacillus, Cornyebacterium*) and fungi (*Aspergillus, Penicillium, Yarrowia*). However, to date, *A. niger* continues to be the most commonly used microorganism, cultivated on agro-industrial wastes such as wheat bran, rice bran, maize bran, apple pomace and others (Soccol *et al.*, 2017). Citric acid has a numerous application in various industries. It is used as a preservative, flavor enhancer and antioxidant. Apart from the food industry, this acid is also used in chemical industry (plasticizer) and detergent industry (replacement of polyphosphates).

Lactic acid is widely used in pharmaceutical, chemical, food, textile and leather industry. In recent years there has been a demand for lactic acid, primarily due to the development of biodegradable lactic polymers used for controlled release of bioactive compounds (Đukić-Vuković *et al.*, 2011). In global world production of lactic acid, it is estimated that the fermentation process participates with 90%, and only 10% of lactic acid is produced by synthesis from lactonitrile. Fermentation process is preferred process because L (+) or D (-) isomer is selectively formed, while the chemical synthesis produces racemate (Datta and

Henry, 2006). Due to its acidity and microbial activity, lactic acid is used as preservative in the food and confectionery industry. In addition, there is a current trend in food industry for the production of lactic acid fermented products, such as fruit-base juices or vegetables that represent extremely healthy products, so called "functional" food products (Ewaschuk *et al.*, 2005; Varadarajan and Miller, 1999). Both fungal and bacterial strains have been used for lactic acid production by SSF. Most utilized are *Rhizopus sp*. and *Lactobacillus sp*. The demand for lactic acid has increased significantly in the last decade. The classical and industrial most commonly used lactic acid production process is a batch process on fermentable sugars (glucose, lactose, xylose, arabinose) as a source of carbon. However this procedure is not economical enough, primarily because of the price of raw materials. When using cheaper raw material, such as agro-industrial waste, it is usually necessary to apply the appropriate pretreatment and complete process is then carried in two phases. The first phase usually involves the liquefaction and saccharification in which fermentable mono- and disaccharides are formed. After the saccharification is complete, lactic acid bacteria is inoculated and the fermentation (Rogers *et al.*, 2005) phase is carried out. Due to the growing need for lactic acid, integrated processes are developed with waste raw materials that are not used in other industries as solid support.

2.2.2 Flavor

Flavor compounds are part of expanding industry and some estimation is that they cover almost a quarter of the food market. Flavor compounds are mostly produces synthetically (over 80% are produced by chemical synthesis), but buyers love to know that the product they buy is of "natural" origin, and therefore they increasingly appreciate the microbiologically obtain product because it is declared as "natural". Also, chemical synthesis has its limitation due to formation of racemic mixtures and lack of substrate specificity (Bhari and Singh, 2019). Extraction of flavor and aroma compounds from plant itself, which is also declared as natural, has its limitation: shortage of plant resources, their variability influenced by the weather and plant diseases and often low recovery of the desired compound (Bhari and Singh, 2019). Aroma and flavor synthesis through bioconversion,

primarily, through fermentation has numerous advantages. First, this process is independent from any climate change, from potential plant diseases or large amounts of pesticide. It is a low cost process, scale up is easy, as is the recovery of product and overall it is a process that is less damaging to the environment. SSF is successfully used for aroma synthesis with both fungi and bacteria.

2.2.3 Enzyme

Cultivation of microorganism on solid support for the production of enzymes has a very long tradition. Historically, this type of fermentation for enzyme production has been used for years in the countries of Far East, and later this technology was transferred to the Western countries. Today, there is a great commercial significance in the production of enzymes such as amylase, cellulase, pectinase, lipases, and others, especially when raw fermented product can be used as enzyme without any separation and purification downstream processes.

However, the production of enzyme on solid support is specific depending of particular enzyme-microorganism system. In general, in addition to the common important factors, for the production of enzymes by SSF keys factors are mechanical pretreatment of the substrate, particle size, moisture content of the substrate, amount of inoculum, temperature control (heat release) and other process parameters, length the duration of the process, the efficiency of oxygen transfer and the removal of carbon dioxide as well as other resulting gases. The influence of process parameters on enzyme biosynthesis depends on the production microorganism and the type of enzyme, even if they are products of the same microorganism. Since the production of enzymes is specific, the properties of the enzymes that are the subject of this doctoral dissertation will be presented in the text below as well as examples of their production.

2.2.3.1 Pectinase

Pectinases are a group of enzymes that hydrolyze pectin that is present in cells of various plant species. The hydrolysis is carried by various mechanisms. Pectin-rich substances are characterized by long chains composed of galacturonic acid units.

These units contain carboxyl grups, and in almost 80% cases are naturally modified by addition of methanol to form methoxy groups. Pectin substances are found in fruit in the amount of 0.5-1.5%, while larger quantities are found in apples and quines. Pectin substances in the plant are found as insoluble protopectin that has a major influence on the structure and consistency of tissue of the fruits. Pectinases belong to the hydrolases family of enzymes and according to the cleavage site can be divided in three gropus: esterase-pectin methyl esterase (EC 3.1.1.11) (Knežević-Jugović, 2008), that break methyl ester linkage, forming methanol and polygaracturonic acid; endo-polygalacturonase (polygalacturonase) the enzyme that catalyzes the random hydrolysis of (1-4)-alpha-D-galactosiduronic linkages in pectate and other galacturonans; pectat lyase (polugalacturonic transeliminase) enzymes that break down pectic substances by the elimination mechanism. Pectinases are produced by microbial fermentation (bacteria, yeast, fungy) of various agro-industrial waste such as wheat bran, soybean meal, apple pulp, lemon and orange peel (Panesar *et al.*, 2016). Pectinases are produced by *Saccharomyces*, *Bacillus*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium* but out of all fungus is preferred for enzyme production due to high yield and stability to temperature, pH and pressure change (Verma *et al.*, 2018).

Therefore, pectin methyl esterases (pectinesterases) act on soluble pectin whereby methanol is released and a product having a highly acidic character (pectic acid) is formed. During the action of these enzymes, the viscosity of the reaction mixture does not decrease because they do not affect the degree of polymerization of the pectin substances. However, if Ca^{2+} ions are present, the viscosity of the mixture can increase because these ions build complexes with segments consisting of a large number of residues of unesterified monomers. At the same time, as the enzyme reaction progresses, the hydrolysis rate decreases because the enzymes have a higher affinity towards substrates with a higher degree of esterification (e.g. apple fruit pectins) (Knežević-Jugović, 2008).

The optimal pH, temperature, stability, and other properties of pectin esterases depend of enzyme source. Generally, these enzymes are activated in the presence of some salts (ammonium sulfate, sodium chloride) and Mg^{2+} ions, while

Cu^{2+} and Hg^{2+} ions, as well as some compounds, D-galacturonates, polygalacturonates and pectates, have an inhibitory effect. As for the mechanism of action of these enzymes on pectin, it differs in pectin esterases originating from plants and fungi. Specifically, plant pectin-esterases separate methoxy groups that are close to the free residues of D-galacturonic acid and after their action, segments with a large number of free galacturonic acid residues are formed, while fungal pectinesterases are less specific acting on methyl ester bonds rather disordered randomly randomly breaking methyl ester linkage.

Endo-polygalacturonases (polygalacturonases) (EC 3.2.1.15) are also complex enzyme that catalyze the random hydrolysis of (1-4)-alpha-D-galactosiduronic linkages in pectate and other galacturonans. For their effective effect on pectin, it is necessary to remove methoxy groups with pectin esterases. It appeared that the enzyme cannot decompose pectin substances that have a higher degree of esterification of 60-70%. In contrast, galacturan 1,4-α-galacturonidase or exo-polygalacturonases (EC 3.2.1.67) act by exo-mechanism on (1-4)-alpha-D-galactosiduronic linkages between galacturonic acid residues from the nonreducing end of the carbohydrate chains releasing D- galacturonic acid at a time. Both types of enzymes primarily act on soluble pectin. The third type of enzymes that catalyze the hydrolytic degradation of pectin are exo-poly-α-galacturonosidases (3.2.1.82), which act on the same bonds in the polysaccharide molecule from the non-reducing end by the exo-mechanism as exo-polygalacturonases, but catalyzes the hydrolysis of the second (1-4)-alpha-D-galactosiduronic linkages, cleaving off two units at a time releasing digalacturonate. It seems that all three groups of enzymes depolymerize pectinic substances causing a decrease in the molecular weight of the macromolecular substrate, but with the participation of water molecules so that they belong to the hydrolases.

Pectat lyases are also compex enzymes that break down pectic polysaccharides of plant tissues into simpler molecules but without the participation of water molecules by the elimination mechanism forming unsaturated product through transelimination reaction. This group of enzymes

comprises at least three different enzymes that present differences in their cleavage mode and specificity: endo-pectatlyasis (EC 4.2.2.2) that acts towards substrate in a random way, exo-pectatlyases (EC 4.2.2.9) that catalyze the substrate cleavage from nonreducing end and endo-pectatlyases (EC 4.2.2.10) catalyzes also the random cleavage of pectin, but preferentially high esterified pectin (Pedrolli *et al.*, 2009).

Pectinases are most commonly used in the fruit industry during the process of juice extraction from fruits to improve extraction and reduce density of the juices and overall improve the quality of juice. Substances rich in pectin are responsible for consistency and viscosity of the fruit juices. Addition of pectinase results in a rapid decline in the viscosity and flocculation of the present micelles, so that the particles can be easily separated by sedimentation or filtration. Pectinases are usually used in combination with cellulases and hemicellulases (Kashyap *et al.*, 2001). These enzymes are also widely used in wine processing. The main role of pectinolytic enzymes in the wine production is during the extraction process, to maximize yield, facilitate filtration and intensify color and taste. However, large concentration of these enzymes can lead to accumulation of large amounts of methanol in wine, due to pectin-esterase activity, so the concentration of this enzyme must be kept low. The use of pectinase is also accustomed in the processing of tea and coffee. Treatment with pectinases accelerates tea fermentation by breaking down the pectin present in cell walls of tea leaves, and also prevents the formation of foam in instant tea powders (Garg and Singh, 2016). During the fermentation of coffee, pectinases are used to remove viscous and gelatinous mucilage from coffee beans, which contains a certain percentage of pectin. The quantity of mucous coating of coffee beans that is converted to sugar determines the quality of the coffee (Murthy and Nidu, 2011). Pectinases are also used in the production of food for ruminants, the activity if the enzyme reduces the viscosity of food and increase the absorbtion of nutrients (Praveen and Suneetha, 2014).

2.2.3.2 Cellulase

Cellulase are a group of enzymes that catalyzes hydrolysis of β-1,4- glycosidic bond of cellulose. It is a multinenzymatic complex that comprises of three different group of enzymes: endocelulase (endo EC 3.2.1.4), exocellulase (EC 3.2.1.91) and cellobiase (β-glucosidases EC 3.2.1.21). The exocelulase act at the ends of the cellulose chain and the end product of hydrolysis is β-cellobiose. The endocellulase randomly attack the internal glycoside bonds resulting in production of glucan chains of different lengths. The cellobiase (β-glucosidases) act specifically on the β-cellobiose disaccharides and produce glucose. This group of enzymes is important because it act on cellobiose which is the endproduct of hydrolysis with exo- and endo-cellulase and acts as an inhibitor (Knežević-Jugović, 2008). Only microorganisms that produce all three groups of enzymes can efficiently degrade cellulose. In another words, depolymerization of cellulose to glucose requires the synergistic action of these three enzymes (Kuhad *et al.*, 2011).

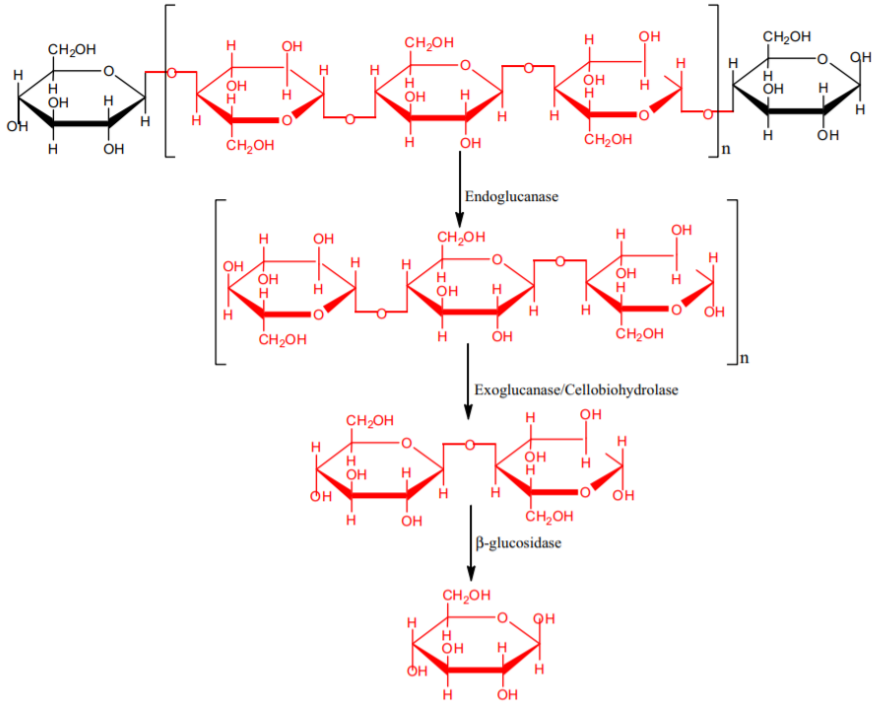

Figure 2.3. Structure of cellulose polymer and the cellulase sites of action on the cellulose polymer liberating glucose (Juturu and Wu, 2014).

Cellulases are expressed by a wide variety of microbes in nature. Mammels do not produce these enzymes, although the food that some herbivores consume is rich in cellulose. Cellulolytic enzymes that can be found in ruminat are actually due to the presence of bacteria, protozoes and fungi that live as saprophytes in the digestive tract of these animals. Cellulases are inducible enzymes synthesized by both fungi and bacteria during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic. The earliest studied cellulases were those produced by aerobic mesophilic fungi *Trichoderma viride*, *T.reesei*, *T. koningii*, *Penicillium pinophilum*, *P. funiculosum*, *Fusarium solani* and *Aspregillus niger*. Only fungi that can concurrently produce all the groups of enzymes can break down cellulose. There is a great interest in cellulases due to their application in various industries such as starch processing, alcohol production, fruit juice extraction, detergent and textile industries. Cellulases also have a crucial role in bioconversion of renewable cellulose biomass to commercial chemicals.

Cellulases play a major role in the food industry. They represent an essential part of the enzymatic complex for maceration consisting of cellulase, pectinase and hemicellulase. This complex is used to extract and clarify fruit and vegetable juices, incrasing yields and reducing process time, improving texture and reducing the viscosity of the pulp (Grassin *et al.*, 1996). Enzymatic saccharification of lignocellulosic materials with cellulases for the production of biofuels is currently the most interesting field of research. Bioconversion of lignocellulosic material is a well know process, however, it is necessary to optimize the process (Kuhad *et al.*, 2010). The price of the enzymes is the crucial step and it can be accessible by cellulase production on cheap support and by developing a catalytic system based on cellulases. Also, attention is paid to the reuse, i.e. recycling of the enzymes (Sun and Cheng, 2002).

2.2.3.3 Amylase

Amylases are a group of enzymes that catalyze partial or total hydrolysis of starch to glucose or oligosaharides. This group includes: α-amylase, β-amylase, glucoamylase, pululanase and isoamylase. This is a very important group of enzymes that is used in many processes in different industries. However, the greatest application of amylase is in the starch industry, for the production of starch syrups, maltodextrins, maltose and glucose syrups, crystalline glucose and others. There are various advantages of enzymatic hydrolysis in comparison to acid hydrolysis, such as improved quality and cost-effectives of production. The large industrial application of amylase began with the discovery of thermostable bacterial α-amylases, which enabled their more efficient application.

α-Amylases belong to group of endo-amylase group because they catalyze the hydrolytic degradation of α-1,4-glycosic bond in the interior of molecule randomly. They are absolutely specific to α-1,4-glycosidic bonds and cannot hydrolyze α-1,6-glycoside bonds but can skip them. α-Amylases are metalloenzymes that comprise ions of calcium as the cofactors that are essential for enzyme stability and activity. The β-amylases and glucoamylases belong to the exo-enzyme group because they catalyze the hydrolytic degradation of α-1,4-linkage next to the nonreducing end of the macromolecules gradually. Maltose is the basic product obtained by the action of β-amylase, while glucose is the basic product obtained by the action of glucoamylase. All amylase form this group have characteristic structure that contains a (β/α)8 barrel or TIM barrel, protein fold that consists of eight α-helices and eight parallel β-strands (Taniguchi and Honda, 2019).

β-Amylase (EC 3.2.1.2) are absolutely specific to α-1,4-glycosidic bonds, however, their activity produces maltose with a β-configuration on the C1 atom, thus obtaining their name. β-Amylase cannot hydrolyze the α-1,6 linkages and this linkage represent an obstacle for further hydrolysis. As a result, only 55% of amylopectin is converted into maltose and the rest is high-molecular-weight branched dextrin (β-limit dextrin).

Glucoamylases (EC 3.2.1.3) have a small specificity in terms of the link they break. This enzyme catalyze the hydrolysis of α-1,3-, α-1,4- and α-1,6-glycosidic bonds in oligosaccharides. The highest rate is for hydrolysis for α-1,4-, then α-1,3 and the slowest rate is for the hydrolysis for α-1,6-glycosidic bonds. Glucose obtained by the action of glucoamylase also has a β-configuration on the C1 atom. Theoretically, this is the only enzyme that can hydrolyze starch completely into glucose. However, in practice that is not the case. Only when there is α-amylase or some other enzyme that can break α-1,4- linkage there can be a complete hydrolysis of starch. That is probably because at high starch concentration, glucoamilase catalyze a condensation reaction that yields maltose and isomaltose and therefore 100% yield of glucose cannot be reached in starch saccharification. Glucomaylase is produced as an extracellular enzyme form fungi, mainly from *Aspergillus* (*A. niger*, *A. awamori*) and *Rhizopus*. Debranching enzymes, pululanases and isoamylases act on α-1,6-glycosidic bonds, but only pululanases can break down the polysaccharide pululane (Knežević-Jugović, 2008).

There are a lot of industrial processes in which amylases can be applied to improve the quality and the yield of final products. However, it is important to optimize the production conditions and catalytic characteristics of new amylases produced from novel microorganisms. Screening a large number of microorganisms for specific amylases with desired and tailored properties combined with process optimization and scale up, can lead to their novel specific application in industrial processes.

2.2.3.4 Proteases

Proteases and peptidases (E.C. 3.4.) are enzymes that catalyze the hydrolysis of a peptide bond in protein and/or peptide molecules. Proteases hydrolyze protein molecules to peptones, polypeptides, dipeptides and to free amino acids. In addition to their function in digestion of protein nutrients, proteases play a large role in other metabolic processes such as posttranslational modification of other proteins, activation of zymogenic forms of the enzyme, spore germination in sporulating microorganisms and as a part of complex metabolic processes (Rao *et*

al., 1998). Proteases can be plant, animal or microbial origin, where proteases of microbial origin have the highest industrial application due to the high growing rate of microorganisms producing them on inexpensive raw materials and with high yields. The most commonly used plant proteases are papain, bromelin and ficin. However, the use of enzymes from plant species requires a great deal of time, as well as large areas for growing these crops, and their industrial application is quite limited. Also the composition of isoenzyme forms in them will depend on the climatic and cultivation conditions, as well as the extraction and purification methods which affect the final quality of these preparations (Grbavčić, 2014).

The best known proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin. Again, the production of animal proteases is limited and depended on the availability of livestock which is influenced by a number of factors, ranging from climate to agricultural. Microbial proteases have by far the largest industrial application, where the majority of commercial proteolytic enzymes are produced using different *Bacillus spp*.

The classification of proteases can be made by a number of criteria such as the postition of peptide bond in the protein molecule on which they act, catalytic domain and pH optimum. Based on the position of the peptide bond in the protein molecule which they hydrolyze, proteases are divided into exopeptidases and endopeptidases. Exopeptidases act at the end of peptide chains gradually separating the individual amino acids and, thus, shortening the chain. It can be done from the N-terminal end (aminopeptidase) or C-terminal end (carboxypeptidase). Aminopeptidase are mainly intracellular enzymes, and so far there has been only one report of an extracellular protease produced by A. oryzae (Rao *et al.*, 1998). Carboxypeptidases are mainly secreted extracellulary and by their catalytic domain they may belong to the group of serine, metal or cysteine proteases. Endopeptidases break peptide bonds of nonterminal amino acids, that is, from within the molecule.

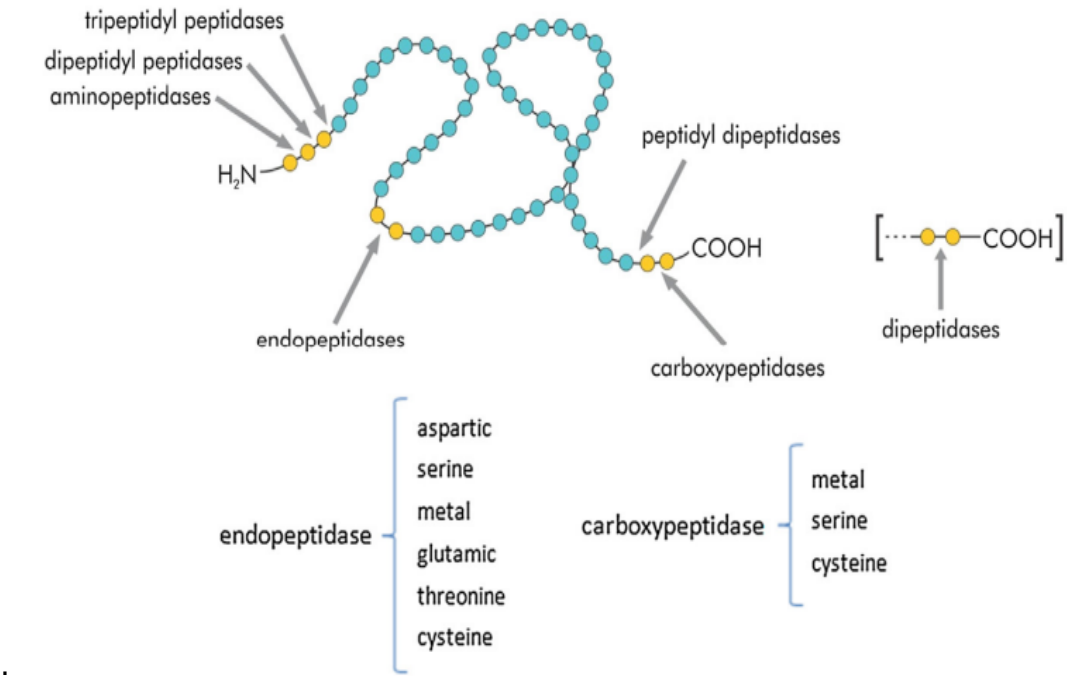

Figure 2.4. Classification of peptidases (de Castro *et al.*, 2018). Dark gray (blue) circles represent amino acids and light gray (yellow) circles indicate the amino acid sequence that will bind to the peptidase. The arrows point to the cleavage site on the protein substrate.

Based on their catalytic domain proteolytic enzymes can be divided into four groups: serine proteases, cysteine proteases, aspartate proteases and metallo-proteases. The type of enzymes can be recognized based on the basis of sensitivity to certain inhibitors.

Serine proteases are characterized by the presence of serine in their active center. They are very widespread in nature and can be found in animal digestion system and in juices and tissues in plants. A large number of microorganisms produces the enzyme extracellulary. Serine proteases are active in neutral and alkaline conditions usually between pH 7 and pH 11. The most important proteases for industry are the alkaline proteases produced by certain types of bacteria and yeast. They are relatively small molecules, (18 to 35 kDa), and the pH of the isoelectric point is generally in the range of 4 to 6. Some of the enzymes from this group are chymotripsin, trypsin, thrombin, elastase and subtilisin. All of the

enzymes from this group have a similar structure and mechanism. Serine proteases are synthesizes in the cells in the form of inactive precursors of enzymes or zymogens which have to be activated. During inactivation conformation and structural changes occur, opening the active site. For example, chymotrypsin is synthesized in pancreas in the form of chymotrypsinogen which is converted in active form of enzyme in the duodenum by trypsin or some other protease. The same regulatory mechanism occurs with other serine protease: trypsin is synthesyzed by the pancreatic cells in the form of inactive trypsinogen, elastase is synthesized in the form of proelastase which is activated by cleavage through trypsin etc. Serine proteases are widely used in detergent industry, especially the proteases with alkaline pH range.

Aspartic proteases are acid proteases with two aspartic acid residues within their active site which are crucial for their catalytic activity (Mamo and Assefa, 2018). Majority of the aspartic proteases show optimal enzyme activity at low pH (pH 3 to 4) and have isoelectric points in the pH range of 3 to 4.5. Pepsin and rennin are the most commonly used animal proteases. They are synthesized in the form of inactive precursor pepsinogen and prorenin. Microbial proteases are preferred over animal or plant proteases due to the easier production and purification of enzymes. Aspartic proteases are found in molds and yeasts but rarely in bacteria and most commonly are produced by *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* spp. Microbial aspartic proteases have a wide range of application in food and beverage industries, namely cheese production and gluten modification.

Cysteine proteases are very common in nature and they are characterized by histidine and cysteine residues present in the active site (Varma *et al.*, 2016). This group of enzymes includes the plant proteases papain, ficin and bromelain, although they are produced by many microorganisms and can be of animal origin. These proteases are active in neutral and slightly acidic environments, and range in size from 20 to 50 kDa. Cysteine proteases are synthesized in the form of inactive precursors which are activated in the presence of reducing factors (cysteine, glutathione, ascorbic acid, hydrogen sulfate).

Metalloproteases are among different types of proteases the most diverse. This group includes proteases that are quite different in nature, structure and origin (Razzaq *et al.*, 2019). A divalent metal ion is in most cases essential for their catalytic activity, most commonly zinc but also other divalent metals such as magnesium, manganese, cobalt, nickel and others.

Proteases are widely used in food production, especially in the meat industry, cheese industry and laundry detergent industry. Due to the new trends in the development of environmentally friendly technologies, their broader application in leather industry and bioremediation is expected. In addition, proteases are extensively used in the pharmaceutical industry for the production of medications such as keratolytic preparations for the removal of scars and debridement of wounds.

2.3 Pretreatment of the substrates

The development of an effective pretreatment that will separate lignin and carbohydrate fractions from the lignocellulosic raw materials is crucial. The addition of a pretreatment step in solid state fermentation increases the susceptibility of the biomass to microbial degradation and could enhance enzyme yields by promoting lignin removal and the destruction of complex biomass structures. The choice of the pretreatment method depends on several factors such as type and composition of lignocellulosic biomass, the intended application, the properties of the production microorganism and the overall economics of the process (Coffman *et al.*, 2014).

Suitable pretreatment should increase specific surface area under energy efficient and operation-easy regime, consequently causing improved accessibility of the nutrients in a manner that prevents excessive leaching of necessary sources of nourishment (Salim *et al.*, 2017). Pretreatments are usually performed in order to enhance the biodigestibility of the substrates by degradation of impenetrable complexes and at the same time changing the physical characteristics of the substrates, i.e. porosity of the residues, thus increasing the accessibility of microorganisms to the media. Preferably, pretreatment process will not cause significant loss of fermentable nutrients, does not require high capital equipment and toxic chemicals demand, nor produce toxic compounds in the process creating a negative environment for microbial growth, and minimizes the use of energy (Salim *et al.*, 2017).

Generaly pretreatments can be divided into physical (grinding, extrusion, hydrothermal, steam explosion), chemical (alkali, acid, oxidative, solvent extraction), physical-chemical (microwave, ultrasound) and biological methods (enzymes). They are based on different approaches for improving biomass yield and, consequently enzyme production, including size reduction to enhance the surface area, lignocellulosic raw material decrystallization, and the removal of hemicellulose and lignin from the cellulosic part.

Each of these methods has its advantages and disadvantages and must be optimized in each particular case raw material/producer/enzyme. For example, an advantage of the chemical method is its high efficiency in removing hemicellulose, however the formation of inhibitors is the major disadvantage of this method as well as the high temperature pretreatment process (Abraham *et al.*, 2020). On the other hand, the biological pretreatments, namely removal lignin and hemicellulose fraction in the raw material by microbial enzymes, is an eco-friendly alternative to physical or chemical processes due to mild process conditions compared to the chemical process, but much longer times are required than other modes of pretreatment (Xu *et al.*., 2019).

Among various methods that establish themselves as efficient methods for substrate pretreatment, ultrasound and microwave assisted methods are gaining increasing attention due to their promising effects on various types of biomass. Microwave is basically a heating method which directly applies an electromagnetic field to the molecule, producing simultaneously physical, chemical and biological changes and reactions. This leads to swelling and fragmentation, decreasing of the polymerization and/or crystalinity (Diaz *et al.*, 2015). Ultrasound pretreatment has been shown to be one of the promising method for processing various materials which is widely studied in recent period due to the fact that cavitation bubbles that are formed in the process cause specific changes in physical and chemical compositions of the treated substrates. It has been indicated that the ultrasonic method is particularly promising in the treatment of lignocellulosic biomass since it apparently affects crystallinity of cellulose, degrades lignin and dissolves the hemicellulose in such way that does not cause a formation of inhibitors of fermentative microorganisms (He *et al.*, 2017; Nakashima *et al.*, 2016). This process appears to be influenced by four main factors: specific energy, ultrasonic frequency, application time, and the characteristics of the substrate (Salim *et al.*, 2017).

2.3.1 Microwave pretreatment

Microwave irradiation is electromagnetic irradiation in the frequency range 0.3 to 300 GHz, positioned between radio waves and IR-waves in the electromagnetic spectrum. It is based on the direct influence of electromagnetic waves on materials that have the ability to covert a portion of electromagnetic energy into heat. Microwave consists of two oscillating, mutually controlling fields, electric and magnetic, which are also responsible for heating. In conventional heating the heat is transferred from (Zhu *et al.*, 2016) the surface to the inner areas of the materials by the means of convection, conduction and radiation, where in microwave heating the heat transfer is more direct, rapid and uniform (Figure 2.5). The very process of microwave heating can be explained by two phenomena: ionic conduction and dipolar rotation. Ion conduction occurs under the influence of an electric field as a result of electrophoretic migration of ions. Basically, migration of ions creates a friction which leads to heating up of the material. Dipole rotation involves the orienting the molecules under the influence of microwaves, in order to align with the direction of electric field. This phenomenon generates heat due to the frictional force between the rotating polar molecule and the surrounding medium (Tsegayea *et al.*, 2019).

Today, there are two commercially available microwave systems, an open and closed system (open vessel, closed vessel system). In the open system reaction is carried out at atmospheric pressure, while in the closed system the reaction takes place in controlled pressure and temperature.

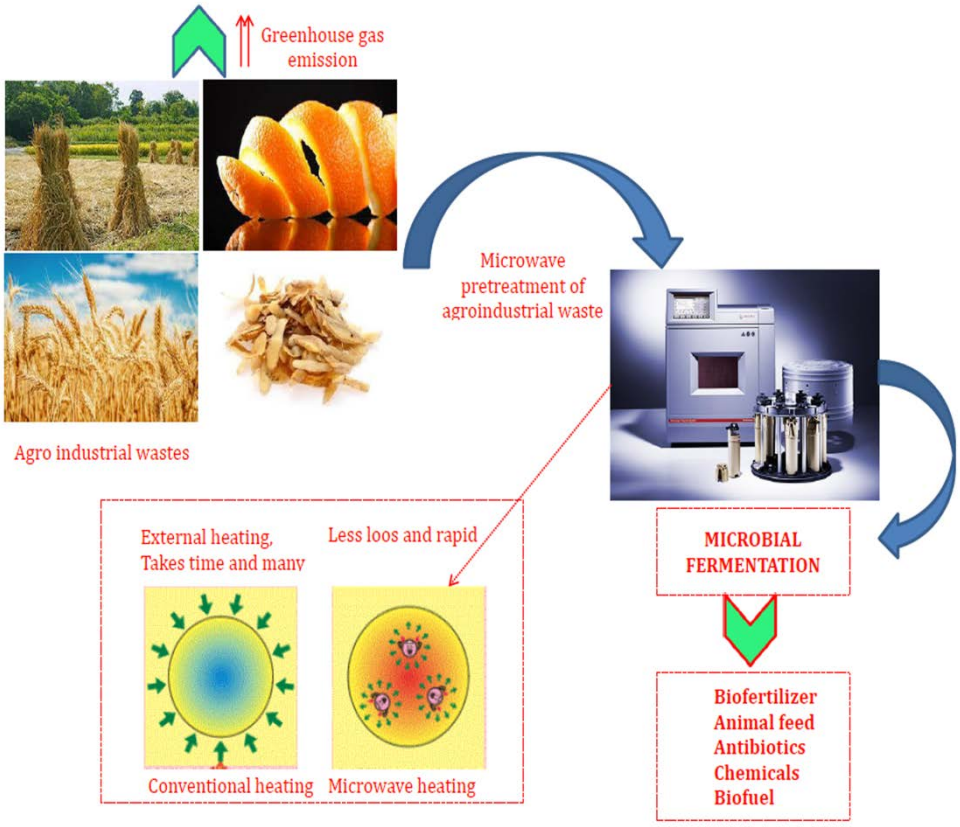

Figure 2.5. Graphical illustration of microwave pretreatment of agroindustrial waste (heat introduction and temperature distribution for conventional heating and microwave heating. While with conventional heating the heat comes from the outside and goes into the reaction mixture by convection currents (resulting in a very hot vessel wall), microwaves go through the almost microwave-transparent vessel wall and directly heat the reaction mixture on a molecular basis.

Today, there are two commercially available microwave systems, an open and closed system (open vessel, closed vessel system). In the open system reaction is carried out at atmospheric pressure, while in the closed system the reaction takes place at controlled pressure and temperature. This decreases reaction time, due to the closed vessel made of chemically inert materials and direct heat in of the sample solution. There are several different solvents that can be used for microwave pretreatment, first of all water, but it is known that solvents with high boiling points such as glycerol greatly enhance delignification (Diaz *et al.*, 2015).

Figure 2.6. Microwave closed vessel system.

2.3.2 Ultrasound pretreatment

An ultrasonic wave is a mechanical wave of frequency above 20 kHz that the human ear cannot hear (Figure 2.7) and it is classified into two categories: low-power ultrasound (high frequency – low power) and high power (low frequency – high power). Ultrasound with frequency range of 5-10 MHz are most commonly used in the diagnostic analysis of food products, where ultrasound frequency range of 20-100 kHz have lethal effect on microorganisms and as such are applied as treatment for storing food.

The treatment, namely pretreatment, of ultrasound on substrates is based on the principle of cavitation. The effect of ultrasound in liquid media results in the spontaneous formation, growth and subsequent collapse of microsize cavities/bubbles (Figure 2.7) (Subhedar *et al.*, 2016).

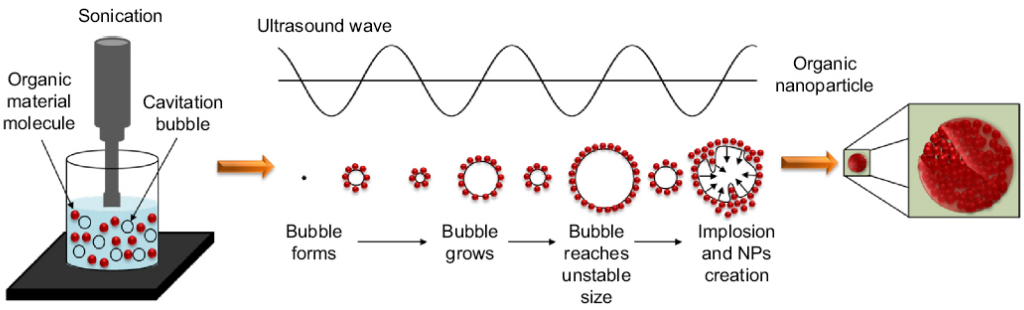

Figure 2.7 The acoustic cavitation phenomenon that occurs under ultrasonic radiation and the nanoparticles creation (Yariv *et al.*, 2015).

The implosion of these cavities produces high temperatures and pressure gradients locally for short duration (microsecond) thus creating the hot-spot effect in the liquid. These changes affect physical as well as chemical composition of the treated materials. The efficiency of cavitation depends on the frequency and intensity of the ultrasonic waves, as well as on the physical properties of the treated medium. Increasing the frequency of ultrasound leads to the creation of more cavitation bubbles of smaller diameter, and the energy release during bubble implosion is minimal.

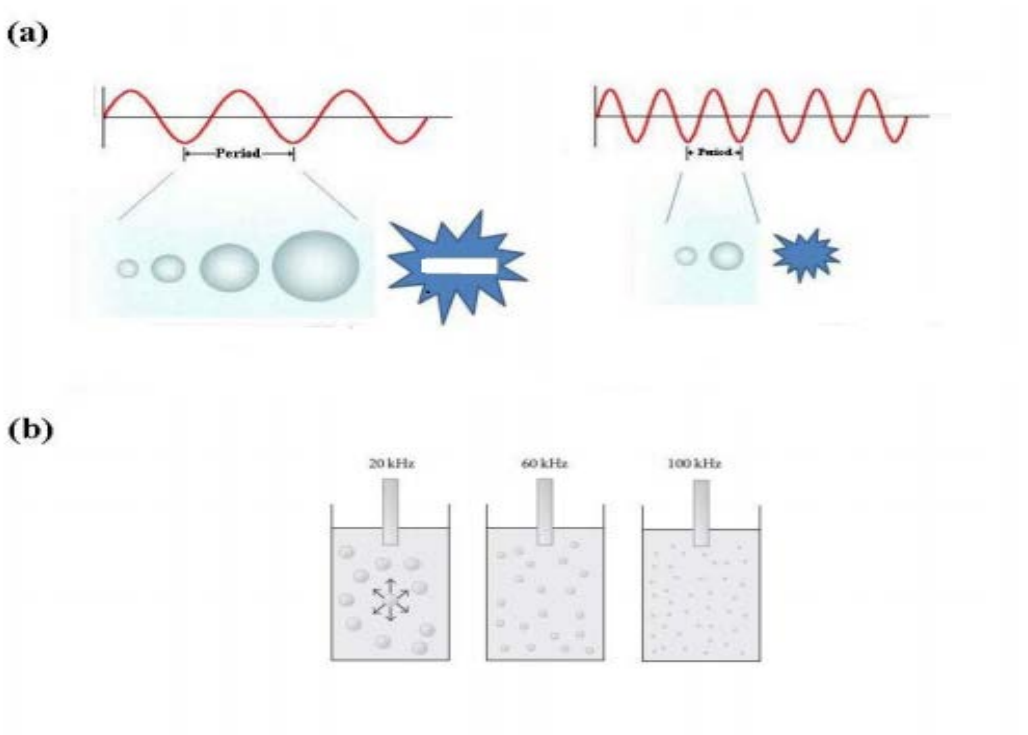

Figure 2.8 The influence of ultrasound frequency on the (a) formation and (b) size of cavitation bubbles (Stefanović, 2017).

In attended use of ultrasound in food industry, it is important to know the energy of the ultrasound which is determined by the power of ultrasound (W), intensity (W/cm2) and density (Ws/m3). Low power ultrasound (W/cm2, f = 5 - 10 MHz) is successfully used for non-invasive detection (process control), for characterization of physic-chemical inherent materials, for stimulation of live cell activity, surface cleaning of food, extraction, crystallization, drying and freezing, while high power ultrasound (10-1000 W/cm^2, f =20-100 kHz) is used for enzyme

and protein extraction, for induction of the reactions of oxidation and reduction, for the inactivation of enzymes, acceleration of reactions and so on.

2.4 Selection of working microorganism

For normal development of metabolic processes of all living cells, including microorganisms, necessity is the production of enzymes. Each individual microbial species produces a large number of different enzymes. However, within the same species there is a number of strains that have been adapted to different environmental conditions and therefore produce different amount of enzymes as well as enzymes with different properties. Primary task for the commercial production of enzymes is the selection of strains that have capacity to produce the largest amount of enzymes with desired properties.

Therefore, when choosing a microorganism for enzyme production, a large number of species from different sources and different habitats have to be examined. For example, extremophiles are good producers of enzymes of improved properties that can resist many industrial processes since these microorganism produce enzymes of extreme thermal and pH stability that actually correspond to their natural habitats. Screening of microorganism is a first step in the selection of enzyme producer and it is usually done by cultivation of potential microbial producer on special types of agar that contain the natural substrates of these enzymes. The ability if microorganism to synthesize the desired enzyme is easily perceived by the formation of clear zones around the emerging colonies, and the diameter of the hydrolyzed zones should correspond to the enzymatic activity.

For example, in the case of mannanase production, the used selection medium as the sole source of carbon offers mannanase isolated from seed rosaries, for pectinase production pectin and for cellulase carboxymethylcellulose. If bacteria is capable of growing on such medium it has to have an enzyme (or group of enzymes whose expression is regulated under the same operon) which enables them to decompose and utilize that product. Microorganism enzyme producers are selected based on the size of the transparent zone around the colonies after 72h,

specifically based on the size of the fluorescent zone after illumination with UV light in the substrate with the addition of Rhodamine B.

In addition to high productivity and desired properties of the enzymes, there are several other criteria that microbial enzyme producers have to satisfy. It is the ability to grow on cheap raw materials, as well as the ability to grow in adapted conditions and the growth has to be fast and abundant. Depending on the desired application, the bacteria has to satisfy certain safety requirements. This is extremely important for the production of enzymes that are intended for the food industry. Such microbial producers must be registered by FAD (America Food and Drug Administration) as a non-pathogenic, non-toxic microorganism that generally do not have any antibiotic activity, which actually implies their GRAS status (Generally Recognized as Safe). Up to date, there are only about 50 microbial types found on the GRAS list. In addition to these requirements on the enzyme producers, the enzyme itself that is intended to be applied in food production has to satisfy a set of strict requirements as defined in the national rules on quality and other requirements for enzyme preparations for food products.

3 NUTRITIONAL AND ANTINUTRITIONAL VALUES OF AGRO-INDUSTRIAL RESIDUES

Agroindustrial residue is the main by-product of agricultural based industries. An increase in the amount of agroindustrial residues is recorded each year. Complex polysaccharides are the main components of agroindustrial residues and can be used as substrates for microbial growth as well as enzyme production. Solid state fermentation (SSF) is superior economic method for the nutritional and antinutritional proportion change in agroindustrial residues which are widely used in animal nutrition. SSF improves nutritional, functional as well as other health promoting properties of substrates used for the fermentation.

3.1 Antinutritional factors in plant material

Plants have several mechanisms of protection from the attacks of insects and pathogens, as well as the impact of unfavorable environmental conditions. The

most important defensive plant mechanism is the production of secondary metabolites so-called antinutritional factors. The antinutritional factors (natural toxicants) can be defined as those substances generated in natural food sources by the normal metabolism which reduces the nutrient utilization and/or food intake of plants or plant products used as human and animal foods, and they play a vital role in determining the use of plants for human and animals (Gemede, 2014).

Antinutritional factors can be classified into few groups (Hajra *et al.*, 2013):

- Antinutritional factors affecting protein utilization and digestion. This group of antinutritional factors includes protease inhibitors, lectins, hemaglutinins, tannins and others;
- The use of minerals in plants is influenced by antinutritional factors such as: phytates, oxalates, glucosinolates, pigments (gossypol);
- Vitamins utilization in plants is affected by antinutritional factors known as antivitamins (antithiamines like pyrithiamine, antiriboflavins like galactoflavin, antiniacin like pyridine-3-sulphonic acid, 3-acetyl pyridine etc., antipantothenic acid like pantoyltaurine);
- Miscellaneous compounds like mycotoxins, mimosine, cyanogens, alkaloids, phytoestrogens are also known as antinutritional factors.

The most important plant species that make up an important part of human and animal nutrition are from the family of legumes. They are important source of protein, carbohydrates, amino and fatty acids, minerals if properly processed. Besides use in nutrition legumes are able to fix the nitrogen from air resulting in increased fertility of soil significantly reducing the use of expensive nitrogenous fertilizers. Generally, despite all advantages (improving of soil fertility, use as food and feed, cheep protein sources), the application of legumes is often limited due to deficiency in sulfur amino acids, poor digestibility and the presence of antinutritional factors (Gebrelibanos *et al.*, 2013).

3.2 Antinutritional factors affecting protein utilization and digestion

The most important in the group of antinutritional factors affecting digestion of proteins are protease inhibitors. Protease inhibitors (PIs) are small proteins, natural antagonists of protease and they interact with their target protease by contact with catalytic site resulting in formation of stable protease-inhibitor complex which has no catalytic function. PIs are widely distributed in nature in all life forms. The protease inhibitors are usually found in storage organs such as seeds (Leguminosae family), tubers (Solanaceae family) but their occurrence in aerial part of plant is also well documented (Shu-Guo and Guo-Jiang, 2015; Olanca and Ozay, 2015) In plant storage tissues, protease inhibitors make up 1-10% of the total content of water-soluble proteins (Shu-Guo and Guo-Jiang, 2015). Plant protease inhibitors can be classified into families such as: Bowman-Birk, Kunitz, Potato I, Potato II, Serpine, Cereal, Mustard and Squash. Although, protease can exert the same function (e.g. inhibit trypsin), the differences in molecular weight, amino acid composition and physical structure originate from the plant source (Table 3.1.).

Table 3.1. Physical and chemical properties of protease inhibitors of Leguminosae, Gramineae, Solanaceae (Friedman, 1986).

Source	Inhibitor(s)	Protease(s) Inhibited	M.W. Daltons	pI	-S-S- bonds /mole	Reactive Site(s)
Leguminosae						
Soybean	Bowman-Birk	T; C;	7848	4.2	7	Lys16-Ser17; Leu43-Ser44.
	C-II	T; C; E;	8282	-	7	
	D-II	T;	8507	-	7	Ala22-Ser23; Arg49-Ser50.
	Kunitz	T; C;	20,083	4.5	2	
Peanut	B-III	T; C;	6716	-	7	Arg24-Ser25;

						Arg51-Ser52. Arg63-Ile64. Arg10-Arg11; Arg39-Ser40.
Gramineae Corn Barley	 - -	 T T	 12,028 13,305	 - -	 5 5	 Arg36-Leu37; Arg33-Leu34.
Solanaceae Eggplant Potato	 - CI-I PTI	 T C C	 5552 9332 5593	 4.7 - -	 4 1 4	 Ar38-Asn39; Met60-Asp61; Arg38-Asn39.

T, C, E are trypsin, hymotrypsin and elastase, respectively.

The synergy of proteases and their corresponding inhibitors is of vital importance to the plant cells as shown in Figure 3.1.

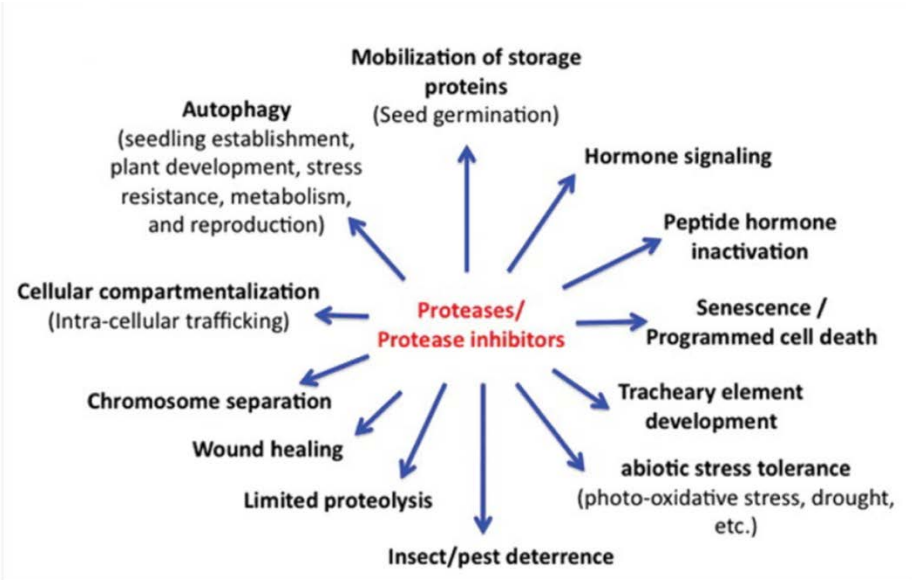

Figure 3.1. Biological significance of protease and protease inhibitors interactions in plants (Rustgi *et al.*, 2018).

The protease inhibitor inhibits the functioning of the digestive enzymes trypsin and, sometimes, have wider spectrum of activity, including the inhibition of other serine proteases such as chymotrypsin. Inhibition of the proteolysis in the intestines as a consequence reduces the absorption of amino acids resulting in reduction of growth and increasing the need for amino acids with methionine and cysteine (Shu-Guo and Guo-Jiang, 2015). The use of plant material in human nutrition can result in breast cancer appearance meanwhile animal feed rich in trypsin inhibitor has proved to have anticancerogenic effects on numerous animal tissues.

3.2.1 Trypsin inhibitor in soybean

Soybean (Glycine max), belongs to the family of leguminose and has become one of the most widely used foods on the world. The nutritive value of soy comes from a large amount of vitamins, minerals, dietary fiber, organic compounds and proteins found in its grains. In addition to the nutrient-rich soybean proteins, there are certain soy proteins that have a detrimental effect on animals if they are used in their diet and are known as protease inhibitors. Plant protease inhibitors originating from the soybean were the most extensively studied among all plant species. Protease inhibitors in soybean reduces the protein digestibility, consequently they represent the main antinutritional factor in soybean. So far, in soybean seeds were found two protease inhibitors: Bowman-Birk inhibitor (BBI) and Kunitz trypsin inhibitor (KTI) and they constitute 6% of the proteins present in soybean (Fanf *et al.*, 2012). Both, Bowman and Kunitz inhibitor belong to the class of serine proteases.

Bowman-Birk inhibitor is a monomeric protein, belongs to the group of double-headed proteases with molecular weight ranging from 8000-10000 Da (Figure 3. 2).

Unusually rich in cysteine, consist of 71 (Figure 3.2) amino acids and a network of 7 highly conserved disulfide bridges (Wei *et al.*, 1979). Disulfide bridges are responsible for maintaining the stability of the molecules in the case of extreme conditions, and have prominent role in the stabilization of structure and

function of the active sites of the molecule (Clemente *et al.*, 2011). The specificity of this protease inhibitor type is in two active sites located on the opposite sides of the molecule. Thus, the Bowman-Birk inhibitor can inhibit both, trypsin and chymotrypsin-like proteases (Srikanth and Chen, 2016).

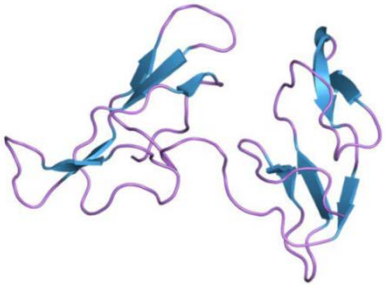

Figure 3.2. Bowman-Birk trypsin inhibitor in soybean.

In the case of chymotrypsin inhibition the Bowman-Birk inhibitor activity is more pH dependent and it was found that its activity is blocked at pH 5.3 and it can be recovered at pH 7.0 (Fang *et al.*, 2012).

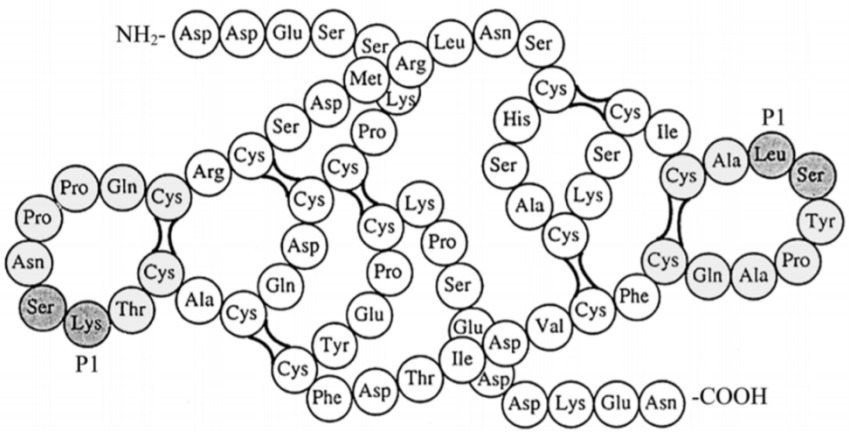

Figure 3.3. Primary structure of Bowman-Birk inhibitor in soybeans (Qi *et al.*, 2015). The two reactive sites consisted of nine residues are shaded and protease binding places are marked as P1, and 7 disulfide bridges between Cys residues are presented.

Bowman-Birk trypsin inhibitor has two independent inhibition sites, one located at the position Lys16-Ser17 for trypsin inhibition and other at the position Leu43-Ser44 for chymotrypsin (Figure 3.4). Bowman-Birk forms 1:1 complex in contact with trypsin or chymotrypsin and ternary complex with both proteases (Birk, 1985). Detailed studies of the structure and function of the Boman-Birk inhibitor found that this protein exists in several isoforms that can be classified into three groups: BBI (which inhibits trypsin/chymotrypsin), iso-inhibitor C (which inhibits elastase/trypsin) and iso-inhibitor D with trypsin/trypsin inhibitor activity (Fang *et al.*, 2015).

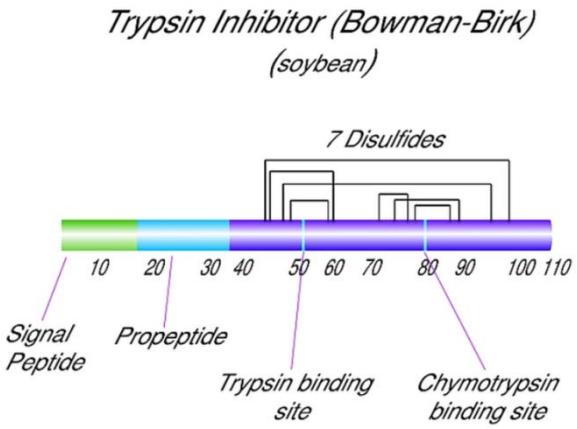

Figure 3.4. Bowman-Birk trypsin/chymotrypsin inhibitor active site (Srikanth and Chen, 2016).

Both active sites in Bowman-Birk inhibitor function independently and at the same time they can bind the same or different proteases for active sites. Soybean Bowman-Birk inhibitor have demonstrated remarkably resistance towards to the action of proteolytic enzymes and severe thermal treatments (A. Clemente and Del Carmen Arques, 2014).

Kunitz inhibitor is another protease inhibitor widely present in soybean seeds (Figure 3.5). Kunitz's inhibitor from soybean was the first isolated and characterized plant protease inhibitor and belongs to the group of non-glycosylated proteins. The molecule has spherical shape with diameter 3-5 nm (Roychaudhuri *et al.*, 2004).

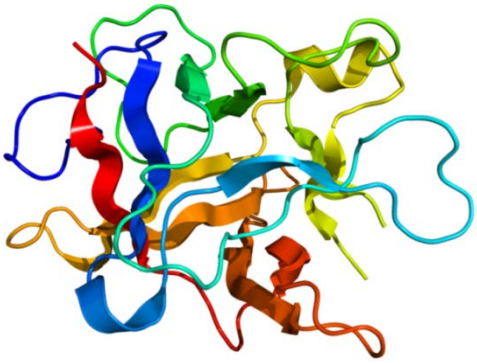

Figure 3.5. Kunitz inhibitor in soybean.

Kunitz inhibitor accounts about 5% of protein in soybean seeds and serves as storage protein and supplies sulfur-containing amino acids to the seeds. It is well known as inhibitor of digestive proteolytic enzymes of mammals and insects.

Unlike the Bowman-Birk inhibitor, Kunitz inhibitor has a higher molecular weight of about 20 kDa and isoelectric point at pH 4.5 (Vagida *et al.*, 2017). It was found that Kunitz inhibitor exists in three isoforms, which are referred to as: Tia, Tib, Tic all three consisting of 181 amino acid residues. Reactive site for all isoforms is found at the position Arg63-Ile64 (Momonoki *et al.*, 2002). Primary structure of Kunitz inhibitor is presented on Figure 3.6.

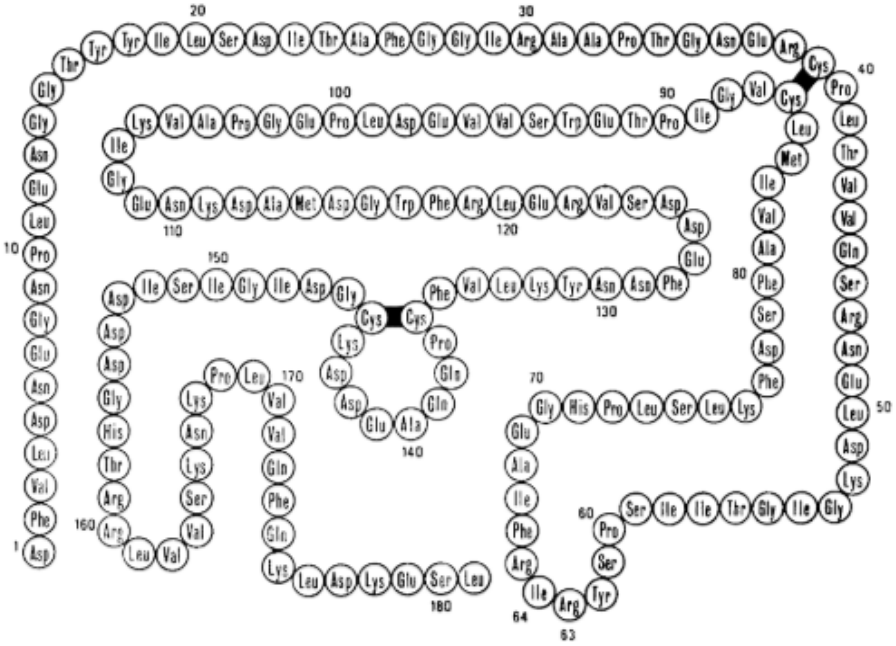

Figure 3.6. Kunitz soybean inhibitor primary structure.

It has only one active site and one or two disulfide bridges in its structure (Figure 3.7). Kunitz inhibitor binds to the protease active site and behaves as a competitive inhibitor.

This type of binding is reffered as reversible but in some cases the inhibitor has such strong affinity towards chosen protease and thus the reaction can be considered as irreversible.

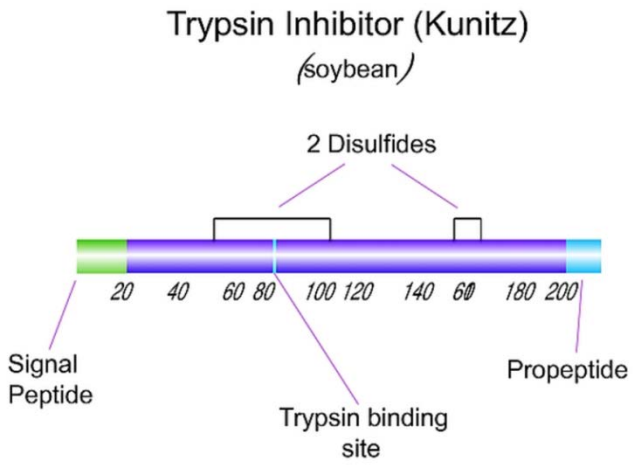

Figure 3.7. Kunitz inhibitor active site in soybean (Srikanth and Chen, 2016).

In the Kunitz inhibitor structure around the loop, where the active center is located, the Cys residues form disulfide bridge necessary for the inhibition (Major and Constabel, 2007). The disulfide bridges are positioned between the cysteine residues Cys36-Cys86 and Cys136-Cys145 and these positions are vital for the inhibitory activity of the molecule (Song and Suh, 1998). This part of Kunitz inhibitor structure is highly resistant to thermal and chemical denaturation (Roychaudhuri *et al.*, 2004). The secondary structure of the Kunitz inhibitor is mainly organized in the form of beta sheets (12 criss-cross antiparalell β strands), but there is also a small contribution of regular sheets. The overall structure of the molecule is stabilized by hydrophobic side chains (Roychaudhuri *et al.*, 2004).

Trypsin inhibitors from soybean reduce the proteolytic activity and increases the fecal loose of pancreatic enzymes rich in sulfur-containing amino acids that can not be compensated by dietary soy protein and thus resulting in animal growth depression. Besides, trypsin inhibitors cause pancreatic

hipertrophy. Inactivation of the trypsin inhibitor is possible if two disulfide bridges break apart which are achieved by various physical, chemical and enzymatic treatments (Vagida *et al.*, 2017).

3.2.2 Phytic acid in soybean

Phytic acid is an antioxidant widely distributed in cereals, vegetables, nuts and natural oils. The amount of phytic acid in the plant material varies in the range 1-5% and it is believed that 60-90% of the total phosphorus in the seed used in the food and feed is in the form of phytic acid. The chemical structure of phytic acid is presented on Figure 3.8. Inositol is the basic unit of the phytic acid structure with 6 phosphate groups attached to the ring. When six carbons are bound to phosphate groups, the compound is known as inositol hexaphosphate (phytic acid, IP6 or myo-inositol-1,2,3,4,5,6-hexaphosphate).

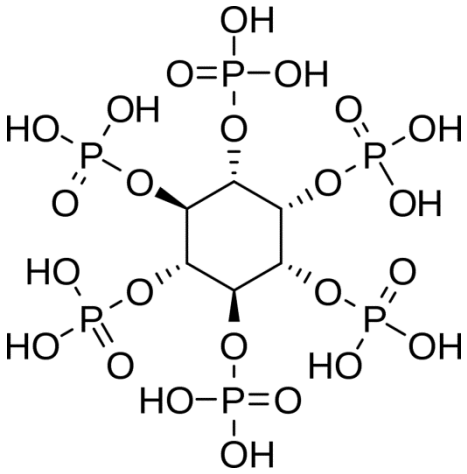

Figure 3.8. Phytic acid in legumes (soybean) structure.

Phytic acid has unique structure where phosphate groups in positions 1,2 and 3 (axial-equatorial-axial) allow the specific interaction with iron, resulting in Fenton reaction inhibition, i.e., the ability of phytic acid to form OH radicals is inhibited. Phytic acid in the seeds is a source of energy, a phosphorus storage and activator of dormancy (Silva and Bracarense, 2016).

Due to its structure, phytic acid is a nutritional and antinutritional factor. Nutritional value of phytic acid is in its antioxidant potential in inhibiting radical oxygen species production (ROS).

The antinutritional effect of phytic acid comes from the chelating ability of its six phosphate groups. Complexing with multivalent cations of calcium, magnesium, iron, and zinc produces insoluble and non-digestible complexes. The affinity of phytic acid towards multivalent cations is as follows: $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$. The most important factor influencing the complexation with multivalent cations are phytate:metal ratio and the pH (Silva and Bracarense, 2016).

Besides interaction with multivalent cations, phytic acid strongly reacts with proteins, enzymes and starches. Namely, a strong interaction between phytic acid and protein is possible when there is a certain charge on the protein surface. This indicates that phytic acid-protein interactions are possible in a pH medium where the pH is above or below the isoelectric point of the protein (Figure 3.9). Negative charge on the dissociated phosphate groups allows protein-phytic acid interaction over the entire pH range (Figure 3.9).

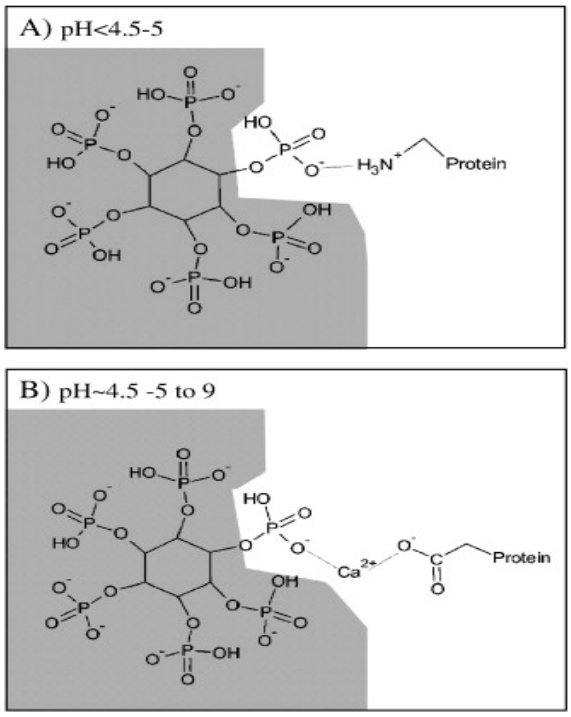

Figure 3.9. Phytic acid interactions with proteins. A) Binary complex: phytic acid-proteins. B) Ternary complex: phytic acid-divalent cations (Ca^{2+})-proteins (Ali *et al.*, 2010).

For a pH below the protein isoelectric point (soybean 4.5-5.5) protein has positive net charge and electrostatic interactions are possible resulting in formation of binary insoluble complex. Above the protein isoelectric point interactions protein-phytic acid-divalent cations ($Ca^{2+,}$ Mg^{2+}) results in ternary complex formation (Figure 3.9) (Ali *et al.*, 2010).

After the protein-phytic acid interaction, the protein properties change significantly. Initially, the proteins become less or completely insoluble, with adverse effects on the functional properties of the protein (viscosity, gelation, emulsifying and foam capacity and dispersibility in aqueous media) (Urbano *et al.*, 2000; (Silva and Bracarense, 2016).

3.2.3 Carbohydrates in soybean

Soybean seeds contains approximately 33% carbohydrates and soluble sugars are up to 16.6% (Ghani *et al.*, 2016). Some of antinutritional factors in soybean are easy to remove because they are thermolabile (protease inhibitors and lectins), but some of antinutritional factors such as non-starch polysaccharides (NSP) and oligosaccharides are thermo-stable and they are not eliminated by thermal treatment (Leske *et al.*, 1993). The carbohydrates in soybean can be classified into two types: soluble oligomers and insoluble polysaccharides. The soluble oligomers are dissacharide sucrose, the trisaccharide raffinose and the tetrasaccharide stachyose and verbascose and they make up one third of the total carbohydrates in soybean (Figure 3.10). The remaining two thirds are complex cell wall polysaccharides. Sucrose is desirable component in soybean seeds and raffinose and stachyose are considered to be antinutritional factors and eliminating or reducing them is beneficial.

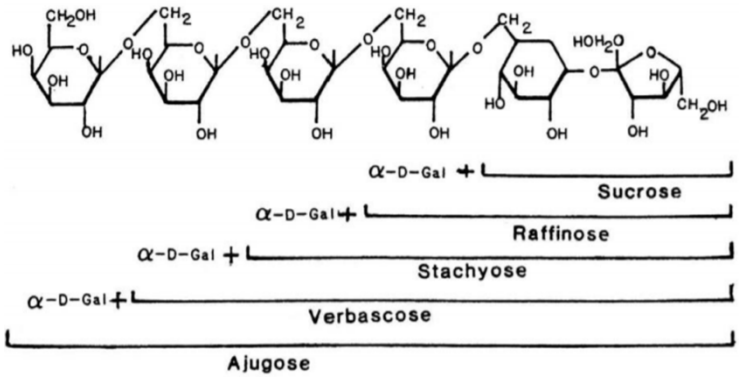

Figure 3.10. The chemical structure of soy carbohydrates (Choct *et al.*, 2010).

Oligosaccharides like raffinose, stachyose and verbascose belong to the group raffinose family oligosaccharides. The first member of this group is raffinose which has one structural unit sucrose and galactose (α-D-galactopyranososyl-(1-6)-α-D-glucopyranosyl-(1-2)-βD-fructofuranoside). Raffinose is found in plants arranged in leaves, root, seeds and rhizomes. Stachyose, α-D-fructoruanoside, tetreasaccharide is distributed in plant leaves. Verbascose results from the addition of third galactose unit. These oligosaccharides, primary, stachyose and raffinose have detrimental effects on the soybean nutritional value and they make 4-6% of dry matter in soybean (Wilcox *et al.*, 2000; Leske *et al.*, 1993). The stability against thermal treatment of oligosaccharides stachyose and raffinose is high so these antinutritional factors can not be eliminated by heat. The antinutritional effect of these oligosaccharides is primary due to lack of α-galactosidase necessary to hydrolyze the α-1,6 linkages present in oligosaccharides stachyose and raffinose. Besides soluble sugars, soybean contains also desirable sugars such as glucose, fructose and sucrose (>1%). This fraction of soybean sugars is known for their sweet taste and easy digestion (Ghani *et al.*, 2016).

3.2.4 Phenolic compounds in soybean

Phenolic compounds are secondary plant metabolites commonly known as antioxidant nutrients. Phenolic compounds have aroused interest due to their remarkable spectrum of biochemical and pharmacological properties. These compounds have the ability to chelate metal ions, ability to donate hydrogen and to inhibit selectively the enzymes responsible for the catalysis of various oxidation

processes (Hounsome *et al.*, 2008; Ozekeri, 1999). In plants phenolic compounds are distributed unevenly. Insoluble phenolic compounds are located in the cell walls meanwhile the soluble phenolics are in cell vacuoles (Naczk and Shahidi, 2006). Phenolic compounds in their structure contain an aromatic ring (one or more) with at least one -OH group as a substituent, including functional derivatives (esters, methyl esters, glucosides etc.) (Ho *et al.*, 1992). Based on their chemical structure phenolic compounds can be divided into at least 10 groups from simple such as phenolic acids to highly polymerized such as tannins (Figure 3.10).

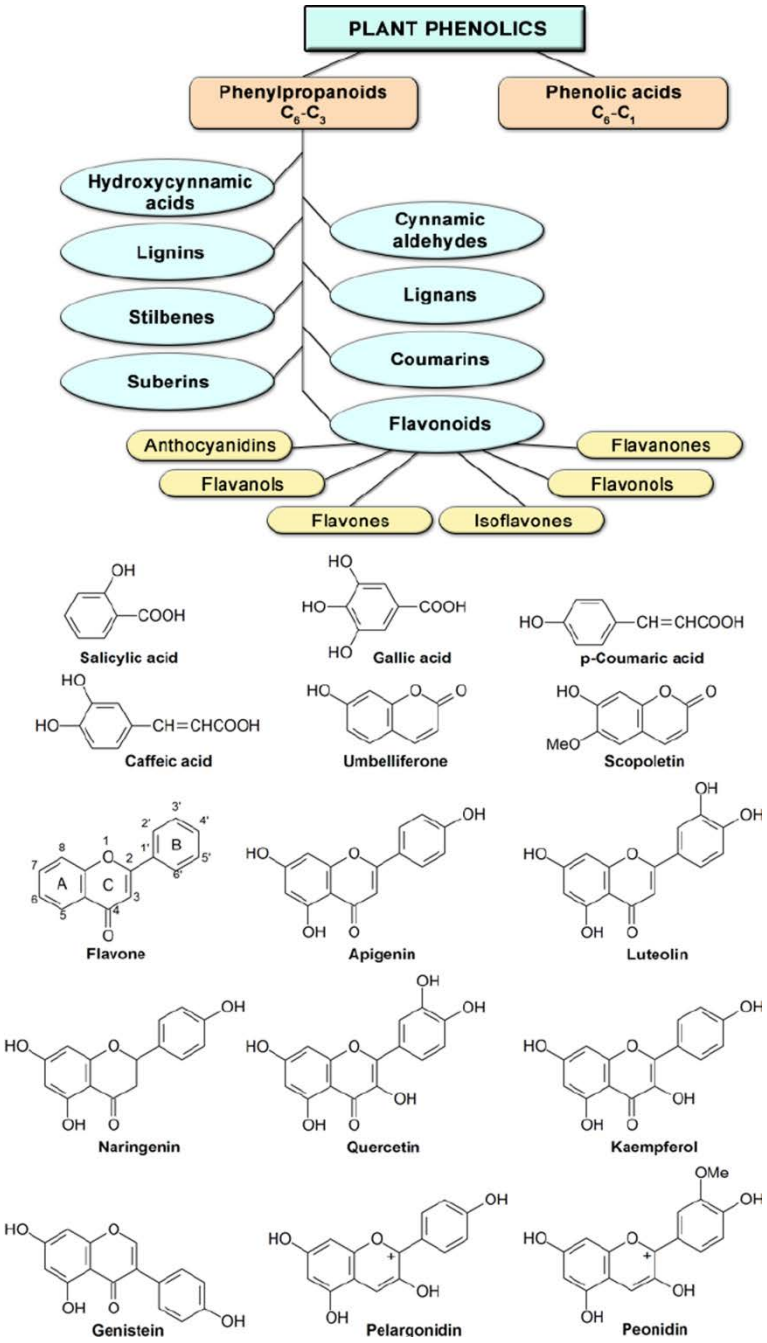

Figure 3.10. Phenolic compounds found in plants (Katerova *et al.*, 2012).

Polyphenols are the compounds with diverse structure, molecular weight and properties and are ubiquitously present in plant-based foods such as tea, coffee, wine, cocoa, soy, fruits and berries. The main dietary phenolic compounds are phenolic acids, flavonoids and tannins.

Phenolic acids can be divided into two groups based on their structure: benzoic acid derivative (C6 to C1) and cinnamic acid derivative (C6 to C3). The most important benzoic acid derivatives are gallic, protocatechuic, vanillic and syringic acid. The most common cinnamic acid derivatives are caffeic, ferulic, p-coumaric and sinapic acid (Balasundram *et al.*, 2006; Cirkovic Velickovic and Stanic-Vucinic, 2018) (Figure 3.11).

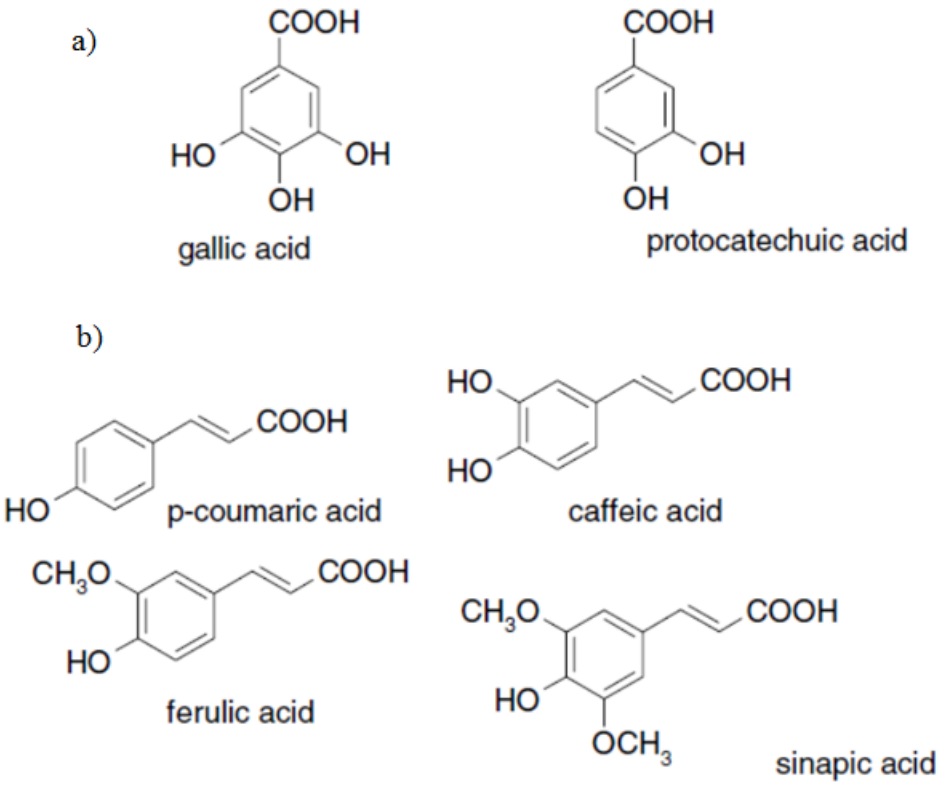

Figure 3.11. Examples of (a) benzoic acid derivatives and (b) cinnamic acid derivatives (Balasundram *et al.*, 2006).

The key factor determining the antioxidant capacity of phenolic acids is certainly their structure. Specifically, monohydroxy benzoic acids with an -OH group positioned in the -ortho or -para position relative to the carboxyl group does not exhibit any antioxidant activity. The antioxidant capacity is directly related with hydroxylation degree. Increasing the number of hydroxyl groups increases the antioxidant capacity as is the case with trihydroxylated gallic acid. In the case of syringic acid the substitution in 3- and 5- position of –OH groups with methoxyl

groups leads to reduction in antioxidant capacity. In general, hydroxycinnamic acid exhibits higher antioxidant capacity compared with hydroxybenzoic acids. The higher activity is related with the presence of the CH=CH-COOH group responsible for increasing the donation capacity of hydrogen and the stabilization of radicals than is the case with the -COOH hydroxybenzoic acid group (Balasundram *et al.*, 2006).

Flavonoids are the second group of polyphenolics found in soybean. The classification of flavonoids is given in Figure 3.12.

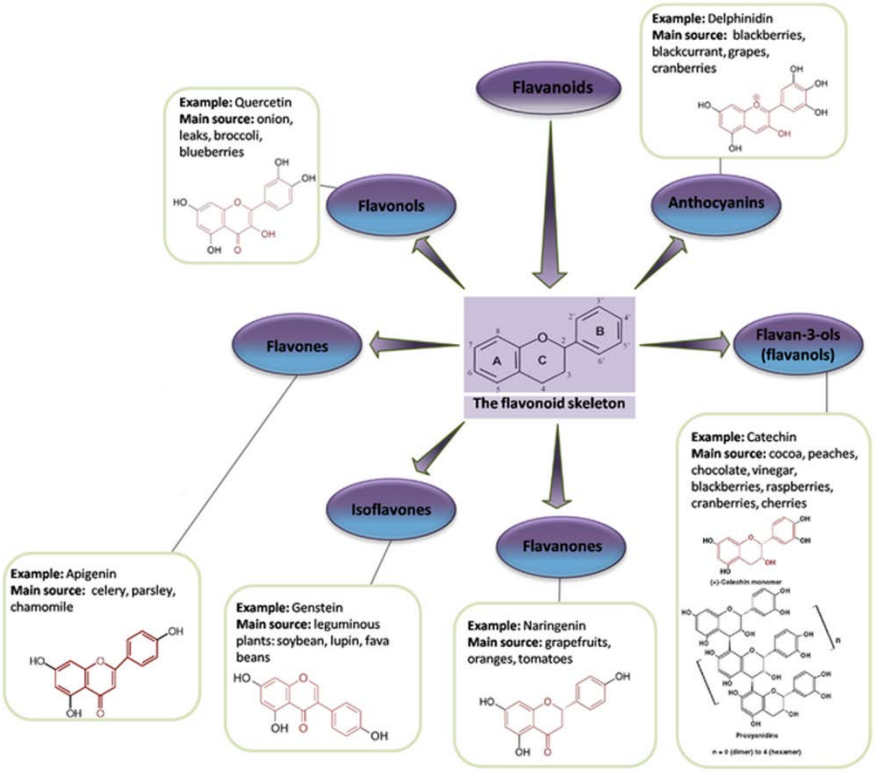

Figure 3.12. Flavonoids in soybean (Goszcz *et al.*, 2015).

This family of flavonoids includes several thousand compounds. This is the group of low molecular weight compounds. Flavonoids have 15 carbon atoms in their basic structure. They are organized in two aromatic rings (A and B) connected with heterocyclic ring C that contains one oxygen atom as presented in Figure 3.12. Two aromatic rings A and B are derived from different metabolic pathways, acetate/malonate and shikimate pathway, respectively (Ghosh, 2015). Different flavonoids are formed during different substitutions on the C ring. Based

on this, flavonoids can be divided into several subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. Glycoside form (O- and C glucosides) of flavonoids is very often. This form allows flavonoids to be more soluble in water and to be less reactive. The antioxidant capacity of flavonoids is strongly related with the substitutions on the B and C ring. It was found that higher hydroxylation degree (ortho-dihydroxyl structure) on the B ring resulted in improvement in antioxidant capacity. Besides, improvement in antioxidant capacity is noted in the case where pyrogallol groups are present in positions 3'-, 4'- and 5' on the B ring. Radical scavenging activity of flavonoids can be enhanced by conjugation of double bond between C-2 and C-3 and 4-oxo group on the ring C. On the other hand, substitution of -OH group with metoxyl group reduces the redox potential of flavonoids (Manach *et al.*, 2004; Iwashina, 2000; Balasundram *et al.*, 2006).

Tannins are the third important class of polyphenolic compounds. They present polymers of flavonoids or phenolic acids and they can be found in hydrolyzable or non- hydrolyzable (condensed) tannins (Figure 3.13).

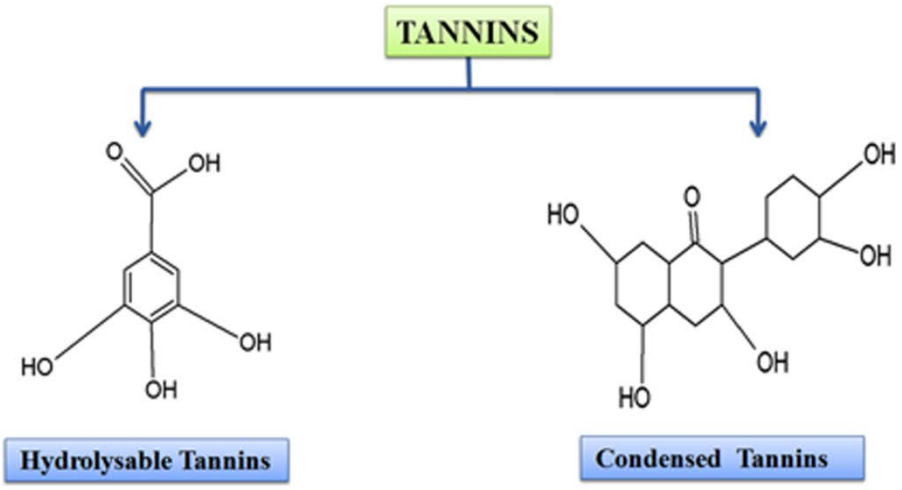

Figure 3.13. Classification of tannins (Ghosh, 2015).

Hydrolyzable tannins are esters of gallic acid, while the condensed tannins are polymers of polyhydroxyflavan-3-ol monomers. Tannins form complexes with dietary proteins and carbohydrates as well as with the enzymes. Besides, it was

found that tannins can reduce the adsorption of minerals such as iron and copper (Watson and Preedy, 2009).

3.3 Methods of reduce the deleterious effect of ANF's

Reduction or removal of antinutritional factors from cereals is vital to maximize their nutritional potential in human and animal nutrition. Various processing techniques can increase the nutritive value of cereals by increasing the content of essential amino acids, the digestibility of proteins, and the utilization of amino acids, all by reducing or eliminating antinutritional factors.

Non-thermal treatment which are used to process antinutritional factors from legumes are wetting, germination and fermentation. Wetting is in most cases the first step in eliminating antinutritional factors and thus largely removing water soluble antinutritional factors. With this non-thermal method it is possible to reduce the content of total sugars, α-galactoside, minerals, phytic acid and proteolytic enzyme inhibitors. Wetting the cereal grains in water often triggers a process of diffusion of Na, K and Mg-phytate, which is then easily removed by decanting water. Germination is another way of removing anti-nutritional factors. During the germination there are significant changes in the biochemical, nutritive and sensory properties of cereals. During germination, the storage proteins are hydrolyzed and the liberated amino acids are transported to the base of the seedlings and thus feed the plant. Also, germination cause activation of the enzyme phytase which catalyzes the hydrolysis of phytic acid whereby an increase in the available inorganic phosphorus is achieved. In addition, it has been found that germination leads to an increase in the content of some minerals and vitamins and that the amount of trypsin inhibitor is reduced (Abbas and Ahmad, 2018; Perlas and Gibson, 2002; Akande and Fabiyi, 2010).

Thermal treatment which implies cooking removes thermolabile antinutritional factors such as trypsin and chymotripsin inhibitors, but not completely. If during the heat treatment water is not removing, very often thermostable antinutritional factors maintain virtually unchanged. Consequently, during thermal treatment in water, it is necessary to remove water and in this way

the vast majority of the thermostable components are removed. Besides, it is necessary to avoid excessive heat treatment because it contributes to the reduction of protein and food quality. In addition to the traditional methods of cooking, the combination of cooking and microwave treatment, followed by reduced fat, total ash, carbohydrate fractions (reduction in the amount of reducing sugar, sucrose, raffinose and starch, while verbascose is completely eliminated in this way). In addition, this combined treatment significantly reduces the amount of trypsin inhibitors, tannin, saponin and phytic acid (Grumezescu and Holban, 2018; Shimelis and Rakshit, 2007).

Fermentation is one of the most commonly used methods of reducing or eliminating antinutritional factors. One of the frequently used fermentation techniques for processing the different substrates in order to reduce/remove antinutritional factors is the solid state fermentation (SSF). This technique is especially important because in this way different agro-industry wastes can be processed. In SSF different types of agroindustrial wastes can be used as solid support, carbon/or nutrient source for the production of a variety of value-added compounds.

Figure 3.14. Positive aspects of microbial fermentation of waste.

SSF can be considered from two aspects. The first aspect is the bioactive compounds and/or enzymes production and the second is antinutritional factors reduction/removal. During fermentation, phytases, enzymes responsible for degradation of phytic acid, are activated. This hydrolysis is of utmost importance because in this way, the 5-phosphate myoinositolphosphates are formed that have no negative effect on zinc absorption and those with 3-phosphate groups that do not inhibit the absorption of iron. During the fermentation process, organic acids of small molecular weights (acetic acid, lactic acid) are formed which increases the absorption of iron and zinc by forming soluble ligands while simultaneously maintaining the pH value of the medium such as to allow optimum activity of phytases present in cereal seed (Mohamed *et al.*, 2011). Besides, the removal of

raffinose and stachyose during the SSF is well documented. The removal of oligossacharides is related with the potential of some lactic bacteria used for the SSF to metabolize these oligosaccharides. On this way the oligossacharides are transformed into absorbable form (Worku and Sahu, 2017).

4 EXPERIMENTAL PLAN

4.1 Materials

Chemicals used in this research were:

- sunflower meal;
- soybean meal (Soja Protein, Bečej, Serbia);
- olive oil cake;
- NaOH (Lach-ner, Neratovice, Czech Republic);
- $C_4H_4KNaO_6$ (E. Merck, Darmstad);
- $CuSO_4 \cdot 5H_2O$ (Zorka Pharma, Šabac, Serbia);
- bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, USA);
- Folin & Ciocalteu reagent (Sigma Aldrich, St Louis, SAD);
- Na_2CO_3 (Zorka Pharma, Šabac, Serbia);
- K_2HPO_4 (LachNer, Neratovice, Češka);
- KH_2PO_4 (LachNer, Neratovice, Češka);
- $MgSO_4$ (LachNer, Neratovice, Češka);
- trichloroacetic acid (E. Merck, Darmstad);
- yeast extract, meat extract
- D-galacturonic acid;
- 3,5-dinitrosalicylic acid (DNS) (Sigma Aldrich, St. Louis, USA);
- azocasein and casein, (Sigma Aldrich, St. Louis, USA);
- Coomassie brilliant blue G-250 (Thermo Fisher Scientific);
- Sephadex G-75 (Sigma Aldrich, St. Louis, USA);
- Toyopearl DEAE (TOSOH Corporation, Shiba, Tokyo);
- carboxymethyl cellulose, (Sigma Aldrich, St. Louis, USA);
- pectin from apple (Sigma-Aldrich);

- locust bean gum, (Sigma Aldrich, St. Louis, USA),
- selective mediums: for proteolytic activity-milk-casein agar, for amylolytic activity-starch based agar, for cellulolytic activity-CMC agar, for pectinase activity pectin agar and for mannanase activity- MS agar;
- genus specific primers- upstream primer UNI16SF 50-GAG AGT TTG ATC CTG GC-30 and the downstream primer UNI16SR 50-AGG AGG (Sigma Aldrich, St. Louis, USA),
- glycerol (Sigma Aldrich, St. Louis, USA),
- 95% ethanol (ZorkaPharma, Šabac, Serbia).

Equipment:

- BANDELIN SONOPLUS Ultrasonic Homogenizer HD 2200 with SG 213G horn and TT 19 probe tip;
- microwave reactor with temperature controlled regime (Whirlpool MWD 321 WH –700 W);
- ELISA spectrophotometer- microplate reader, Thermo Scientific™;
- Thermo Scientific Microcentrifuge with refrigerated unit;
- Hoefer Scientific SE-600 Series Vertical Slab Gel Electrophoresis Unit SE600;
- QB-24 PCR cycler, LKB;
- Incubator Shaker KS 4000;
- Ultraspec 3300 pro UV/Visible spectrophotometar
- Preparative Chromatography Systems consisting of 2 x C-601 Pump Modules 10 bar, C-615 Pump Manager, injection valve (simple), UV Vis Dectetor Model SPD 20 AV, Fraction Collector C-660, SepacoreControl Software, cartridge holder and set of Sepacore silica flash cartridges.

4.2 Methods

4.2.1 Screening of enzymes' producers by a selective plates method

A screening approach on selective mediums for proteolytic, amylolytic, cellulolytic, pectinase and mannanase activity was used to select efficient producing microorganisms with extracellular enzyme activity. Several microorganisms from the Collection of cultures of the Faculty of Technology and Metallurgy, University of Belgrade were screened for enzymatic activities on selective agar plates. These included a large number of microorganisms, mostly bacteria, collected from various food, soil and waste samples taken from distinct sources, and also some commercially available microorganisms. The microorganisms were cultivated on agar slants containing: peptone 5 g/L, yeast extract 5 g/L, peptone I 10 g/L and bacteriological agar 15 g/L. The potential of microorganisms to produce particular enzyme was evaluated using following selective mediums: for proteolytic activity-milk casein agar (per liter: semi skimmed milk (50%, v/v), casein 5 g, yeast extract 3 g, dextrose 1 g and agar 15 g); for amylolytic activity-starch based agar (per liter: meat extract 3 g, soluble starch 10 g and agar 15 g); for cellulolytic activity-CMC agar (per liter: CMC 1 g, yeast extract 3 g, K_2HPO_4 3 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g and agar 15 g); for pectinase activity pectin agar (per liter: meat extract 3 g, apple pectin 10 g and agar 15 g); and for mannanase activity - MS agar (per liter: galactomannan 4 g, K_2HPO_4 7.54 g, KH_2PO_4 2.32 g, $MgSO_4$ 0.2 g, agar 20 g). After selective agar mediums were spread on Petri dishes, a loopful of each different microorganism from nutrient agar slants was transferred and placed in the center of the agar plates. Petri plates with screened microorganisms were incubated for 72 h at 30°C. After 3 day cultivation, milk-casein agar plates were soaked by 10% of trichloroacetic acid, while starch agar, CMC agar and pectin agar plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water). A clear zone of hydrolysis gave an indication of protease/amylase/cellulase/pectinase/mannanase-producing strains (Casana *et al.*, 2008). The largest halo zone and the largest halo zone to colony ratio detected microorganisam with high extracellular enzyme activity and they were selected as potential producing microorganisms.

4.2.2 The identification of selected microorganism

Several tested strains, particularly a strain named TMF-1 presented promising activities on all tested selective mediums. The isolate was identified as *Bacillus* sp. according to the methods described in Bergey's Manual of determinative Bacteriology (Gibson and Gordon, 1974). Further identification on the basis of the 16S rDNA sequence analysis was conducted by amplification of DNA fragment by using the upstream primer UNI16SF 50-GAG AGT TTG ATC CTG GC-30 and the downstream primer UNI16SR 50-AGG AGG TGA TCC AGCCG-30. The PCR amplification was conducted under the following conditions: first denaturation at 95 °C for 5 min, then 30 cycles of 96 °C for 30 s, 55 °C for 60 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. DNA sequencing was conducted by Macrogen (Amsterdam, Netherlands). The resulting DNA sequence of 1484 bp obtained by 16S rDNA sequencing was analyzed with Blast through the NCBI server.

4.2.3 Solid state fermentation

Several substrates including soybean meal, sunflower meal, wheat bran, olive oil cake, maize bran and corn pericarp were used in solid state fermentation. Untreated substrates were dried overnight in the vacuum oven (60 °C), grinded and sieved to obtain substrates with particle sizes between 200 and 800 µm. Solid state fermentation of all samples was performed in 150 ml Erlenmeyer flasks to investigate the effects of several physio-chemical parameters on the produced enzyme activity by various microorganisms. The media in flask (5g of dry substrate) were autoclaved at 121 °C for 20 min (0.12 MPa autoclave pressure). Unless otherwise mentioned, SSF was carried by inoculating the medium (initial moisture content adjusted to 50%) with 500 µl of inoculums followed by incubation. The fermentation was carried out at 30 °C for 72 h since preliminary study showed that the optimal activities of desired enzymes on the majority of the studied substrates occured under these conditions. The water added with the inoculum was also considered in moisture content. The samples are aseptically withdrawn at various time intervals (1, 2, 3, 4 and 5 days) for the determination of enzyme activity and biomass yield. Extraction of the enzymes was carried out by

the addition of distilled water to know quantity of the fermented media (5:1 w/w) with mixing on a rotary shaker (180 rpm, 30 min, 30 °C). After that, the extract was centrifugated at 8000 rpm for 10 minutes. The resulting supernatants were considered as a crude enzyme extract and evaluated for secreted protease, cellulase, α-amylase, mannanase and pectinase activities.

4.2.4 The effect of moisture content of untreated agro-industrial waste materials on solid-state fermentation

The influence of moisture content on solid state fermentation was examined as follows. Erlenmeyer flasks containing 5 g of dried and sterilized substrates were moistened with different amounts of sterilized water prior inoculation to achieve a ratio of 1:0.5; 1:1; and 1:1.5 g of dry substrate: g of water. An aliquot of liquid (0.5 mL) introduced through inoculation was also considered in moisture content. In addition, different moisture content was also calculated in enzymes' extraction from fermented substrate part. Prior to the assessment of different moisture content range, water holding capacity (WHC) of each substrate was determined in order to establish the maximum amount of water which can be added to the substrate without water in the free form. This maximum capacity of water absorption in the substrate which defines the solid-state fermentation was evaluated by a method described previously (Moftah *et al.*, 2012).

4.2.5 The effects of pretreatments

The chemical pretreatment of the substrates was performed by the addition of 50 mL of 3% sulphuric acid or 50 mL of 3% NaOH to 10 g of substrate. After 2 h incubation at room temperature, substrates were thoroughly washed with distilled water to remove traces of acid or base, dried and further processed. Ultrasound treatment was performed using BANDELIN SONOPLUS Ultrasonic Homogenizer HD 2200 with SG 213G horn and TT 19 probe tip. Substrates (10 g) were soaked in 50 mL of distilled water and treated with ultrasound. The ultrasound treatment was performed at different amplitudes setting to 25, 35 or 50% for 5 min ensuring constant cooling of the system. After treatment, substrates were dried, measured, sterilized, moistened and inoculated as formerly presented.

The microwave pretreatment was performed in similar manner as follows. The substrates (10 g) were soaked in distilled water and/or 85% glycerol (50 mL) overnight which and subjected to irradiation in a microwave (Whirlpool MWD 321 WH –700 W) for 2 min. Glycerol was used as studies showed that solvents with higher boiling points improve delignification (Diaz *et al.*., 2015). Samples soaked in glycerol were thoroughly washed with distilled water.

4.2.6 Enzymes' extraction

Two different approaches in enzyme extraction from fermented substrate were attempted in order to maximize the recovery of the produced enzymes and ease further purification steps, namely: 1) the conventional procedure for obtaining crude extracts that it is, an treatment of the fermented solid phase solubilized in water at the time through orbital shaking; and 2) the novel method applied in this work, that it is the ultrasound extraction (UAE) consisted in an treatment of the fermented solid phase through high intensity ultrasonication.

4.2.6.1 The conventional method for enzyme extraction

The convencional procedure for producing crude enzyme extracts was conducted in Erlenmeyer flasks (150 mL) by addition of the sodium phosphate buffer (50 mM) according to the experimental design given in Table 4.1. The flasks were termostaed at 30 ^{o}C in the orbital shaker (IKA® KS 4000 i control). Subsequently, the Erlenmeyer contents were centrifuged at 8,000 rpm for 10 min (4 °C). The obtained supernatants were considered as a crude enzyme extract and evaluated for secreted protease, cellulase and α-amylase activities.

Modifications of the conditions of the standard procedure were performed by response surface analysis. All experiments were done in duplicate or triplicate to ensure reproducibility. The reproducibility was estimated as satisfactory since standard deviation in all cases was less than 5 % of the mean. The statistical analysis was done with the mean vauels of the results obtained using Design Expert® (Stat-Ease, Inc.; Minneapolis, USA; version 7.0.0). Box-Behnken experimental design was selected to study the enzyme extraction from the fermented solid by response surface analysis. Investigated factors and their range

were solvent pH (7–9), stirring rate (50–550 rpm), time (15 to 45 min) and solid/liquid ratio (1:20–5:20). The response variables were the protease/cellulase/α-amylase hydrolytic activity in the crude extract expressed as a totally obtained yield from 5 g of fermented substrate.

4.2.6.2 The ultrasound assisted extraction of the enzymes from the SSF substrate

The ultrasound assisted extraction was performed using BANDELIN SONOPLUS Ultrasonic Homogenizer HD 2200 with SG 213G horn and TT 19 probe tip. Fermented substrates (5 g in 150 mL Erlenmeyer flasks) were soaked in 50 mL of sodium phosphate buffer (pH 7-9, 50 mM) and subjected to ultrasonication at frequency of 20 kHz setting the admissible amplitude to 10 % up to 15 minutes while ensuring constant cooling of the system. Every 2.5 minutes 5 mL aliquots were taken, centrifuged and studied for the enzymes' activities.

4.2.7 Enzymatic assays

4.2.7.1 Protease activity assay

The proteolytic activity was determined using azocasein as a substrate according to the method described by Sarath *et al*. The method is based on the reaction of the enzyme with azocasein. Briefly, enzyme sample of 75 µL was added in 125 µL 2% azocasein solution in Tris-HCl buffer (50 mM, pH 9.0) and the reaction mixture was incubated at 37 ^{o}C for 30 min. Then, 600 µL of 10% TCA solution was added to the reaction mixture. After cooling in an ice bath, samples were centrifuged at 8000 rpm for 10 min, and 600 µL of the resulting supernatants was mixed with 700 µL 1 M NaOH. A blank reaction (the value for the mixture at zero time) was prepared by mixing samples with azocasein substrate and the reaction was immediately stopped with TCA solution, cooled and further treated same as the sample, just before measuring the absorbance differences. One unit of proteolytic activity was defined as the quantity of enzyme that produced a unitary difference in absorbance at 440 nm between the reaction blank and the sample under the assay conditions.

4.2.7.2 Cellulase activity assay

The cellulase activity was measured according to the method that is based on estimating the amount of reducing sugars liberated from carboxymethyl cellulose (CMC) by the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959). Namely, 0.5 mL of crude enzyme extract was incubated with 0.5 mL of 1% CMC prepared in 0.05 M sodium acetate buffer, pH 4.8 at 50 °C for 30 min. The enzyme reaction was stopped by the addition of 1 mL DNS reagent (dinitrosalicylic acid 1 g, NaOH, 16 g, potassium sodium tartrate 300 g, and distilled water up to 1 L) to the above 1 mL reaction mixture, boiled in capped glass tubes for 5 min, cooled and then absorbance was measured at 540 nm (Ultrospec 3300 pro Amersham Bioscience). The calibration curve for determination of the cellulase activity was obtained with glucose. One unit of cellulase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose equivalents per min (Sadhu *et al.*., 2014).

4.2.7.3 α-Amylase activity assay

The activity of α-amylases were determined as follows. A sample of 250 μL of crude enzyme extract was added in 250 μL of substrate (1% soluble starch in 20 mM phosphate buffer with 6.7 mM NaCl, pH 6.9) and incubated 30 min at 37 °C. One unit of enzyme activity was defined as the amount of produced reducing sugars equivalent to 1 μmol of D-glucose/min under the assay condition estimated according to the previously described DNS method.

4.2.7.4 Pectinase activity assay

Pectinase activity assays were determined by mixing 250 μL of crude enzyme extract with 250 μL of substrate and incubated 30 min at 37 °C. Pectin substrate for this assay was prepared by diluting pectin from apple (Sigma-Aldrich) in 2 mL ethanol using the magnetic stirrer, followed by further dilution to 100 mL with 0.1 M citrate buffer (pH 5.8). The release of reducing sugars was determined by the DNS method using a standard curve obtained with D-galacturonic acid (Biz *et al.*., 2016). One unit (IU) of pectinase activity corresponds to the release of 1 μmol of D-

galacturonic acid equivalents per minute (Biz *et al.*, 2016). All enzyme activities were expressed on the basis of the mass of dry substrate (IU g^{-1}).

4.2.8 Total phenol content determination

The method for determining the content of total phenols was developed at the Department of Biochemical Engineering and Biotechnology at the Faculty of Technology Metallurgy, starting from the available literature data (Wardhani *et al.*, 2010; Pavlović *et al.*, 2013). Prior to extraction of total phenols from unfermented and fermented soybean meal, the samples were ground using a ball mill. Afterwards, the total phenols were extracted as follows: 1g of dry sample was resuspended in 9 g of ethanol (20%) and subjected to microwave treatment (household microwave oven, LG MC7849HS) during 35 s and 160 W. Afterwards, the samples were centrifuged, 3 min, 5000 rpm and the obtained supernatant was discarded and submitted to analytical assay.

50µl of the supernatant was mixed with 3.950 ml of distilled water and 250 µl of FCR (Folin&Ciocalteu) reagent. The solution was thoroughly mixed with 750 µl of saturated Na_2CO_3. Afterwards, the samples were incubated for 2h in dark at room temperature. After the incubation the change of absorbance was measured against the reagent blank at 765 nm.

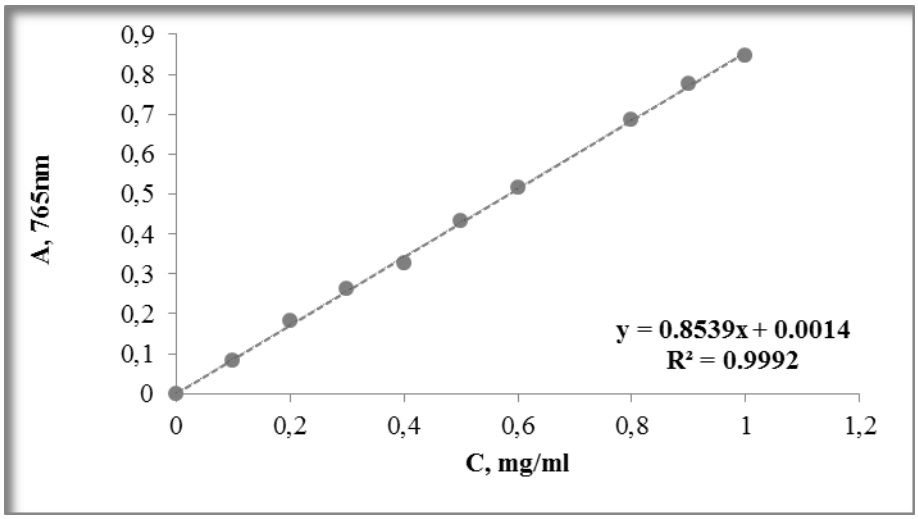

Figure 4.1. Standard curve for the total phenol content determination (galic acid as a standard).

The total phenolic content was calculated using the standard curve constructed with the gallic acid standard (Figure 4.1).

4.2.9 Phytic acid content determination

Phytic acid content was determined using method of Dragičević et.al with some modifications. The extraction of phytic acid from fermented and unfermented samples was performed as follows: 40 mL of 0.5 MHCl was added to 0.1 g of dry sample and incubated at room temperature 3 h under constant shaking, 200 rpm. Supernatant was recovered by centrifugation (3 min, 5000 rpm) and used for phytic acid quantification. 0.5 mL of sample (diluted if necessary (supernatant after the extraction process) was added to 0.3 mL of Wade reagent solution (0.03% $FeCl_3$ and 0.3% sulfosalycilic acid prepared in distilled water). Absorbance of the mixture was read at 500 nm. The same procedure was used for the construction of standard curve using phytic acid as a standard.

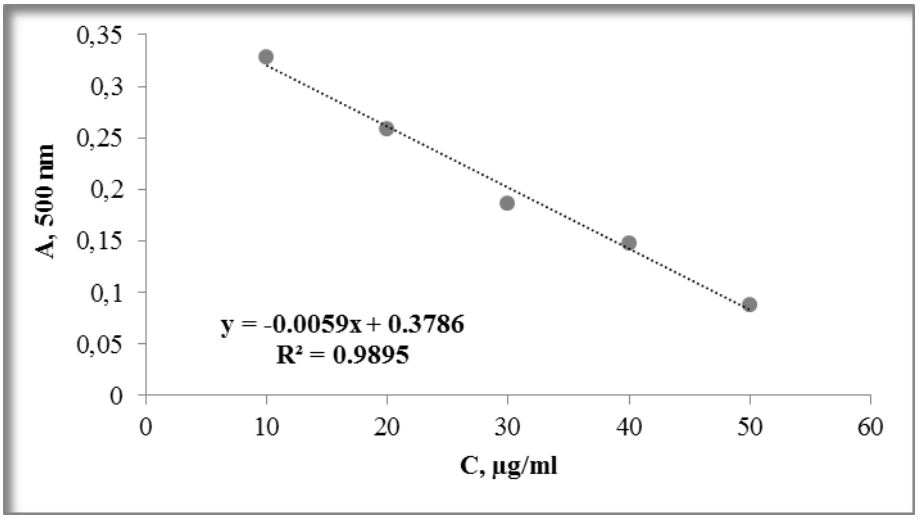

Figure 4.2. Standard curve for the phytic acid content determination.

4.2.10 Trypsin inhibitor content determination

The content of trypsin inhibitor in soybean meal was determined prior and after the fermentation. The content of trypsin inhibitor was determined using the standard Sigma procedure. For the extraction step the procedure was developed at the department of Biochemical Engineering and Biotechnology. Namely, 1g of the sample was resuspended in 50 mL of 0.01N NaOH. Afterwards, the pH in the

suspension was set at 9.4-9.6 using the 1N HCl and the samples were incubated at room temperature for 3 h with constant mixing. Further, the sample was transferred into the volumetric flask and made up to 100 mL with distilled water. After 10 min of incubation at room temperature the sample was used for the determination of trypsin inhibitor as follows. An aliquot of 0.5 mL of extract was mixed with cold 0.001N HCl and 0.250 mL of trypsin (1mg/mL), filed with 0.01N HCl to get the final volume of 5mL in test tubes and thoroughly mixed. After the incubation 5<t<6 min, 0.1 mL of the sample was mixed directly in the cuvettes with 3 mL of substrate (0.25 mM BAEE, freshly prepared in cold 67 mM sodium-phosphate buffer, pH 7.6) and 0.1 mL of 0.01N HCl. The change in absorbance was monitored at 253 nm, 5 minutes each 1 minute against buffer. The standard curve for the content of trypsin inhibitor was calculated using the trypsin inhibitor from egg white (Figure 4.3).

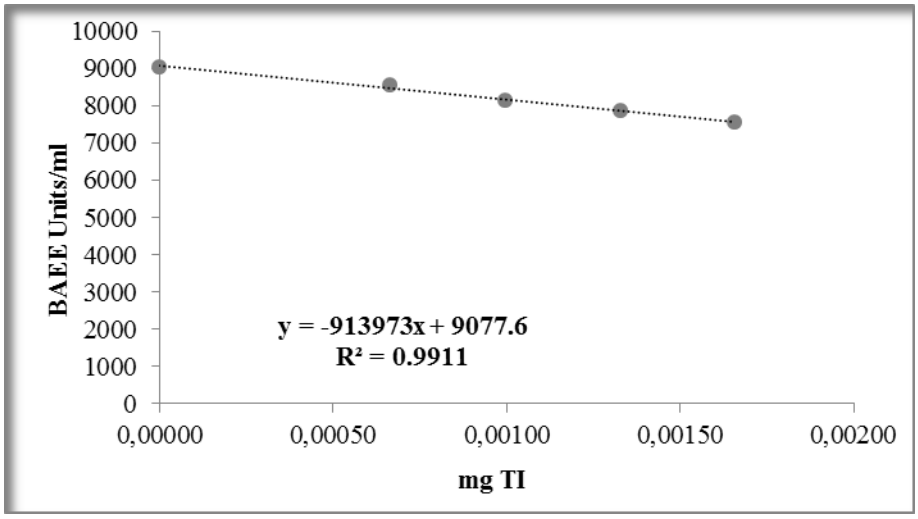

Figure 4.3. Standard curve for the trypsin inhibitor content determination using trypsin inhibitor from egg white as a standard.

The amount of trypsin inhibitor was calculated using the following equations:

$$Trypsin\ activity\ in\ BAEE \frac{Units}{ml} enzyme = \frac{(\Delta A_{253nm}/\min Test - \Delta A_{253nm}/\min Blank)(df)(10.0)}{(0.001)(0.10)(0.5)} \quad (4.2.1)$$

$$mg\ Trypsin\ inhibited\ by\ 1\ mg\ of\ trypsin\ inhibitor = \frac{mg\ \frac{Trypsin}{RM}\ (normalizing\ factor)}{(mg\ Trypsin\ inhibitor)} \quad (4.2.2)$$

$$Normalizing\ factor = \frac{BAEE\ Uinits\ of\ Uniinhibited\ Trypsin\ per\ mg\ solid}{10000\ BAEE\ Units\ of\ Trypsin\ per\ specification} \quad (4.2.3)$$

where:

df = dilution factor,

0.001 = The change in A_{253nm}/minute per unit of Trypsin at pH 7.6 at 25°C in a 3.2 mL reaction mixture,

0.10 = Volume (in milliliters) enzyme used,

10.0 = Total volume in milliliters of assay,

0.5 = Volume (in milliliters) of enzyme used.

4.2.11 Antioxidant activity determination

The antioxidant activity before and after the fermentation was determined using two different methods, DPPH and ABTS. The alcoholic extracts of soybean meal (SBM) (unfermented and fermented) were obtained after the extraction of 1g of dry sample with 70% ethanol (1:10, SBM:ethanol) during 6h at room temperature under constant mixing, 500rpm. After the extraction, the samples were centrifuged (3 min, 8000 rpm) and an antioxidant capacity was determined.

4.2.12 Antioxidant activity determination using the DPPH assay

For the antioxidant activity determination using DPPH assay, DPPH solution 0.15 mM was prepared in methanol. Afterwards, the sample 0.1 mL was mixed with 0.9 mL of DPPH solution and incubated in the dark at room temperature for 30 min. The control was prepared by mixing 0.1 mL of methanol and 0.9 mL of DPPH solution, meanwhile the blank sample was prepared by mixing 0.1 mL of sample and 0.9 mL of methanol. Apsorbance changes in regard to methanol were read

after incubation at 517 nm using a double beam UV/VIS spectrophotometer (Ultrospec 3300pro, Amersham, Biosciences, Sweden). The percentage of inhibition of DPPH radicals is calculated according to the equation:

$$RSA(\%) = \left[1 - \left(\frac{A_s - A_b}{A_c}\right)\right] \times 100\% \quad (4.2.4)$$

where A_s is the absorbance of the sample, A_b absorbance of the blank and A_c absorbance of the control.

4.2.13 Antioxidant activity determination using the ABTS assay

ABTS assay is based on the reaction between the sample and radical cation ($ABTS^{\bullet+}$). Radical cation was prepared by mixing the ABTS solution (7mM) and $K_2S_2O_8$ solution (140mM) until the final concentration of ABTS 2.5 mM, is reached. The prepared solution was incubated in the dark during 16 h to give the ABTS radical cation. Thereafter, the solution was diluted with PBS buffer (5 mM, pH 7.4) until the absorbance of 0.7±0.02 was measured at 734 nm against the same buffer. 1mL of diluted solution was mixed with 10 µl of the sample, incubated at room temperature for 5 min and the change in absorbance was monitored (A_s). Control was prepared in the same way only using buffer instead of the sample (Ac). Radical scavenging activity was calculated using the equation:

$$RSA(\%) = \left(\frac{A_c - A_s}{A_c}\right) \times 100\% \quad (4.2.5)$$

where A_s is the absorbance of the sample and A_c absorbance of the control.

5 RESULTS AND DISCUSSION

5.1 Production of enzymes by solid state fermentation on agricultural by products – the evaluation of substrate pretreatment methods

The first step in analyzing the possibility of a microorganism to produce desired enzymes is the initial screening of a large number of different commercial and natural strains. Different strains, commercial and natural isolates from different habitats, have been tested for the ability to produce extracellular proteases, cellulases, amylases and pectinases. For this purpose the selective agar medium plates have been used. These selective agar medium plates are plates that contain casein, CM-cellulose, starch or pectin as one of the exclusive nutrients. Such mediums require extracellular enzyme activity to enable successful hydrolyzation and utilization of the nutrients from the media and allow microbial growth. Myriad of different strains have been cultivated on these plates and the appearance of the clear zones surrounding grown microbial colonies indicates that the desired enzymes have been produced. Extracellular enzymes enable microbial growth by hydrolyzation and utilization of the nutrients from the selective medium.

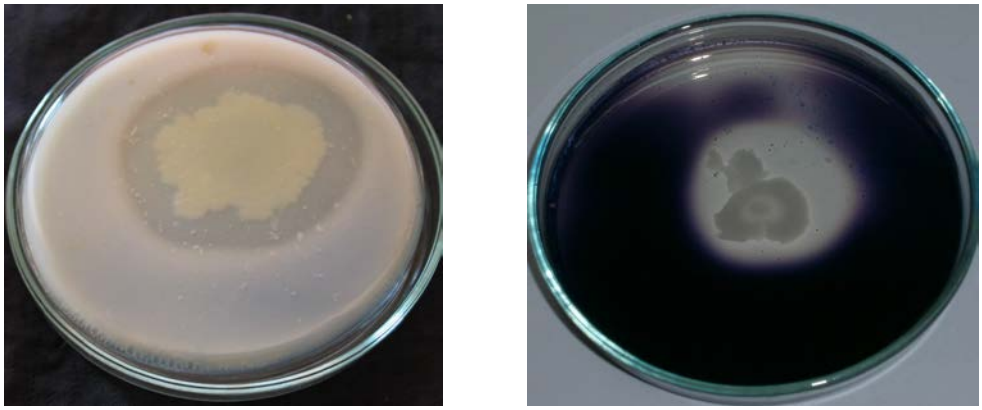

Figure 5.1. Exibited activities on selective mediums: a.) Milk-casein agar b.) Starch agar overlaid with KI solution (cultivated 72 h, on 37 ˚C).

Several *Bacillus* spp. strains have shown to be a good source of desired enzymes, especially one of the strains which was isolated from putrid proteinaceous food samples. This strain showed to be a prospective microbial source of several desired enzymes since it showed significant halo zones on previously tested agar plates (Figure 5.1). After the selection, the strain had to be identified. The strain was not commercially available, therefore the identification must be performed. The first step is the preliminary microscopic identification. It was observed that it was a spore forming microorganism and the colonies on the solid medium were irregularly shaped, large spreading, white and flat. This appearance suggested that the most likely the microorganism was a member of *Bacillus* spp. However, further identification has to be carried. It was conducted by sequencing 16SrRNA encoding gene and subsequent analysis of the obtained sequence using the BLAST (Basic Logical Alignment Search Tool). It enabled the comparative analysis of genomic DNA sequence of 16S rRNA compared to the NCBI public database. A 16s rRNA-based phylogenetic tree showing the position of the selected strains using ARB database and software (www.arb-silva.de) is given as Figure 5.2.

The obtained and isolated strain was used in the solid-state fermentation process by using different types of agro-industrial waste materials such as wheat bran, soybean meal, olive oil cake, sunflower meal. The objective was to examine the possibility of the selected microorganism to produce desired enzymes by growth on different type of agro-industrial waste. This is a rather new method that has several benefits namely the substrates being cheap materials in most cases considered as wastes. Basically these materials have already passed some type of processing methods in which the nutrients are already exhausted, but still have an high organic load.

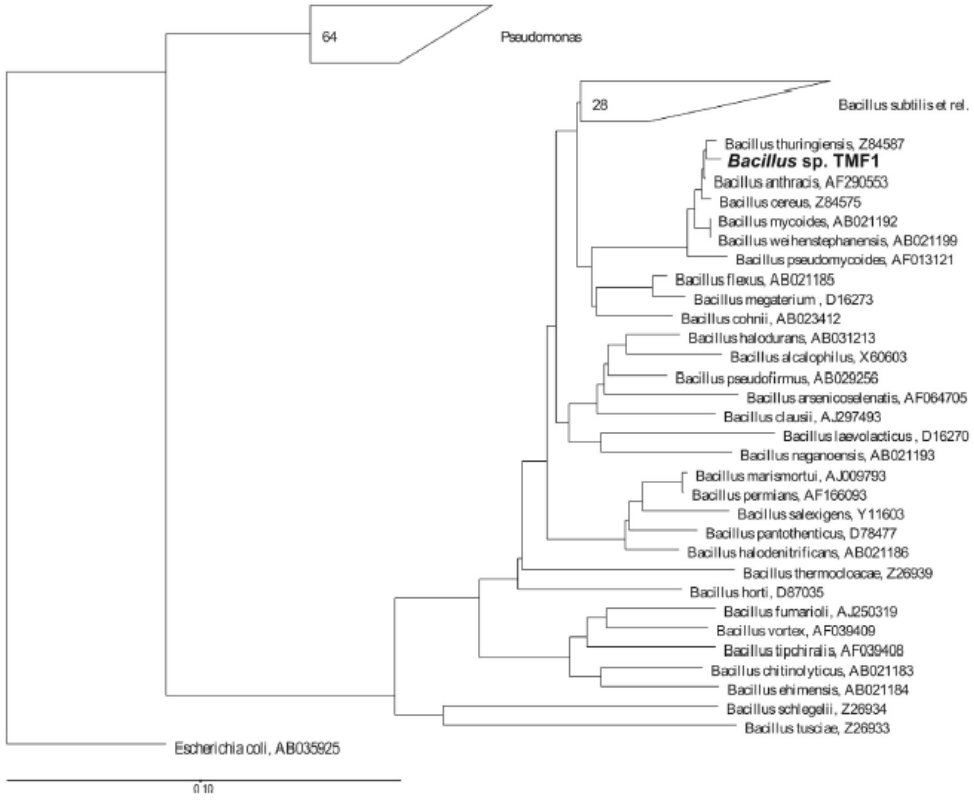

Figure 5.2. The position of the selected producer in the phylogenetic tree determined on the basis of homology 16S geonmic sequences

Different types of waste differ in composition but usually contain large amount of protein, sugar and mineral as well as bioactive compounds such as polyphenols, carotenoids and dietary fibers. Beside the advantage of using cheap waste material as substrates, other benefits are better productivity, less capital and lower energy requirements. The first parameter that had to be evaluated is the water content. This is very important parameter since water greatly influences the enzyme production by the SSF. If the water content is excessive, there can be changes in particle structure and therefore it can decrease nutrients diffusion and oxygen transfer, and it can lead to stickiness which causes reduction in substrate porosity. On the other hand, insufficient water content leads to reduced solubility of nutrients, and may cause accumulation of inhibitors which can affect microbial growth (Prakasham *et al.*, 2006; Uyar and Baysal, 2006; Veerabhadrappa *et al.*, 2014).

Tables 5.1 and 5.2 show the effect of different initial moisture content on enzyme production on several different substrates: soybean meal, sunflower meal, olive oil cake, wheat bran, maize bran and corn pericarp. The preliminary results show that almost all substrates have the ability to be used as substrates for enzyme production, namely: proteases, cellulases, amylases and pectinases. It can be concluded that the highest enzyme activities were achieved by fermentation on soybean and sunflower meal for pectinase and cellulase, while in addition to these substrates, oil cake and corn pericarp have been shown to be suitable media for the production of α-amylase and proteases. What these results also show is that the careful analysis of the process parameters for each enzyme and substrate needs to be established, in order to obtain the desired enzymatic activity. For example, as it can be seen in Table 5.1 and 5.2, for all of the enzymes (amylase, protease, cellulase and pectinase) soybean meal seems to be the best choice to be used as the substrate. High water content was beneficial for amylase and cellulase secretion while modest water content favored protease production. Similar, high water content promote the production of amylolytic enzymes when *Bacillus* sp. TMF-1 was cultivated on sunflower meal, olive oil cake and wheat bran, but low moisture content stimulated the cellulases' secretion on olive oil cake. But also amount of cellulolytic acitivity was high when cultivation was performed on sunflower and soybean meal with relatively high moisture content.

Table 5.1. Amylase, cellulase and pectinase activity (IUg^{-1} of substrate) achieved by SSF fermentation on different solid substrates (after 72 h at 30 °C, 0.5 mL inoculums) at different initial moisture content of the substrate

	Amylase			Cellulase			Pectinase		
	Substrate:water ratio								
Substrate (WHC, g_{H2O}/g_{subst})	1:0,5	1:1	1:1,5	1:0,5	1:1	1:1,5	1:0,5	1:1	1:1,5
Soybean meal (4.20)	4.48	12.31	0.81	0.16	0.95	0.38	0.00	64.27	29.7
Sunflower meal (4.26)	2.94	0.09	20.48	0.00	0.12	1.98	14.67	22.55	126.30
Olive oil cake (1.06)	4.41	9.95	15.76	0.56	0.45	0.01	7.92	0.13	0.00

Wheat bran (4.97)	2.94	0.18	2.90	0.02	0.02	0.02	13.75	21.26	13.38
Maize bran (5.46)	0.66	4.36	1.25	0.00	0.01	0.01	7.23	14.11	0.32
Corn pericarp (4.00)	1.12	1.48	0.48	0.04	0.01	0.01	6.67	14.31	6.34

Table 5.2. Proteolytic activity (IUg^{-1} of substrate) achieved by SSF fermentation on different solid substrates (after 72 h at 30 °C, 0.5 mL inoculums) at different initial moisture content of the substrate

	Protease		
Substrate (WHC (g_{H2O}/g_{supst})	Substrate:water ratio		
	1:0.5	1:1	1:1.5
Soybean meal (4.20)	14.63	2.99	7.82
Sunflower meal (4.26)	0.00	3.22	0.24
Olive oil cake (1.06)	0.72	0.96	0.18
Wheat bran (4.97)	3.79	1.46	4.31
Maize bran (5.46)	1.44	2.34	3.59
Corn pericarp (4.00)	17.17	30.36	24.58

There are several explanations for such discrepancies, one of the most logical one is the differences in chemical composition of carbohydrate fraction as well as the significant changes in water holding capacity values. Other researches also concluded that substrates with high concentrations of cellulose and hemicellulose such as wheat bran (~20% cellulose and 50% hemicellulose) have an inhibitory effect on production and secretion of enzymes (Merali *et al.*, 2015). Such results lead to conclusion that solid state fermentation on waste material as substrates would benefit from pretreatment of the substrates with the aim that selected pretreatments would disrupt carbohydrate structures and therefore increase the accessibility of the nutrients to microbial degradation. Pretreatments are usually conducted towards enhancing the biodigestibility of the substrates by degradation of impenetrable complexes along with changing the physical characteristics of the substrates, namely porosity of the residues, thus enhancing the accessibility of microorganisms to the media.

There are several methods that can be employed, and the choice of the pretreatment method depends on several factors such as type and composition of biomass, the intended application and process economics (Coffman *et al.*, 2014). Suitable pretreatment should increase specific surface area under energy efficient and operation-easy regime concurrently causing improved accessibility of the nutrients in a manner that prevents excessive leaching of necessary sources of nourishment (Salim *et al.*, 2017). Also, pretreatments should not employ toxic chemical or produce one during the pretreatment.

Several different approaches have been applied: alkaline pretreatment, acidic pretreatment, ultrasound (different amplitudes) and microwave (in water and in glycerol). All the results for soybean meal, sunflower meal and olive oil cake presenting the influence of different pretreatments on levels of produced enzymes by *Bacillus* sp. TMF-1 are presented in Tables 5.3-5.5.

Table 5.3. Effect of various chemical and physical pretreatments on the production of hydrolytic enzymes by fermentation with *Bacillus* sp. TMF-1 on soybean meal at 30 ^{o}C for 72 h and at substrate: water ratio 1: 1.

Enzyme	Control	Acid	Alkaline	Ultrasound, amplitude %			Microwave	
				25%	35%	50%	Water	Glycerol
α-Amylase	12.31	18.90	18.38	2.32	1.84	1.96	0.99	0.00
Pectinase	64.90	13.57	4.58	12.50	4.58	3.00	11.36	1.65
Cellulase	0.95	0.21	0.32	0.43	0.21	1.20	0.23	0.12
Protease	3.00	6.44	15.07	10.24	13.31	6.29	5.78	1.48

Table 5.4. Effect of various chemical and physical pretreatments on the production of hydrolytic enzymes by fermentation with *Bacillus* sp. TMF-1 on a sunflower meal at 30 ^{o}C for 72 h at a substrate: water ratio of 1: 1.

Enzyme	Control	Acid	Alkaline	Ultrasound, amplitude %			Microwave	
				25%	35%	50%	Water	Glycerol
α-Amylase	0.09	24.40	18.02	0.61	0.13	0.00	0.00	0.18

Pectinase	22.58	31.53	9.58	6.02	5.56	6.12	17.98	8.62
Cellulase	0.12	0.33	0.04	0.06	0.10	0.13	0.06	0.04
Protease	3.22	1.54	18.10	10.61	11.54	13.24	2.93	1.24

Table 5.5. Effect of various chemical and physical pretreatments on the production of hydrolytic enzymes by fermentation with *Bacillus* sp. TMF-1 on olive oil cake at 30 ^{o}C for 72 h at substrate: water ratio 1: 1.

Enzyme	Control	Acid	Alkali	Ultrasound, amplitude %			Microwave	
				25%	35%	50%	Water	Glycerol
α-Amylase	9.95	39.97	30.96	6.82	6.73	3.68	0.00	0.00
Pectinase	0.13	15.44	10.13	5.78	15.92	18.57	2.75	8.07
Cellulase	0.45	0.27	0.09	0.43	0.40	0.30	0.38	0.11
Protease	0.96	1.88	28.20	2.39	4.15	1.90	2,93	1.24

As it can be assumed, pretreatment differently affected substrates. This is due to the differences in chemical composition as well as physical appearances of the agricultural wastes used as substrates. For example, soybean meal is rich in protein (app. 45%) and carbohydrate (app. 35-40%) with small amount of oil (1%), whereas almost 15% of the carbohydrates are pectins, 10% are free sugars mainly sucrose, raffinose and stachylose), app. 8% cellulose and 1% starch (Choct *et al.*, 2010). This composition of the soybean meal could indicate that soybean meal would be a good choice of substrate for pectinase production and that was confirmed in our experiment (Table 5.3). The highest yield of pectinase in the whole study was achieved when *Bacillus* sp. TMF-1 was cultivated on this medium (Table 5.6). However, for this substrate and enzyme, none of the pretreatments had the beneficial effect on pectinase production. The explanation for such results possibly lies in the fact that some pretreatment methods such as microwave and chemical pretreatments may cause leaching of the pectin as well as decomposition of the pectin molecule (Yeoh *et al.*, 2008). When there is a degradation of cell structures and increase of capillary-porous structures as a consequence of certain

pretreatments, a better penetration of the solvent into the tissue is enabled. When this happens there is a significant loss of pectin through leaching. The regulatory mechanisms of enzyme synthesis are such that there is a need for pectin for the induction of enzyme production, so when there is a lack of pectin as a consequence the pectinase production is decreased (Teixeira *et al.*, 2000). As noted before, there is a need for careful selection of pretreatment for every specific substrate as well as for desired enzyme. In another words, the pretreatment that is beneficial for one substrate and enzyme will not be desired for another substrate. On the same substrate, the soybean meal, the alkaline/acid pretreatment significantly improved protease and amylase production, and had a negative effect on cellulase production. The levels of produced cellulase were highest for the untreated meal. The ultrasound pretreatment only improved the protease production.

Table 5.6. Pectinase production by SFF on untreated and treated substrates (*B. thurgensis,* 1:1 moisture, 72 h, 30°C).

	Chemical pretreatment			Ultrasound amplitude, %			Microwave	
	Pectinase, IU g^{-1}							
Substrate	Control	Acid	Alkaline	25%	35%	50%	Water	Glycerol
Soybean meal	64.90	13.57	4.58	12.50	4.58	3.00	11.36	1.65
Sunflower meal	22.58	31.53	9.58	6.02	5.56	6.12	17.98	8.62
Wheat bran	21.26	20.90	7.37	0.37	1.47	1.96	59.40	4.95
Maize bran	12.62	0.96	19.91	8.25	13.20	2.90	2.75	3.85
Corn pericarp	14.11	39.96	18.25	0.21	0.36	0.98	14.30	4.00
Olive oil cake	0.13	15.44	10.13	5.78	15.92	18.57	2.75	8.07

The treatment, i.e. pretreatment of ultrasound on substrates is based on the principle of cavitation. The ultrasound in liquid media results in the spontaneous formation, growth and following the collapse of microsize bubbles (cavities). When these bubbles, cavities, explode, the specific changes in physical and chemical composition in materials occur. The efficiency of cavitation depends on the frequency and intensity of the ultrasound waves, as well as on the physical properties of the treated medium. In attended use of ultrasound in food industry, it

is important to know the energy of the (Stefanović, 2017) ultrasound which is determined by the power of ultrasound (W), intensity (W/cm^2) and density (Ws/m^3) (Stefanović, 2017; Leite *et al.*, 2016). Literary data shows that the ultrasound is beneficial for the pretreatment of lignocellulosic material since it influences cristallinity of cellulose and degradation of lignin (He *et al.*, 2017; Nakashima *et al.*, 2016). By the usage of ultrasound hemicellulose is degraded and dissolved in such a way that inhibitors of fermentative microorganisms are not formed. The ultrasound also had beneficial effect on protease production by solid state fermentation on sunflower meal (Table 5.7). There was a four fold increase in protease levels: 3.22 IUg^{-1} for control and 13.24 for ultrasound pretreatment with 50% ultrasound amplitude. Sunflower meal is rich in protein, the dehulled sunflower meal used in our study contains app. 33% protein, 21% cellulose and 8% lignin (Sredanovic *et al.*, 2006). It is possbile that the majority of the nitrogen is linked to lignocellulose so these types of substrates have poor solubility. The ultrasound has probably affected the protein structures in the substrates since several studies have confirmed that this kind of treatment of substrates rich in protein can lead to exposure of hydrophobic groups, leading to redistribution of secondary structure and changes in microstructure. But this type of pretreatmant wasn't beneficial for other substrates. The ultrasound pretreatment was evaluated as unsatisfactory for processing the majority of the studied substrates. Similar study was conducted by Yang *et al.*. where the ultrasound was used for treatment of rice hulls for the production of cellulases and xylanase by *Aspergillus japonicus.* Ultrasound waves caused significant structural changes namely, loss of hemicellulose fraction, resulting in slight improvement of enzymatic production. Another group of authors studied the influence of ultrasound on the productivity of *A. niger* on olive pomace. They have analyzed two differently treated substrates, crude olive olive pomace and exhausted olive pomace, for the xylanase and cellulase production. Results have indicated that there has to be a careful selection of process parameters in order to direct production towards the one or the other enzyme.

Table 5.7. Protease production by SFF on untreated and treated substrates using *Bacillus* sp. TMF-1 (*B. thuringiensis*) at 30 °C for 72 h at substrate: water ratio 1: 1.

	Chemical pretreatment			Ultrasound amplitude, %			Microwave	
	Protease, IU g^{-1}							
Substrate	Control	Acid	Alkaline	25%	35%	50%	Water	Glycerol
Soybean meal	3.00	6.44	15.07	10.24	13.31	6.29	5.78	1.48
Sunflower meal	3.22	1.54	18.10	10.61	11.54	13.24	6.51	3.03
Wheat bran	1.46	0.59	0.50	6.88	15.14	13.31	2.12	0.44
Maize bran	2.34	0.51	2.71	1.11	0.44	0.63	0.44	0.29
Corn pericarp	30.36	2.76	50.51	3.00	1.84	0.40	4.54	2.85
Olive oil cake	0.96	1.88	28.20	2.39	4.15	1.90	2.93	1.24

It appeared that only chemical pretreatment favored α-amylase production, which resulted in an increase in enzyme activity of 1.5 fold for soybean meal, and over 200 times for sunflower meal. Ultrasound and microwave pretreatments appeared to have negative impact on the production of this enzyme. As α-amylases are inductive enzymes, it is likely that in the case of chemical treatment, partial hydrolysis of soluble and insoluble carbohydrates, especially cellulose and hemicellulose, resulted in an increase in the content of reducing sugars that induce α-amylase biosynthesis. The effect of pretreatment of maize bran and corn pericarp containing higher content of cellulose and hemicellulose on α-amylase production has also been studied and the results are presented in the Table 5.8. In this case, the acid treatment did not appear to be sufficient to decompose the lignocellulosic structure. In this case, the alkaline pretreatment gave the best results in terms of α-amylase yield.

Table 5.8. Amylase production by SFF on untreated and treated substrates using *Bacillus* sp. TMF-1 (*B. thurgensis*), at 30 °C for 72 h at substrate: water ratio 1: 1.

	Chemical pretreatment	Ultrasound amplitude, %	Microwave
	Amylase, IU g^{-1}		

Substrate	Control	Acid	Alkaline	25%	35%	50%	Water	Glycerol
Soybean meal	12.31	18.90	18.38	2.32	1.84	1.96	0.99	0.00
Sunflower meal	0.09	24.40	18.02	0.61	0.13	0.00	0.00	0.18
Wheat bran	0.18	20.43	7.38	0.03	0.18	0.06	2.13	1.32
Maize bran	4.64	6.42	38.46	0.00	0.00	0.00	0.00	0.00
Corn pericarp	1.48	32.59	50.75	0.01	0.14	0.03	0.92	1.50
Olive oil cake	9.95	39.97	30.96	6.82	6.73	3.68	0.00	0.00

Good choice of substrates for the production of cellulases, amylases and pectinases was sunflower meal, but the only treatment that had beneficial influence on the production of these enzymes for all studied substrates was acid treatment (Tables 5.6, 5.8 and 5.9). Also, the same pretreatment had beneficial influence on the production of cellulases, amylases and pectinases by *Bacillus* sp. TMF-1 on wheat bran. Overall, wheat bran was not the best choice of substrate for enzyme production among evaluated substrates with this microorganism. Although the enzyme activity was observed, it was rather moderate. But significant improvement was observed when acidic pretreatment was used. However, our results collide with the literature data. Namely, the data shows that the acid pretreatment of the substrates that have a high percentage of hemicellulose could result in formation of monomers, furfural, hydroxymethylfurfural and other volatile byproducts. These substances along with lignin produce an environment that is not suitable for the growth of microorganisms. Bearing in mind that wheat bran contains approximately 50% of hemicellulose, 20% of cellulose and 10% of lignin, it was expected that the acidic pretreatment would have a negative effect on enzyme production on that substrate. But in contrast, our study suggested that this particular treatment induced amylase and cellulase production in wheat bran. It must be kept in mind that not all microorganisms favor the same substrates; therefore it is difficult to compare results of different studies where different microorganisms have been used. For example, in another study, *Aspergillus niger* NS-2, produced less cellulolytic enzymes on chemically treated wheat bran, and it was also noticed that other environmental factor also had more pronounced influence (Bansal *et al.*, 2012).

Table 5.9. Cellulase production by SFF on untreated and treated substrates using *Bacillus* sp. TMF-1 (*B. thurgensis*) at 30 ^{o}C for 72 h at substrate: water ratio 1: 1

	Chemical pretreatment			Ultrasound amplitude, %			Microwave	
	Cellulase, IU g^{-1}							
Substrate	Control	Acid	Alkaline	25%	35%	50%	Water	Glycerol
Soybean meal	0.95	0.21	0.32	0.43	0.21	1.20	0.23	0.12
Sunflower meal	0.12	0.33	0.04	0.06	0.10	0.13	0.06	0.04
Wheat bran	0.02	0.21	0.01	0.00	0.00	0.00	0.67	0.01
Maize bran	0.01	0.16	0.19	0.12	0.01	0.13	0.09	0.04
Corn pericarp	0.01	0.29	0.37	0.01	0.00	0.00	0.58	0.09
Olive oil cake	0.45	0.27	0.09	0.43	0.40	0.30	0.38	0.11

Another substrates that are also rich in hemicellulose (>70%) are the products of corn processing: corn brain and maize pericarp. Corn pericarp also consists of some amount of cellulose (~12%), lignin (~8%), starch (~5%) and protein (~5%), and corn bran contains smaller amount of lignin (~0.1%) and a higher content of cellulose component (~25%). This substrate was an excellent choice for the production of protease with *Bacillus* sp. TMF-1, but alkali pretreatment improved even more the proteolytic activity. Namely, proteolytic activity reached 30.4 IUg^{-1} for the untreated corn pericarp and 50 IUg^{-1} for alkali pretreatment. This pretreatment was also beneficial for cellulase activity which has significantly improved with alkali pretreatment. It seems that especially beneficial treatment for enzyme production was alkali pretreatment (Dien *et al.*, 2005; Yoshida *et al.*, 2012).

Although emerging as a promising new method, microwave irradiation was generally evaluated as inadequate method for substrate processing. In almost all cases, the microwave pretreatment had a negative effect on most of the substrates, most prominently for the production of α-amylase. For example, there was no amylolytic activity detected for several microwave pretreated substrates. It is assumed that the catalytic repression occurs due to the significant depletion of simple sugars that was caused by the pretreatment. Literary survey indicates that

microwave irradiation of corn bran can enhance the levels of inhibiting substances release. These substances are probably part of the plant defense system.

During the production of olive oil, large quantities of waste are generated such as waste water and other liquid waste, olive oil cake (obtained after extraction), leaves and other parts of the plant. Olive oil cake obtained after the extraction has a relatively high content of nutritionally valuable substances including lipids, carbohydrates and proteins, as well as a significant amount of other valuable substances (polyphenols, unsaturated fatty acids). In some cases, olive oil cakes can be used as animal feed but this is rather limited due to the low digestibility of olive oil cake in animal rumen. But largest quantities are together with waste water dumped into environment which represent serious environmental problem. Thus, very small quantities of this important industrial by-product are valorized. It is still poorly exploited substrate but with great potential. However, this kind of substrate seems to be suitable for the solid state fermentation. The olive oil cake is relatively high in protein (~4%), residual fat (~10%) content, and it also contains high amount of insoluble fiber (>60%), lignin (>25%) and relatively high ash content (>2.5%) (Moftah *et al.*, 2012). Olive oil cake was successfully used as a substrate for the SSF of *Bacillus* sp. TMF-1 for the production of proteases and amylases. The best pretreatment for the proteases production by far was the alkaline pretreatment. As for the amylases production the best were again alkaline and acidic pretreatment. Similar results were obtained in the study of SSF on olive oil cake for lipase and protease production with *C. utilis,* where fermentation processes and enzymes' production were significantly promoted by the alkaline treatment of the substrate (Moftah *et al.*, 2012). Literary survey showed that the alkaline pretreatment is beneficial to the substrates with high content of lignin, because with the release of lignin, solubilization and modification in the crystalline state of cellulose occur. It should also be noted that ultrasound pretreatment had a positive influence on the pectinase production (table 5.6). Namely, the pectinase production on olive oil cake was enhanced over 100-fold.

Table 5.10 Substrate/pretreatment yield of the highest enzymes' activities obtained

S. No.	Enzyme	Substrate	Pre-treatment	IUg^{-1}
1.	Protease	Corn pericarp	Alkaline	50.51
2.	Cellulase	Maize bran	Alkaline	1.19
3.	α-amylase	Corn pericarp	Alkaline	50.75
4.	Pectinase	Soybean meal	No-treatment	64.90

Table 5.10 shows the best achieved results. It represents substrate/pre-treatment/yield of the highest enzymes' activities obtained. Additionally, statistical analysis reformed by the two –way ANOVA revealed that the pretreatment of substrates had significant influence on the yield of amylase ($p<0.01$) and protease ($p<0.05$) production, while the type of the substrate showed insignificant influence on the same output. However, neither type of substrate nor its treatment had the significant influence on the yield of cellulose and pectinase production. Ultrasound pretreatment showed limited or no effects, while microwave radiation was efficient only for treatment of wheat bran. Generally it can be concluded that for the majority of the substrates the best pretreatment were acidic and alkaline. However, it has to be acknowledged that these chemical pretreatments have several disadvantages such as extensive biomass washing and the need for the equipment to be made with corrosion-resistant materials. All these parameters have to be taken into consideration of the process feasibility.

5.2 Enzyme production by solid-state fermentation on soybean meal: a comparative study of conventional and ultrasound-assisted extraction methods

It has previously been shown that by the proper selection of microorganisms, it is possible to produce industrially significant enzymes by fermentation on solid agro-industrial waste. After fermentation and enzyme production, it is crucial to leach the enzyme from the substrate in a yield as high as possible. Therefore, the first step in downstream process in SSF is the selection of the most suitable extraction technique. More recently, several different approaches and techniques have been applied to leach out the enzymes produced as efficiently as possible and to reduce the cost of the process. Among all innovative techniques, ultrasound technology has the highest potential for application in extraction processes. Ultrasound-assisted extraction is an incomparably more efficient process in terms of yield of the target component. In addition, the process takes place at much lower and significantly milder reaction conditions than conventional extraction techniques so it can be considered as eco-friendly (Jovanović *et al.*, 2017; Chung *et al.*, 2010; Heffels *et al.*, 2015). Any ultrasound-assisted extraction must be optimized in detail in terms of extraction time, ultrasound frequency, specific energy, and substrate characteristics (Leitte *et al.*, 2016). Consequently, herein the extraction method of protease/α–amylase/cellulase from the SBM fermented with *B. subtilis* was estabilished based on yields of enzyme recovery. The yield of enzyme recovery of conventional methods was compared with ultrasound-assisted methods.

5.2.1 Optimization of the recovery of protease/α–amylase/cellulase obtained by SSF by experimental design technique

In order to find the optimal extraction conditions for the enzymes produced on the soybean meal, the experimental design technique was applied. In planning the experiment, the most important extraction factors were captured and tested: pH, solid-liquid ratio, mixing rate, and extraction time. We have adopted the

starting point, the central point and the test range of four independent variables as presented in Table 5.10. The entire set of experiments is performed to determine the relationship between mass transfer and yield and the activity of the extracted enzyme. Also, the series of experiments is designed so that the solid-liquid ratio and extraction time are such that the process can easily be translated to a larger scale, which means economic and environmental friendliness, sustainability and validity as well as practicability of purification. As stated previously, the extraction process was examined with respect to the application of conventional and ultrasound techniques and compared with each other. A summary of responses (activity of protease, α-amylase and cellulase) under each of 29 sets of statistically designed experiments are presented in Table 5.10.

Table 5.10. The real and coded values and respective responses of independent variables used in the experimental design for the optimization of protease/α–amylase/cellulase extraction from SBM fermented by *B. subtilis.*

Independent variables					Responses		
Run	Stirring	pH	Solid:liquid	Time	Protease	α-amylase	Cellulase
no.	rpm		S/L	Min	IU	IU	IU
1	0 (150)	0 (8)	0 (0.15)	0 (30)	142.75	376.92	1.78
2	0 (150)	1 (9)	-1 (0.05)	0 (30)	160.4	533.33	0.25
3	0 (150)	0 (8)	1 (0.25)	1 (45)	140.04	110.37	1.44
4	0 (150)	-1 (7)	0 (0.15)	1 (45)	166.69	150.62	4.52
5	0 (150)	-1 (7)	-1 (0.05)	0 (30)	89.11	618.11	4.72
6	-1 (50)	0 (8)	0 (0.15)	1 (45)	159.60	358.44	1.20
7	1 (250)	0 (8)	1 (0.25)	0 (30)	105.60	340.90	2.12
8	0 (150)	0 (8)	1 (0.25)	-1 (15)	148.16	86.42	3.86
9	-1 (50)	1 (9)	0 (0.15)	0 (30)	194.18	242.80	1.25
10	1 (250)	-1 (7)	0 (0.15)	0 (30)	154.72	444.44	5.50
11	0 (150)	-1 (7)	1 (0.25)	0 (30)	108.26	197.53	6.35
12	-1 (50)	0 (8)	0 (0.15)	-1 (15)	199.60	72.43	0.11
13	0 (150)	0 (8)	0 (0.15)	0 (30)	123.69	228.70	1.95
14	0 (150)	0 (8)	0 (0.15)	0 (30)	144.6	288.48	1.57
15	0 (150)	0 (8)	0 (0.15)	1 (45)	207.04	160.49	1.05
16	-1 (50)	0 (8)	-1 (0.05)	0 (30)	126.5	791.36	0.01
17	-1 (50)	-1 (7)	0 (0.15)	0 (30)	135.66	303.70	4.06
18	-1 (50)	0 (8)	1 (0.25)	0 (30)	98.95	161.48	3.33
19	0 (150)	1 (9)	0 (0.15)	-1 (15)	200.39	63.12	1.87
20	1 (250)	1 (9)	0 (0.15)	0 (30)	146.74	372.84	3.94
21	0 (150)	0 (8)	-1 (0.05)	-1 (15)	123.69	402.10	1.00
22	1 (250)	0 (8)	-1 (0.05)	0 (30)	75.81	807.12	2.92
23	0 (150)	-1 (7)	0 (0.15)	-1 (15)	182.65	201.48	5.04
24	0 (150)	0 (8)	0 (0.15)	0 (30)	153.84	369.88	2.11
25	0 (150)	0 (8)	-1 (0.05)	1 (45)	156.94	807.41	0.05
26	0 (150)	0 (8)	0 (0.15)	0 (30)	158.71	264.98	2.22
27	0 (150)	1 (9)	1 (0.25)	0 (30)	127.68	215.06	1.71
28	1 (250)	0 (8)	0 (0.15)	1 (45)	185.76	393.83	1.97
29	1 (250)	0 (8)	0 (0.15)	-1 (15)	165.05	304.94	6.00

The statistical analysis of the results presented in Table 5.10 enabled the introduction of appropriate coded empirical models (Eq. 5.2.1-1; Eq. 5.2.1-2 and Eq. 5.2.1-3) to describe the activity of the enzymes as a function of the factors examined:

$$\text{Protease activity} = +144.98 - 6.41 \times R + 16.61 \times pH - 16.62 \times R \times pH + 13.35 \times R \times S/L + 15.20 \times R \times T - 12.97 \times pH \times S/L - 10.34 \times S/L \times T + 12.85 \times pH^2 - 38.19 \times S/L^2 + 33.10 \times T^2 \quad (5.2.1\text{-}1)$$

$$\alpha\text{-amylase activity} = +270.46 + 64.91 \times R - 238.18 \times S/L + 70.89 \times T - 95.34 \times S/L \times T + 93.25 \times R^2 + 152.9 \times S/L^2 - 93.21 \times T^2 \quad (5.2.1\text{-}2)$$

$$\text{Cellulase activity} = +1.78 + 1.06 \times R - 1.68 \times pH + 0.96 \times S/L - 0.77 \times T - 1.45 \times R \times S/L - 1.68 \times R \times T + 1.57 \times pH^2 \quad (5.2.1\text{-}3)$$

Where all activities are represented as total recovered enzyme from 5 g of fermented SBM, and equation variables are coded values for stirring rate (R), pH (pH), solid to liquid ratio (S/L) and time (T).

Accuracy of the fit, variance analysis as well as adequacy of the model were done using ANOVA. Besides, Fisher F-test for protease 30.4, α-amylase 21.25 and cellulase 17.5 indicate the significance of the model.

The model related to protease extraction, based on the p-value level ($p<$ 0.0001), indicates that linear coefficients, pH and quadratic term coefficients S/L ratio and time are the most significant. Also, this model gives values of several coefficients that show an interesting common dependence between the examined factors. Recovery of enzyme from the fermented substrate by selecting of the appropriate buffer is one of the key aspects of SSF fermentation. The ideal buffer should extract the enzyme selectively and completely at room temperature, with minimal contact time and ideally at the pH of cultivated substrate (Han-Qing *et al.*, 2011). Generally, it can be seen that extraction of protease in alkaline buffer effects positively on the enzyme activity and the whole extraction process (Figure 5.3.).

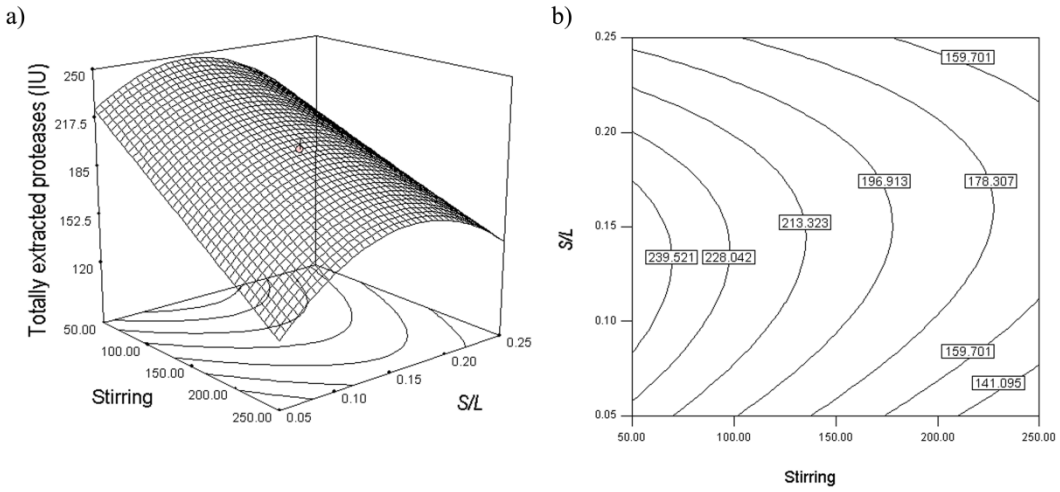

Figure 5.3. a) Responses and b) contour analyses *versus* process conditions, stirring rate and *S/L* ratio for totally extracted protease from 5g of FSBM (15 min, 50 mM phosphate buffer pH 9).

According to the mathematical and graphical analysis it can be seen that both *S/L* ratio and pH have negative interactive coefficient, which further implies that *S/L* ratio must maintained at a low range to achieve maximal protease secretion. The solid-liquid ratio is one of the key parameters when optimizing the extraction process. Specifically, the low *S/L* ratio indicates that a large amount of fluid is required to allow the transfer of mass, which is based on the existence of a concentration gradient, from liquid to solid phase. Large amounts of extract in terms of volume after extraction represent an obstacle or aggravating circumstance for further steps of isolating and purifying of the target enzyme. Based on the predicted model, optimal conditions were determined, pH 9 of the extraction buffer, low agitation and low *S/L* ratio (0.07), extraction time of 15 minutes with approximately 240 IU of proteolytic activity recorded in the extract. The conditions for protease extraction produced by *Rhizopus oryzae* on wheat bran differ significantly from the optimal conditions for protease extraction produced on soybean meal using *B. subtilis*. Namely, the extraction of the protease produced on wheat bran requires a considerably longer time, that is, the maximum yield was recorded after 2 hours of extraction (520 U) (Aikat and Bhaattacharyya, 2000). In addition to differences in extraction time, significant differences in *S/L* ratio were observed. For example, the *S/L* ratio of 5 proved to be optimal for the extraction of

protease produced on wheat bran, while the *S/L* ratio of 0.07 was quite sufficient to achieve the maximum yield of the protease produced on soybean meal in the extract. The assumption is that such differences can be attributed to the different water holding capacity of wheat bran, which ranges from 4-7.3 g/g_{dm} (Onipe *et al.*, 2017), while the water-holding capacity of soybean meal is smaller and, according to the literature, is approximately 5 g/g_{dm} (Ngoc and Lindberg, 2012). In addition, repeated extraction has been shown to have no effect on increasing yield, which means that most of the protease produced is extracted in one step (Aikat *et al.*, 2000).

Analyzing the predicted model for α-amylase defined by Eq. 5.2.1-2 concludes the different effect of the *S/L* parameter on the extraction yield of this enzyme. Specifically, a high *S/L* ratio was found to favor a higher yield of amylolytic activity in the extract (Figure 5.4). Higher amylolytic activity in the extract is a consequence of better solubilization of α-amylase present in the cultivated solids.

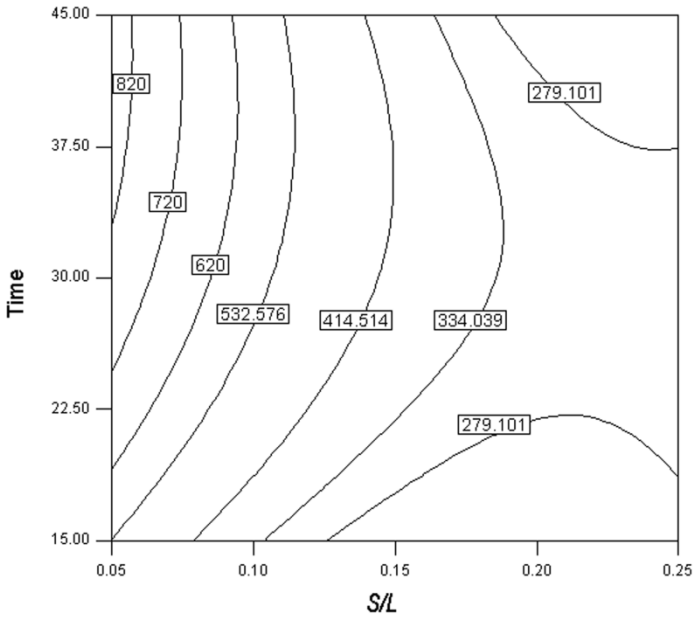

Figure 5.4. Contour analyses for totally extracted α-amylase as a function of time and *S/L* ratio from 5g of FSBM (stirring rate 250 rpm, 50 mM phosphate buffer pH 7).

Furthermore, negative interactive coefficient between T and *S/L* ratio was established by contour plot given on the Figure 5.4. This indicates that prolonged extraction time can have a positive effect on the extraction of α-amylase from FSBM. In conclusion, the highest activity of α-amylase of 820 IU was achieved under the optimized conditions: extraction time 45 minutes, stirring rate 250 rpm, solid to liquid ratio, *S/L*=0.05, and pH 7 of extraction buffer.

The recovery of cellulase for different variables could also be predicted by contour plot, Figure 5.5.

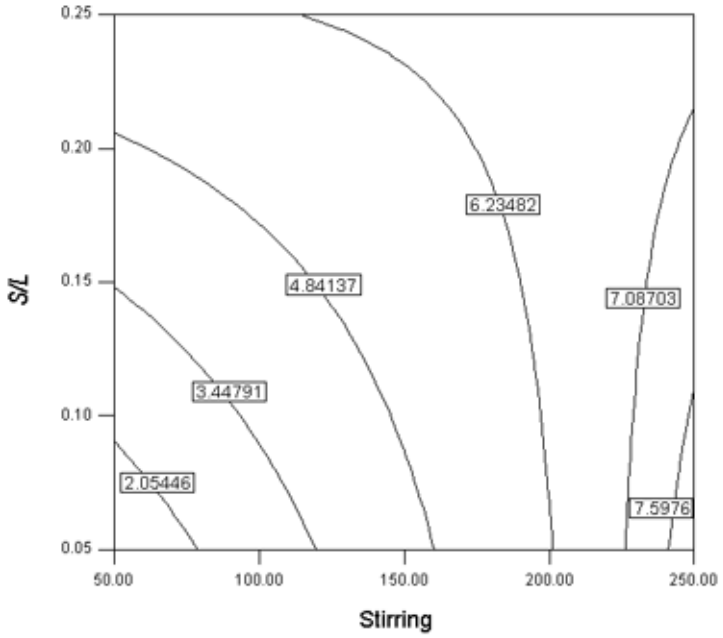

Figure 5.5. Contour analyses for totally extracted cellulase as a function of stirring time and *S/L* ratio from 5g of FSBM (extraction time 30 min, 50 mM phosphate buffer pH 7).

As can be seen, with careful optimization it is possible to achieve an extraction yield of 70%. During the extraction of the cellulase from the FSBM, the choice of stirring rate proved to be the most important parameter. Contour curve given on the Figure 5.5 indicates that cellulase recovery is affected positively and dominantly by stirring rate without being much influenced by *S/L* ratio. It is assumed that a higher stirring rate is necessary because the cellulases are firmly bound to the fermentation substrate. This indicates that *B. subtilis* is not only the

producer of extracellular cellulases but also those that are bound to cellular components/intracellular enzymes. This is not peculiar to *B. subitlis* although there is evidence that intracellular cellulases produced by a number of other bacteria and fungi. Tightly bound enzymes to the substrate on which they are produced are a common data found in the available literature (Kim *et al.*, 2012). Besides, a satisfactory yield of cellulases could be obtained at low *S/L* ratio which results in a more concentrated product. The similar results were found in the literature for the xylanase extraction from the wheat bran fermented by *A. niger*. In this study the highest xylanase recovery was found under the high agitation rate and very low extraction volume (Pan and Khanum, 2010). Furthermore, the extraction time could negatively affect the enzyme recovery (Pal and Khanum, 2010). Namely, it was found that prolonged extraction time led to the loss of enzyme activity as evidenced in this study as well. For instance, decrease in cellulolytic activity from 5.04 to 4.5 IU after the extraction time prolongation from 15 to 45 minutes was observed in this study.

5.2.2 Ultrasound assisted recovery of enzymes from fermented soybean meal (FSBM)

As stated previously, the extraction of enzymes from the substrate is a crucial factor in terms of yield and cost-effectiveness of solid state fermentation. Consequently, work is constantly underway to find a technique that meets both criteria. Today is a very common phenomenon that low-frequency or power ultrasound is the part of the process equipment of food or pharmaceutical industry. During ultrasound treatment, cavitation bubbles are formed and collide asymmetrically on the substrate surface. In this way, a solvent jet is formed which penetrates the substrate at high speed and thus the mass transfer is intensified. Besides mass transfer intensification, disruption of biological cell wall and liberation of intracellular components is the second benefit of ultrasound assisted extraction (Sabo *et al.*, 2015). Therefore, we have evaluated is it possible to dramatically improve the hydrolytic enzymes recovery from the cultivated substrate by using the technology of ultrasound.

5.2.2.1 Ultrasound assisted recovery of protease from FSBM

In order to examine the protease recovery from FSBM, the cultivated substrate was soaked in different buffers. The sonication influence i.e. sonication time was varied, meanwhile the ultrasound amplitude was kept constant, 10%. Besides, the system is constantly cooled to avoid the denaturing effects of increased temperature caused by ultrasonication. The protease recovery in terms of activity is depicted on Figure 5.6.

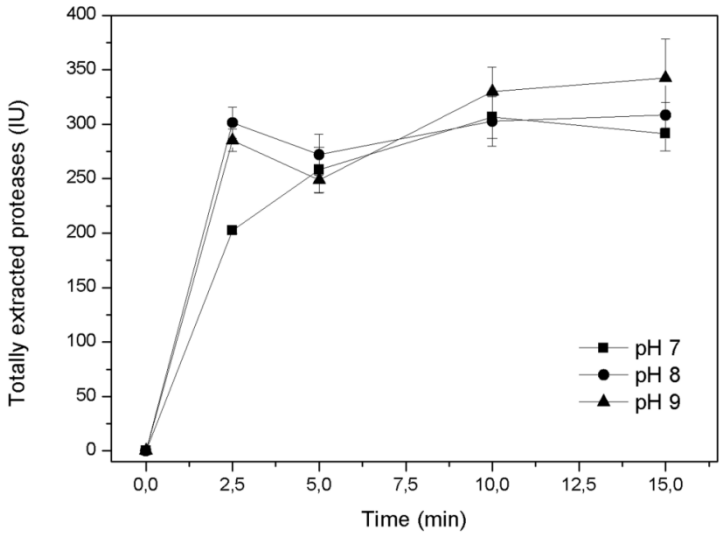

Figure 5.6. Sonication time influence on protease recovery from 5g of FSBM (0.1 *S/L* ratio, 50 mM phosphate buffer, pH 7-9).

Data on ultrasound-assisted extraction of enzymes from fermented substrates are scarce in the literature. Accordingly, the results presented in this study are a significant contribution to this scientific field. The Figure 5.6. clearly demonstrates that there are some differences in the extraction yield aided by influence of ultrasound. Thus, after 2.5 minutes of sonication, a proteolytic activity of approximately 285 IU was recorded in the extract. Prolonged sonication time in buffers at pH 7 and 8 did not significantly affect protease extraction. On the other hand, a completely different trend of ultrasound assisted protease extraction in buffer pH 9 was observed. A gradual increase in proteolytic activity in the extract

was observed over the entire test range of 2.5–15 minutes, and the maximum was observed after 10 minutes of sonication and amounted to 330 IU. Thus, the influence of pH and sonication time on ultrasound-assisted protease extraction is evident. Significantly, ultrasound extraction increased the yield of the protease compared to conventional extraction on orbital shaker where, after 15 min, the proteolytic activity observed in the extract was ~210 IU. As can be seen, the ultrasound did not affect the enzyme activity, which means that ultrasound did not had "sonochemical" effect which is manifested unambiguously by the decline in enzymatic activity (Sabo *et al.*, 2015). Extraction is a process where it is necessary to allow good contact between the solid and liquid phases and the concentration gradient. Obviously, ultrasound contributes to the disintegration of the fermented substrate fibers, that is, to a reduction in particle size, making the solid-liquid contact much better. In this way, the mass transfer is intensified, which is eventually detected by the increased yield of the recovered enzyme. This effect of ultrasound has been observed during the extraction of lignolytic and hydrolytic enzymes obtained by fermentation of flax fiber as a lignocellulosic substrate by *Aspergillus oryzae* Sabo *et al.*, 2015).

5.2.2.2 Ultrasound assisted recovery of α-amylase from FSBM

Ultrasound also proved to be a successful method for extracting the produced amylase (Figure 5.7).

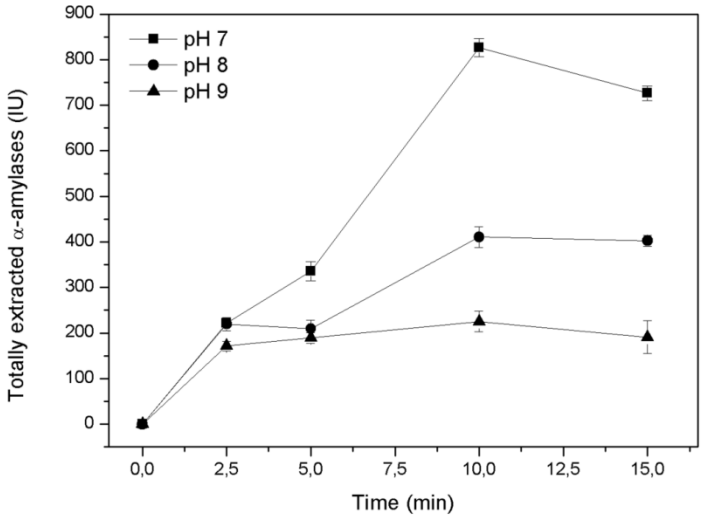

Figure 5.7. Sonication time influence on α-amylase recovery from 5g of FSBM (0.1 *S/L* ratio, 50 mM phosphate buffer, pH 7-9).

As one can conclude, there are major differences in the ultrasonic assisted extraction of amylase under the influence of different pH. The highest recovery of α-amylase, ≥ 825 IU was obtained during 10 min of ultrasonication in buffer at pH 7. Although an approximate result is achieved by conventional technique, the ultrasonic treatment is preferred. Conventional extraction takes 45 min, while a large amount of amylase can be extracted with ultrasound for twice as long, which implies that economically, ultrasound would be justified. Furthermore, conventional treatment requires significantly higher amounts of extractant, which means that the final product will be diluted, which is a difficulty in optimizing and performing the isolation and purification of the target enzyme and the difficulty of processing large quantities of wastewater. This influence of ultrasound is also attributed to the destruction of the substrate matrix and the release of a large amount of enzyme embedded inside the matrix. However, there are also cases where the enzyme extraction does not only occur under the influence of buffers or shear forces created by mixing. In this case, there is a hypothesis that explains the effect of ultrasound in different ways. One of them is assumed that the transport of the enzyme and increased hydrolytic activity is attributed to ultrasonically induced

metabolic response of cells by modifying cellular metabolism and facilitating nutrient utilization (Guanghui *et al.*, 2009).

5.2.2.3 Ultrasound assisted recovery of cellulase from FSBM

Besides protease and α-amylase, herein we consider the ultrasound-assisted recovery from the FSBM of another industrially important enzyme, cellulase. The same parameters of extraction were varied and the ultrasound efficiency in terms of enzyme recovery was evaluated and the results are presented on Figure 5.8.

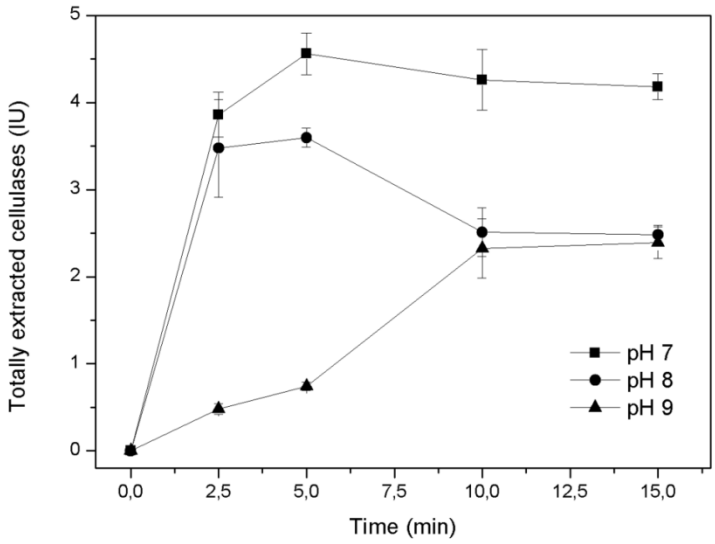

Figure 5.8. Sonication time influence on cellulase recovery from 5g of FSBM (0.1 *S/L* ratio, 50 mM phosphate buffer, pH 7-9).

When it comes to the effect of ultrasound on cellulase extraction, the results obtained here are different and, in contrast to the results obtained for the influence of ultrasound on protease and amylase extraction. It is clear that increasing the exposure of the substrate to ultrasonic waves has a negative effect on cellulase extraction. Specifically, sonication of fermented soybean meal for 5 min recorded the activity of cellulase 4.5 IU. Conventional shaking treatment on an orbital shaker for 15 min, 250 rpm yielded a cellulolytic activity≥7.5 IU and a much more concentrated product was obtained. In the conventional extraction process, the

liquid comes in contact with the surface layers of the substrate, so the velocity gradient in the layers close to the surface is extremely small, and the mass transfer is driven only by diffusion. On the basis of the result obtained, it can also be said that the stirring rate was correctly determined because high cellulase activity in the extract was achieved. However, during the application of ultrasound certain convective flows occur, thereby the mass transfer is intensified. In addition, the mechanical effect of acoustic bubbles on the inner layers also significantly improves the influence of ultrasound on enzyme recovery. However, sonication can also impair the activity of the enzyme. The abruptly increase in local temperature, pressure and shear forces as a result of acoustic cavitation often negatively affects the activity of the enzyme, which is assumed to be the case with the effect of sonication on cellulase extraction. Most often, the loss of enzyme activity is associated with dissociation into subunits caused by cavitation bubble collisions, expressed shear forces, and high temperatures. Literature survey indicates that sonication has a positive effect on the extraction of individual enzymes. For example, by applying an amplitude of 60%, it is possible to increase the yield of filterpaperase, lignin peroxidase and laccase by 167, 129 and 209%, respectively. With a further increase in ultrasound amplitude up to 80%, the yield of these enzymes is increased up to four times in the extraction medium (Sabo *et al.*, 2015). In a study by Szabo *et al.*., a 170% increase in polygalacturonase yield was achieved by ultrasonic flax fiber extraction (Huang *et al.* 2017). By comparing the literature data and the results of our study, it is concluded that when it comes to cellulose extraction by ultrasonic treatment, it is necessary to analyze the process parameters in more detail.

5.3 Improvement in bioactivity and anti-nutritional factors profile in soybean meal after solid-state fermentation with *B. subtilis*

Based on data from the SoyProtein company, which is considered to be the largest producer and exporter of soybean and soybean products, the annual production of soybean meal in Serbia exceeds 380 000t. Due to its high protein content (43-48%) and good amino acid profile (especially rich in lysine, 2.5-2.8%), soybean meal is an integral component in the preparation of high protein animal feed (Li *et al.*, 2019). Although protein-rich, soybean meal also has proteins that are considered undesirable in its composition, and are called anti-nutritional factors (ANFs). ANFs lead to allergic reactions in patients with atopic dermatitis and reduce the digestibility and absorption of nutritional elements of soybean meal. In this group of ANFs protease inhibitors are included, a trypsin inhibitor, pancreatic protease inhibitor, which inhibits proteolysis and protein absorption (Chun-Hua and Seong-Jung, 2016). In addition, soybean meal contains high percentage of alergenic proteins, β-conglycinin and glycinin, about 30%. Besides to the anti-nutritional components of protein origin, soybean meal also contains phytic acid, non-digestible oligosaccharides, such as stachyose and raffinose, and lectins (Sang-Hyun and Seong-Jun, 2016). The ANFs content in soybean meal should be reduced or removed in order to apply this in human and animal feeding. There are different techniques and approaches for the removal of ANFs from the soybean meal, such as toasting, chemical treatment and alcohol extraction. However, the soybean meal product obtained after the application of these techniques has practical drawbacks, low digestibility, unpleasant palatability, unacceptable dark color and the level of ANFs is still above the allowed limits (Zhao *et al.*, 2018; Yin *et al.*, 2019). Apparently, SSF is a promising tool regarding the removal of ANFs from soybean meal. Fermentation can reduce the ANFs from the soybean meal, thus the immunoreactivity and allergic reactions caused by soy products are reduced (Sang-Hyun and Seong-Jun, 2016).

Bacillus subtilis is most widely used in the food industry due to its probiotic properties, low cost and ability to produce numerous enzymes such as proteases, lipases and carboxypeptidases (Yin *et al.*, 2019). The change in the protein profile of soybean meal by *B. subtilis* cultivation has been demonstrated in the previous sections, so the aim of this experimental series is to determine the effect of *B. subtilis* on the profile of anti-nutritional components of soybean meal.

5.3.1 Trypsin inhibitor profile in SBM fermented with *B.subtilis*

Biological detoxification such as fermentation of various agroindustrial substrates is the technique of choice when it comes to eliminating anti-nutritional factors such as trypsin inhibitor. *Lactobacillus fermentum* and *Lactobacillus plantarum* have been successfully applied towards reduction of trypsin inhibitors in *Vigna sinensis* flour (Doblado *et al.*, 2004). Also, a decrease in trypsin inhibitory activity was observed in de-oiled rice bran after fermentation with *Rhyzopus oryzae*. *Jatropha* seed cake is a by-product in the production of biodiesel extremely rich in protein and as such an exceptional substrate for the cultivation of microorganisms (Ranjan *et al.*, 2019). However, a large percentage of anti-nutritional factors limit its use. Fermentation with *Aspergillus versicolor* therefore reliably improved the profile of this by-product in terms of reducing the trypsin inhibitor content so that it could be used as a feed component (Veerabhadrappa *et al.*, 2014). The diversity of substrates and microorganisms has led us to examine the potential of the *B. subtilis* strain in the elimination of trypsin inhibitors from soybean meal. For this purpose, the fermentation was performed under two different moisture regimes, soybean meal:water 1:0.5 and 1:1 and the trypsin inhibitor activity in fermented soybean meal was evaluated. The results obtained are shown in Figure 5.9.

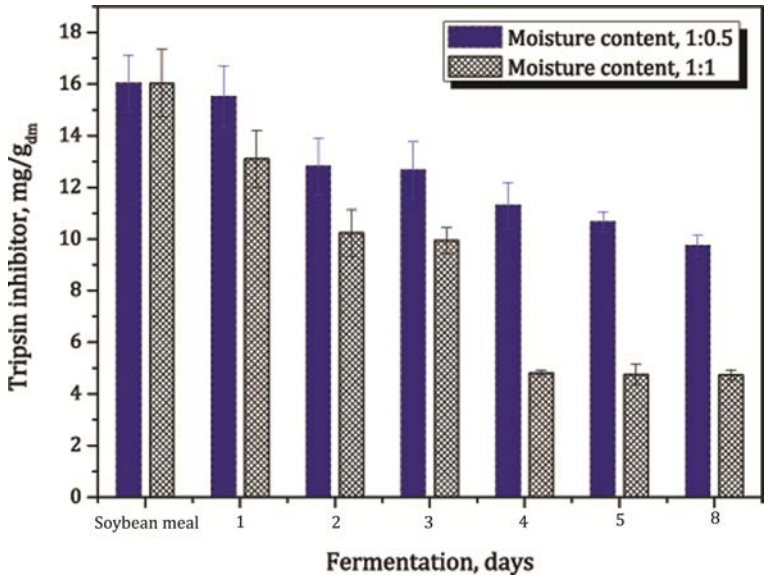

Figure 5.9. Trypsin inhibitor activity profile in soybean meal after fermentation with *B. subtilis.*

Apparently, the fermentation remarkably decreased the protease inhibitor content. A gradual decrease in protease inhibitor content is observed during all fermentation days in both moisture content regimes. After 8 days of fermentation under fermentation conditions where the moisture content was 1:0.5 a decrease in trypsin inhibitor content from 16.01±1.3 to 10.66±0.4 mg/g_{dm} was observed. However, in the case of 1:1 moisture content, a greater decrease was observed after 8 days of fermentation, thus a decrease to approximately 4.74±0.2 mg/g_{dm} was observed under these conditions, indicating that in the fermented soybean meal the trypsin inhibitor content was reduced by 75%. As can be seen water activity had significant influence on the trypsin inhibitor content reduction. Similar results regarding the effect of moisture content during fermentation on the level of trypsin inhibitor removal were obtained by Yuo-Ling *et al.*. Trypsin belongs to the class of serine proteases and it is essential for the animal and human nutrition. Trypsin inhibitor binds to the serine protease, trypsin and chymotrypsin interfering the normal functioning of pancreatic proteolytic enzymes in non-ruminants (Veerabhadrappa *et al.*, 2014). Reduction in trypsin inhibitor is related with production of large amount of different acid proteases by *B. subtilis* during fermentation which obviously reduced the trypsin inhibitor content (Phengnuam

and Suntorsuk, 2013). Generally, it is known that *Bacillus* sp. is a potent extracellular protease producer, so it is not surprising that the results obtained are consistent with the literature. De-oiled rice bran is an agro-industrial waste that is used in the diet of cattle, fish and poultry but is rich in anti-nutritional factors. It has been shown that fermentation by *Rhyzopus oryzae* can reduce the trypsin inhibitor content by 24.8% (Ranjan *et al.*, 2019). Increased interest in the use of peas in human and animal nutrition increases the need for appropriate processing of this raw material in order to reduce anti-nutritional factors. Thus, using a commercial mixture of microorganisms *B. subtillis* and *B. licheniformis* known in the market as BIOPLUS 2B®, is possible to reduce the trypsin inhibitor content from the peas using SSF and thus increase the nutritional value of this legume (Boroojeni *et al.*, 2018). Moreover, it was found in a previous study that it is possible to remove the trypsin inhibitor by solid-state fermentation using *B. subtilis*in a high percentage up to 96% (Teng *et al.*, 2012). In addition to bacteria and fungi, there are data on the reduction of trypsin inhibitor content in yeast fermentation media. Thus, soybean meal was used to grow yeast *S. cerevisiae* and the trypsin inhibitor content was monitored. It was established that after 48h of fermentation it was possible to reduce the trypsin inhibitor content by 10% in this way (Hassaan *et al.*, 2015). Based on the available literature data and the results presented in this study, it is clear that fermentation can increase the nutritional value of various agro-industrial wastes in terms of reduction of trypsin inhibitor content. The level of solid-state fermentation efficiency is determined by the type of microorganism and the composition of the substrate, and surely the process parameters need to be aligned with them.

5.3.2 Phytic acid profile in SBM fermented with *B.subtilis*

In plants, phosphorus is in the highest percentage, 60-80% stored in the form of phytic acid and in this form monogastric animals cannot utilize it. The inability to utilize the phosphorus that is in the phytic acid complex is due to the fact that pigs, turkeys, chickens and other non-ruminants do not have a phytic acid degradation system, i.e.phytase (Neira-Velma *et al.*, 2018). Phytase catalyze the degradation of phytic acid to lower forms of myo-inositol phosphates and

inorganic phosphorus. Since phytin is not degraded it passes through the digestive system of non-ruminants and reaches the environment where it is the main cause of eutrophication. Consequently, there is a great need for supplementation of animal feed with commercially available phytase that are estimated to cost $2–$3/ metric ton of feed (Bala *et al.*, 2014). Phytase are produced by numerous plants, animals and microorganisms, but microbial phytase have supremacy in the commercial industry (Rani and Gosh, 2011). In particular, this advantage is reflected in the cultivation of microorganisms on a suitable agro-industrial residue having an impermissible level of phytic acid, where the simultaneous production of phytase brings the levels of phytic acid and inorganic phosphorus within the permissible limits. Herein the phytic acid content in soybean meal was moitored during 8 days of fermentation with *B. subtilis* and it was established whether this strain was potent for enzymatic system production responsible for the phytate depletion from the substrate.

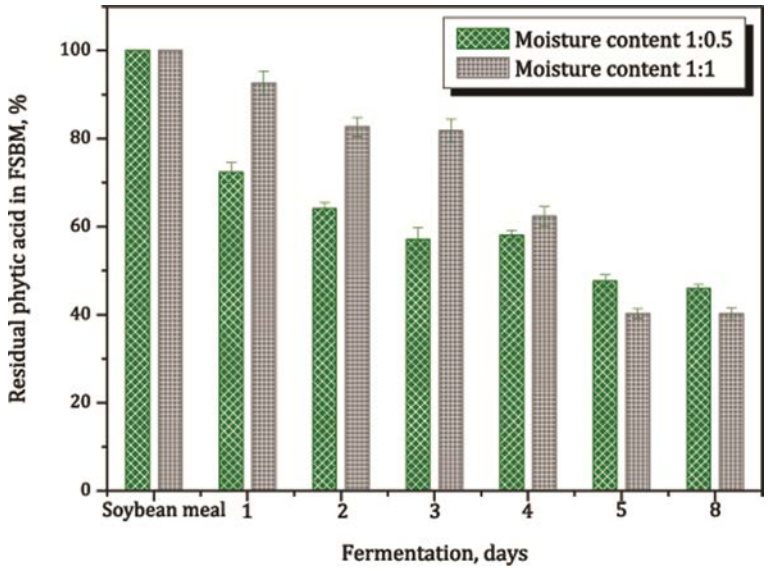

Figure 5.10. Phytic acid profile in soybean meal after fermentation with *B. subtilis.*

First of all, based on the data given in Figure 5.10., it can be concluded that the level of phytic acid reduction is different depending on the initial moisture content of the substrate. It is justified to assume that higher moisture content in the substrate was (1:1) the trigger for better diffusion or increased enzyme level, which resulted in a higher reduction of phytic acid level, probably due to higher

degree of substrate particles swelling and water tension, rather than in the system with moisture content 1:0.5 (Bala *et al.*, 2014). Similar findings during solid state fermentation of rapeseed meal with *Aspergillus niger* were reported by El-Batal et. During 8 days of fermentation one can observe gradual decrease in phytic acid content in soybean meal. Although the decrease in phytic acid content was slightly more intense and more pronounced during the first days of fermentation in the system with less moisture content, it was finally shown that after 8 days of fermentation in soybean meal, it was approximately 47% behind the initial amount of phytic acid. In a system with a higher moisture content, this trend is slightly more uniform, but on the fifth day of fermentation a more drastic decrease can be observed, where we can assume that the enzymatic system responsible for the phytate degradation reaches maximum of activity. Finally, in a system with higher moisture content, the decrease of about 60% of initial phytic acid content was achieved. Results in the available literature confirm our findings. *H. nigrescens* was chosen for the fermentation of different agro-industrial residues, rice bran, citrus peel, mustard oil cake, wheat bran and sugarcane bagasse. Among all tested substrates, it was shown that wheat bran was the best choice due to large amount of inducer present in wheat bran for phytase production, phytic acid. After cultivating *H. nigrescens* the 50-60% reduction in phytic acid content was observed in wheat bran (Bala *et al.*, 2014).

The following data found in the literature can further elucidate the mechanism of reduction of phytic acid content in agro-industrial wastes. Specifically, wheat bran was used as a substrate for growing a commercial starter culture containing *Lactobacillus bulgaricus* and *Streptococcus thermophiles*, LAB and active dry yeast. The highest degree of phytic acid removal, 30% from wheat bran, was achieved by inoculation with LAB and dry yeast as a mixture. The removal of phytic acid by phytase is a strictly pH dependent process, so acidifying the substrate to pH 5 can significantly contribute to the hydrolysis of phytic acid. Besides phytase, phosphatase were also found in microorganisms and are responsible for the hydrolysis of phytic acid. According to the significantly lower degree of phytic acid removal obtained in this study compared with previous

studies (phytic acid removal >90%), it can be concluded that phytic acid degradation with endogenous phytase present in wheat bran has missed. The main explanation for this is that when autoclaving the substrate, endogenous phytase loses activity (Zhao *et al.*, 2017).

Soybean residue (okara) was used for cultivation of *Kluyveromyces marxianus* and the changes in anti-nutritional composition of soybean meal residue was determined. Namely, after the fermentation of soybean residue at 28°C during 44h decrease in phytic acid content from 16.8±0.1 to 6.4±0.1 mg/g_{dm} was recorded (Zhou *et al.*, 2019).

Vig et. al. found that it is possible to detoxify rapeseed meal with solid state fermentation. To be specific, rapeseed meal was used as a substrate for cultivation of fungus *Rhyzophus oligosporus* and potential of this strain was examined in terms of phytase production. The results obtained after 10 days of fermentation revealed significant decrease in phytic acid content compared with unfermented rapeseed cake, 42.4% (Pal Vig and Walia, 2001).

Soybean meal was used in another study, where it was tested experimentally whether *Aspergilus niger* in low humidity conditions could be applied for biotransformation of proteins, carbohydrates, saponins and phytates. It is already a known fact that phytates (hexaphosphates of myo-inositol, $InsP_6$) reduce the digestibility of proteins and have the ability to chelate with di- and trivalent mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+} and Fe^{3+}, resulting in decreased availability of these ions for animals (Ranjan *et al.*, 2019). Obviously, *A. niger* is a good producer of phytase because it has been reported to drop over 90% in phytic acid content of fermented soybean meal. By the action of phytase, inositol hexaphosphates, $InsP_6$ are hydrolyzed towards lower $InsP_5$, $InsP_4$ and $InsP_3$. It is established that higher inositol phosphates $InsP_6$ have strong inhibitory effect on iron and zinc absorption. Withal, monitoring of the content of inositol phosphate revealed a significant decrease and absence of lower inositol phosphates, which indicated that dephosphorylation of phytic acid in soybean meal was done, and by examination of the mineral composition it was confirmed (Jacobsen *et al.*, 2018).

5.3.3 Total phenols profile of SBM fermented with *B.subtilis*

Secondary metabolites such as antibiotics, alkaloids, mycotoxins, pigments, plant growth factors and phenols are produced in small quantities and are classified as nutritive constituents in plants (Martins *et al.*, 2011). In recent years, these bioactive compounds have been found to exert a myriad of positive effects on human health, such as a positive effect on cardiovascular diseases but also degenerative diseases such as cancer and diabetes. The anti-mutagenic, antioxidant, anti-microbial, anti-inflammatory, anti-allergic effect of these compounds has been confirmed in countless studies (Martins *et al.*, 2011). Consequently, the efforts of the researchers are directed towards finding plants, fruits, vegetables and agro-industrial residues as rich sources of bioactive phenolic compounds. Solid-liquid extraction with organic solvents in heat reflux systems as well as submerged fermentation are very often used as a method of extraction of these bioactive compounds (Martins *et al.*, 2011). However, there is a growing trend today towards the application of solid state fermentation, which has proven to be a much more efficient method in terms of yield of these compounds.

So far, it has been confirmed that the probiotic strain *B. subtilis* used, is a good producer of numerous hydrolytic enzymes, thus enriching the nutritional profile of soybean meal in terms of increased protein content. Then, the nutritional value of soybean meal was increased by decreasing trypsin inhibitor and phytic acid content, which was evident at the end of fermentation. Now, under this chapter, we examined the phenolic profile after fermentation, namely whether the fermentation influenced the intensive production of phenolic compounds. The experiments were performed with varying amounts of moisture, and the obtained results are graphically shown in Figure 5.11.

Figure 5.11. is a clear indication that the initial moisture content of the substrate is certainly an important parameter in terms of testing the optimal conditions for increasing the yield of bioactive phenolic compounds by fermentation of solid agro-industrial waste. More specifically, by increasing the

moisture content from 1:0.5 to 1:1, the content of phenolic compounds in the fermented soybean meal doubled. The increase in the content of phenolic compounds in the fermented soybean meal is clearly visible in the first 3 days of fermentation at an initial moisture content of 1:1. Amount of 949.64±45.12mg GAE/100g_{dm} is the highest content of phenolic compounds recorded on the third day of fermentation. On the fourth day of fermentation a slight decrease was observed, thus the content of phenolic compounds in soybean meal after the eighth day of fermentation was 750.23±20.1mg GAE/100g. This decrease in total phenol content after third day of fermentation is probably due to the Maillard reaction occurring between phenols and carbonyl compounds (Yang *et al.*, 2019). Furthermore, the reason for this decrease could be attributed to the microbial biodegradation of phenolic acids. Namely, some *Bacillus strains* can transform ferulic acid as well as *p*-coumaric to vanillin and p-hydroxybenzoic acid (Peng *et al.*, 2003).

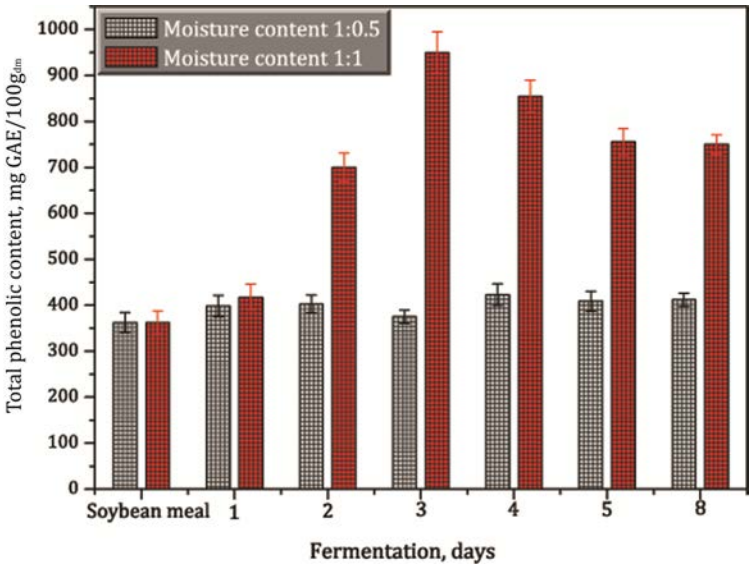

Figure 5.11. Total phenolic profile in soybean meal after fermentation with *B. subtilis.*

The lower initial moisture content of the substrate apparently had a negligible effect on the content of phenolic compounds in the fermented soybean meal. There was an insignificant increase in the content of total phenols from

362.57±21.4 to 412±14.5mgGAE/100g_{dm} after 8 days of fermentation. Consumption of foods rich in polyphenolics is important due to countless beneficial health effects. However, these compounds have to be liberated in a form of bioactive compounds and to be easy available and absorbed in order to manifest its positive effects. According to the data obtained in this study, solid state fermentation with *B. subtillis* contributed to improvement in this biomolecules content in fermented soybean meal.

Previous studies have found that microorganism such as *B. subtillis*, is extremely effective in increasing the biological capacity of soy products (Shin *et al.*, 2014). Another study reported an increase in phenolic compounds from 3.0±0.27 to 14.7±0.62 mg GAE/g, indicating that fermentation had increased bioactive capacity fivefold with respect to phenol after fermentation (Dai *et al.*, 2017). Predominant soybean varieties in the Sikkim Himalayan region, yellow and black soybeans were used to grow two *B. subtilis* strains, MTGG 5480 and MTGG 1747. Apparently, cooked and dehulled yellow and black soybean varieties have proven to be an excellent substrate for the production of *β*-glucosidase during fermentation.*β*-glucosidase are mainly responsible for the dramatic increase in free phenolic acids from 1.93 and 1.64 mg GAE/g to 8.4 and 7.5 mg GAE/g after fermentation in yellow and black soybeans, respectively (Sanjukta *et al.*, 2015).

It is firmly established that the mobilization of phenolic compounds during solid-state fermentation occurs due to the complex system of enzymes that make up α-amylase, β-glucosidase, lacasse, tannin acyl hydrolase, ellagitanin acyl hydrolase which are mainly found in different types of fungi (Martins *et al.*, 2011). Therefore, the potential of different fungi to produce phenolic compounds on soybean products has been examined in numerous studies. Thus, for example, the entire fermentation process on the defatted soybean flour of fungi *Aspegillus oryzae* and *Monascus purpureus* was optimized. Although both selected microorganisms are members of fungi, parameters such as pH, defatted soy flour:water ratio and fermentation temperature differently affect the yield of polyphenolic compounds depending on the fungus selected. Thus, for example, the content of phenolic compounds remained almost unchanged during fermentation

with the fungus *M. purpureus*. However, a different trend was observed in the sample fermented with *A. oryzae*. During the fermentation with *A. oryzae* the phenolics content gradually increased. The assumption is that *A. oryzae* has the enzymatic system able to hydrolyze complex polyphenols into simpler, biologically more active ones with a greater capability to reduce Folin's reagent (Handa *et al.*, 2019). The literature also contains data on the use of soybean meal, which is a high source of protein, as an additive for solid state fermentation of other agro-industrial wastes. Thus, for example, *Xylaria nigripes* was grown on a mixture of wheat bran and soybean meal (ratio, 1:1) and an extremely high yield of phenolic compounds, 25.4 mg GAE/g_{dm}, was observed (Divate *et al.*, 2017).

Nonetheless, a complex enzyme system responsible for the mobilization of phenolic compounds during fermentation has also been found in bacteria. *Bacillus pumilus* HY1 was used for the fermentation of traditional soybean food in Korea, *cheonggukjang* (soybean cook) and significant increase of gallic acid and flavanols was recorded, meanwhile the content of isoflavone glycosides, malonylglycosides and flavanol gallates was reduced during the fermentation. The results obtained are elucidated and assigned to bacterial esterase and *β*- glucosidase activities (Cho *et al.* 2009).

In addition to the use of soybean meal as a substrate for the cultivation of microorganisms on solid media, there is also information on the use of soybean meal in submerged fermentation (SmF). Following the selection and screening of microorganisms *B. amyloliquefaciens* SWJS22 was found to have an excellent and efficient enzyme system thermally stable over a wide temperature range (10-60°C), pH stability over a wide range of pH 5-11, and high salt tolerance and accordingly this microorganism is ideal for fermentation. Afterwards, the submerged fermentation of soymeal was performed and increase in total phenolic content during first 54h of fermentation wherefrom the declining trend in the amount of total phenols in the fermented soybean is recorded. In parallel, the activity of bacterial proteases and glucosidases was monitored and was shown to have the same pattern as the phenol content, i.e after 54 hours of fermentation the activity of proteases and β-glucosidase decreased significantly, hence the content

of phenolic compounds is directly related to the activity of these enzymes produced by *B. amyloliquefaciens* SWJS22.Over the period 12-60h of fermentation it was estimated that total phenol content increased from 3.40 to 7.11mg GAE/g_{solid} (Yang *et al.*, 2019). If we compare the results of submerged soybean fermentation (7.11 mg GAE/g) and solid state fermentation presented in our study (~9.5 mg GAE/g), it is clear that solid state fermentation is a more appropriate technique for increasing the content of phenolic compounds and obtaining the added-value soybean meal product.

5.3.4 Antioxidant capacity of SBM fermented with *B. subtilis*

Although there are data about antinutritional factors reduction and nutritive profile improvement by *B. subtilis* the more information are necessary about nutritive profile of SBM after the fermentation. The bioactivity profile in terms of DPPH scavenging and ABTS activity was followed during the SBM fermentation with *B. subtilis* under two different SBM:water ratios and the data are presented in Figure 5.12.

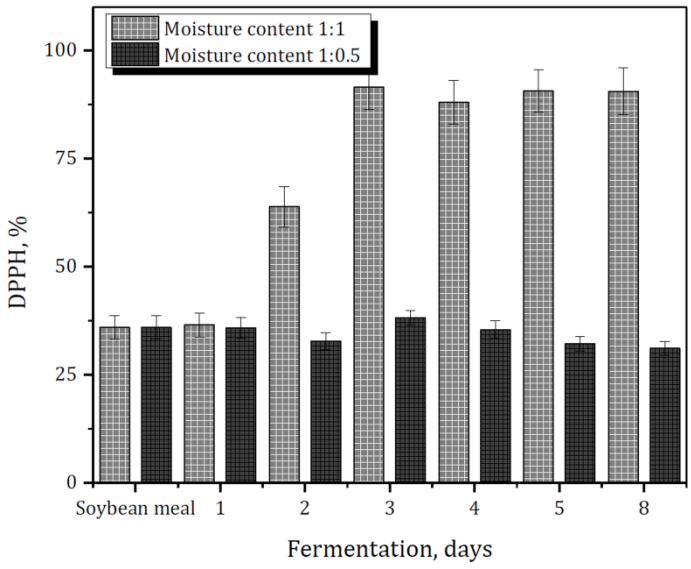

Figure 5.12. DPPH scavenging rate as a function of fermentation time for two different SBM:WATER ratios-1:1 and 1:0.5.

The DPPH scavenging ability pattern coincides completely with the phenol production pathway in soybean meal throughout the fermentation time. As can be seen from the Figure 5.12, the fermented soybean meals exhibited different anti-oxidative abilities. Increase in DPPH scavenging ability from 35.91±2.71 to 90.56±5.39% after 8 days of fermentation under the SBM:water ratio 1:1 was observed. Maximum of DPPH scavenging ability is observed on the third day of fermentation, 91.49±5.21%. The third day of fermentation was also optimum in terms of phenol production under the same moisture conditions. In this way, the literature claims that soybean meal has phenols that have antioxidant activity and can reduce free radicals is confirmed (Chun-Hua and Seong-Jung, 2016). Alongside to phenol, the antioxidant capacity of soybean meal after fermentation may also be due to the production of bioactive peptides (Chun-Hua and Seong-Jung, 2016). Significantly lower DPPH scavenging ability was observed during fermentation on soybean meal at a SBM:water ratio of 1:0.5. Specifically, DPPH scavenging ability was slightly increased after three days of fermentation 38.18±1.67%, while remaining almost unchanged on other days. This result is also consistent with the presented results of phenol production under the given conditions. The fermentation conditions in terms of moisture content significantly influences the activity of microorganism as previously determined during soybean meal fermentation with *Lactobacillus* and *Saccharomyces* (Romero *et al.*, 2004; Marazza *et al.*, 2012).

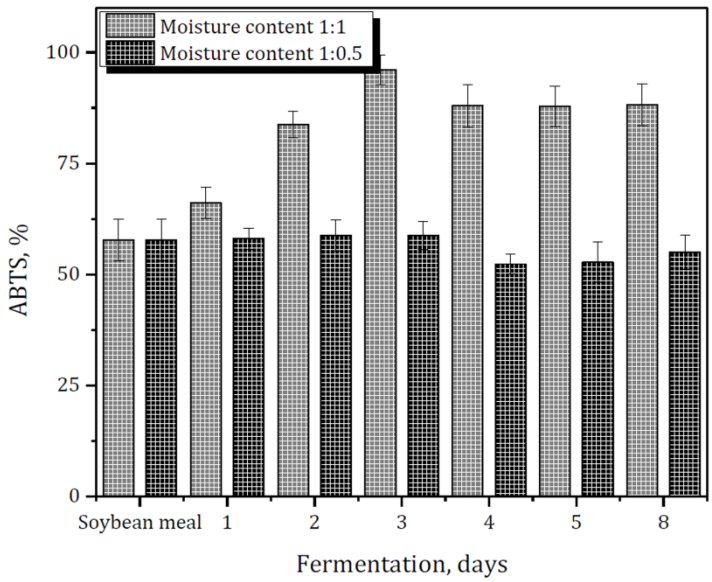

Figure 5.13. ABTS scavenging rate as a function of fermentation time for two different SBM:WATER ratios-1:1 and 1:0.5.

The antioxidant potential of the samples evaluated by ABTS radical cation scavenging ability is shown in Figure 5.13. Like the DPPH, the changing trends of ABTS scavenging ability are in agreement whit dose of total phenolic content. The ABTS cation radical increases rapidly until the third day of fermentation (57.72→ 96.11%), wherefrom there is no significant increase with the prolongation of fermentation time. On the other hand, the lower initial moisture content of the soybean meal obviously affected the activity of the microorganism. Under these conditions there is no significant change in ABTS radical cation scavenging ability. Intensive research in this area has led to the realization that antioxidant capacity is not only associated with phenol content. Specifically, it has been confirmed that the antioxidant potential is significantly influenced by the chemical structure and nature of aglycones as well as the presence of different types of conjugates (Santos *et al.*, 2018).

Deffated soybean meal was fermented using *A. oryzae*, and afterwards the obtained fermented samples were hydrolysed using proteases. It turned out that after 24h of deffated soybean meal fermentation and 34h of hydrolysis the DPPH scavenging ability in the sample was 42.95% (Zhao *et al.*, 2018). If we compare

these results with the results obtained in this study, it is clear that *B. subtilis* is a very interesting microorganism in terms of enhancing the biological profile of fermented soybean meal. Soybean *okara* was fermented using *S. cerevisiae* in order to increase the bioactivity profile of this by-product. It appears according to DPPH and ABTS values that fermentation improved bioactivity of soybean *okara*. Namely, after 72h of cultivation increase of 67 and 112% in DPPH and ABTS activities respectively was observed (Santos *et al.*, 2018). *Y. lipolytica* was exploated for the fermentation of soybean production residue, *okara*. The increase in antioxidant activity in this case is explained by several hypotheses, some of which agree with the explanations in previous research. Soy protein are considered as one of the biggest food allergens, more precisely they belong to the group of „Big 8" food allergens. These allergens cause different alergic reactions in humans as well as hypersensitivity in animals. It is well established that during solid state fermentation different types of proteases are produced. They use allergen proteins and hydrolyse them into peptides with lower molecular mass. These peptides are easily absorbed by animals, and different specific bioactivities such as antioxidative and metal-chelating are related with them (Chun-Hua and Seong-Jung, 2016).Therefore, the first hypothesis is that during fermentation some aromatic and hydrophobic aminoacids originated from soybean *okara* proteolysis, which have antioxidant activity. The second hypothesis is that during fermentation *β*-glucosidase produced by *Y. lipolytica* cleaved bonds in glycoside conjugates forming more easly absorbed aglycone forms, and thus increase in bioactivity is observed (Wong *et al.*, 2016).

Lee et. al examined the nutritional profile and fluctuations in phenol composition and antioxidant capacity during fermentation of soybean seed using *T.matsutake* mycelia. Herein, it was reported that with increase in fermentation time ($0 \rightarrow 9\ days$) the total aglycone content increased remarkably. This is of great importance given that aglycones have been found to be better absorbed and have a positive biological effect on cancer and coronary heart disease. Furthermore, it was established that antioxidant capacities are related with isoflavone profile in

fermented product, and it was also observed that the fermented soybean had a slightly higher affinity towards ABTS than the DPPHradical (Lee *et al.*, 2019).

Previous studies have shown that, during fermentation, the microorganism produces enzymes that break down the bonds between phenolic compounds and other substituents in the conjugate molecules, thereby improving the antioxidant capacity of the final product. In addition, there is a great need for testing different microorganisms, as for each of them the ability to produce specific enzymes is linked with the corresponding bioactive compounds and the biological profile of the final fermented product. *M. purpureus* and *A. oryzae* were used for the fermentation of deffated soybean meal (deffated soybean meal:water ratio, 1:1), and the bioactivity profile of fermented soy product was evaluated. The results which refer to DPPH and ABTS radical scavenging ability indicated that aglycone isoflavones content in deffated soybean meal increased during the fermentation and contributed largely to the antioxidant activity by mechanism of hydrogen donation (DPPH) (Handa *et al.*, 2019). A similar result in the combined process, ultrasound-assisted liquid state fermentation of soybean, was obtained. In this case, in addition to fermentation, the further influence of ultrasound on DPPH scavenging activity was observed. Specifically, the highest DPPH scavenging activity recorded is attributed to the increase in the number of hydrogen donors that react with the DPPH radical and form stable products, so that they can scavenge more free DPPH radicals (Ruan *et al.*, 2019).

The literature provides data related to the fermentation of soybean powder products, which are in complete contradiction with the results presented so far. Namely, during *Lentinus edodes* cultivation on soybean meal powder the total phenol profile does not follow the antioxidant capacity profile of the fermented product.Monitoring of the total phenol content showed a steady increase in the fermented soybean product. On the other hand, the antioxidant capacity of the first days of fermentation has dropped dramatically, but in the late stages of fermentation this trend reverses.The authors explain this result with the structure of soybean powder phenolics.Phenols in soybeans have been found to exist in the form of polymer matrices crosslinked with lignin, carbohydrates, cellulose,

hemicellulose and starch. Lignin is thought to have significant antioxidant capacity, so by producing fungal enzymes, these plimeric lignin-phenol complexes are broken down into smaller molecules. The decrease in biological capacity is explained by the fact that the resulting smaller molecules have a reduced antioxidant capacity compared to lignin despite the increased content of total phenols in the fermented soybean (McCue *et al.*, 2004).

6 CONCLUSION

Within the scope of this doctoral dissertation, the possibility of utilizing agro-industrial waste as a substrate for the growth of microorganisms and the production of industrially significant hydrolytic enzymes were examined. A detailed research and careful analysis of the results obtained can lead to the following conclusions:

- Using selective casein, CM-cellulose, starch and pectin enriched agar plates, the producers of the desired hydrolytic enzymes were selected from a rich collection of natural and commercial strains of the Department of Biochemical Engineering and Technology. Among all tested *Bacillus* spp. isolated from putrid proteinaceous food samples proved to be the strain with the highest potential for the production of proteases, α-amylases, cellulases and pectinases on various agro-industrial wastes. The identified and sequenced strain was named *Bacillus* sp. TMF-1,
- *Bacillus sp.* TMF-1 has great potential for enzyme proteases, α-amylases, cellulases and pectinases production on agro-industrial wastes such as: wheat bran, soybean meal, olive oil cake, sunflower meal under the different moisture content,
- The highest enzyme activities were achieved by fermentation on soybean and sunflower meal for pectinase and cellulase, while in addition to these substrates, oil cake and corn pericarp have been shown to be suitable media for the production of α-amylase and proteases,
- For α-amylase, protease, cellulase and pectinase production by *Bacillus sp.* TMF-1 soybean meal is the best choice to be used as the substrate,
- High water content is beneficial for amylase (12.31 IUg^{-1}) and cellulase (0,91 IUg^{-1}) secretion while modest water content in soybean meal favored protease production (14.63 IUg^{-1}),
- High water content promote the production of amylolytic enzymes when *Bacillus* sp. TMF-1 was cultivated on sunflower meal (20.48 IUg^{-1}), olive oil

cake (15.76 IUg^{-1}) and wheat bran (2.90 IUg^{-1}), but low moisture content stimulated the cellulases' secretion on olive oil cake (0.56 IUg^{-1}).

- Pretreatments of the agro-industrial residues enhanced the biodigestibility of the substrates by degradation of impenetrable complexes and concomitantly changing the physical characteristics of the substrates as evidenced by the increased yield of the target enzymes in the fermented medium.
- Of all the methods of pretreatment of soybean meal (18.90 IUg^{-1}), sunflower meal (24.40 IUg^{-1}) and olive oil cake (39.97 IUg^{-1}), chemical treatment proved to be the best option for α-amylase production.
- Ultrasound-assisted pretreatment (50% amplitude) is the best choice for the production of pectinase on olive oil cake where 18.57 IUg^{-1} of pectinolytic activity was recorded. On the other hand, chemical pretreatment (acid) od sunflower and soybean meal gave the pectinolytic activity of 31.53 IUg^{-1} and 13.57 IUg^{-1}, respectively. Microwave pretreatment of wheat bran resulted in pectinase activity of 59.40 IUg^{-1}.
- The highest cellulase yield is achieved on soybean meal pretreated with ultrasound (50% amplitude), 1.20 IUg^{-1}. After ultrasonic pretreatment of olive oil cake (25% amplitude) cellulolytic activity of 0.43 IUg^{-1} is recorded, while after cultivation of *B. subtillis* TMF-1, 0.33 IUg^{-1} is recorded on acid-pretreated soybean meal.
- A significant increase in protease production on soybean meal, sunflower meal and olive oil cake was observed after alkaline pretreatment of these substrates. Increase in proteolytic activity after alkaline pretreatment from 0.96→28.20 IUg^{-1} on soybean meal, from 3.22→18.10 IUg^{-1} on sunflower meal and from 3.0→15.07 IUg^{-1} on olive oil cake is estabilished. Apparently, alkaline pretreatment is a good method for corn pericarp pretreatment due to the fact that proteolytic activity of 50.51 IUg^{-1} in extract is measured.
- Among all the substrates tested and the techniques of pretreatment, it is concluded that soybean meal has the highest potential for use in animal

nutrition, and accordingly, this substrate has been further examined in terms of the effect of fermentation on substrate characteristics.

The selection of extraction technique in solid state fermentation downstreaming process proved to be extremely important. Different techniques from conventional to novel technologies such as ultrasound technology in order to recover as much as posibble of enzyme from fermented media were examined and the following conclusions can be drawn reliably:

- Examining the optimal parameters for the extraction of proteases from the pro-fermented medium by conventional extraction techniques, it was found that pH of extraction buffers, *S/L* ratio and extraction time have the greatest influence. Optimal conditions for the protease extraction are: pH 9 of the extraction buffer, low agitation and low *S/L* ratio (0.07), extraction time of 15 minutes and approximately 240 IU of proteolytic activity are recorded in the extract.
- Prolonged extraction time can have a positive effect on the extraction of α-amylase from fermented soybean meal. The highest activity of α-amylase of 820 IU was achieved under the optimized conditions: extraction time 45 minutes, stirring rate 250 rpm, solid to liquid ratio, *S/L*=0.05, and pH 7 of extraction buffer.
- Cellulase recovery is affected positively and dominantly by stirring rate without being much influenced by *S/L* ratio. Besides, decrease in cellulolytic activity from 5.04 to 4.5 IU after the extraction time prolongation from 15 to 45 minutes was observed.
- The influence of pH and sonication time on ultrasound-assisted protease extraction is evident. Ultrasound extraction increased the yield of the protease compared to conventional extraction on orbital shaker where, after 15 min, the proteolytic activity observed in the extract was ~210 IU.
- Ultrasound also proved to be a successful method for extracting the produced α-amylase. The highest recovery of α-amylase, ≥ 825 IU was obtained during 10 min of ultrasonication in buffer at pH 7.

- Increase in the exposure of the substrate to ultrasonic waves has a negative effect on cellulase extraction. Specifically, sonication of fermented soybean meal for 5 min recorded the activity of cellulase 4.5 IU.

Although protein-rich, soybean meal also has proteins that are considered undesirable in its composition, and are called anti-nutritional factors. Following the profile of the anti-nutritional factors in soybean meal during *B.subtilis* fermentation, the following conclusions can be drawn:

- The fermentation remarkably decreased the protease inhibitor content. A gradual decrease in protease inhibitor content is observed during all fermentation days in both 1:0.5 and 1:1 moisture content regimes.
- After 8 days of fermentation under fermentation conditions where the moisture content was 1:0.5 a decrease in trypsin inhibitor content from 16.01±1.3 to 10.66±0.4 mg/g_{dm} is recorded.
- In the case of 1:1 moisture content, a greater decrease was observed after 8 days of fermentation, thus a decrease to approximately 4.74±0.2 mg/g_{dm} is recorded under these conditions, indicating that in the fermented soybean meal the trypsin inhibitor content is reduced by 75%.
- After 8 days of fermentation in soybean meal, it was approximately 47% behind the initial amount of phytic acid in the system with lower moisture content (1:0.5).
- In a system with higher moisture content, the decrease of about 60% of initial phytic acid content is achieved.

The biological capacity of soybean meal after fermentation was also monitored. A detailed analysis of the results obtained can lead to the following conclusions:

- The initial moisture content of the substrate is certainly an important parameter in terms of testing the optimal conditions for increasing the yield of bioactive phenolic compounds by fermentation of solid agro-industrial waste.

- By increasing the moisture content from 1:0.5 to 1:1, the content of phenolic compounds in the fermented soybean meal doubled.
- The increase in the content of phenolic compounds to 949.64±45.12mg GAE/100g_{dm} in the fermented soybean meal is clearly visible in the first 3 days of fermentation at an initial moisture content of 1:1.
- The lower initial moisture content of the substrate apparently had a negligible effect on the content of phenolic compounds in the fermented soybean meal. The content of total phenols at the regime of lower moisture content did not change significantly, so an insignificant increase was observed during the 8 days of fermentation 362.57±21.4 to 412±14.5mg GAE/100g_{dm}.
- The fermented soybean meals exhibited different anti-oxidative abilities. Increase in DPPH scavenging ability from 35.91±2.71 to 90.56±5.39% after 8 days of fermentation under the SBM:water ratio 1:1 was observed. Maximum of DPPH scavenging ability is observed on the third day of fermentation, 91.49±5.21%.
- Significantly lower DPPH scavenging ability was observed during fermentation on soybean meal at a SBM:water ratio of 1:0.5, 38.18±1.67%,
- The ABTS cation radical increases rapidly until the third day of fermentation (57.72→ 96.11%), wherefrom there is no significant increase with the prolongation of fermentation time,
- On the other hand, the lower initial moisture content of the soybean meal obviously affected the activity of the microorganism. Under these conditions there is no significant change in ABTS radical cation scavenging ability.

After careful analysis of the results obtained in the course of this dissertation it can be concluded that raw material that is considered as waste through the application of certain biotechnological processes can be transformed into a value added material with great potential for application in animal nutrition. To achieve this it is necessary to apply a number of different steps in order to optimize the whole process such as the selection of the most favorable microorganism producer, the selection of agroindustrial waste suitable for growth, the selection of substrate

preparation methods which involves the application of different pretreatment techniques. It is especially important after production to adequately isolate the target biomolecules in as high a yield as possible, with as little impact on the environment and economy of the whole process.

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8 BIOGRAPHY

Abdalla Ali Salim, MSc, was born on April 12, 1985. In Misrata, Libya, where he finished primary and secondary school. He completed his studies at the University of Misrata (Science Faculty) in the school year 2009. He completed his master's academic studies at the Faculty of Technology and Metallurgy, Department of Biochemical Engineering and Biotechnology in 2014, defending his master's thesis entitled: *"Influence of pretreatment type on hydrolysis of protease-catalyzed protein from Bacillus licheniformis - process kinetics and mathematical modeling"*.

He enrolled in doctoral academic studies at the Faculty of Technology and Metallurgy, University of Belgrade, in the 2014/2015 school year. years at the Department of Biochemical Engineering and Biotechnology by advisor of mentor full professor Zorica Knežević-Jugović. He passed all the exams provided by the curriculum of doctoral studies with an average grade of 9.64. He speaks Arabic and English. He has completed several Microsoft Windows courses.

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