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OPTIMIZATION OF SUBMERGED FERMENTATION CONDITIONS FOR GLUTEN-DEGRADING ENZYME PRODUCTION USING *BACILLUS SUBTILIS* ISOLATE

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Abstract

In modern times wheat gluten has drawn attention to many research groups. Wheat gluten represents one of the most widely used proteins in the food industry. It is a byproduct of the starch industry and has a higher percentage of protein content compared to other plant-based protein sources. In order to help reduce the allergenicity of wheat gluten, bacterial enzymes have been proven to have beneficial results in wheat gluten treatment. In search for an extracellular peptidase producing strain we have tested *Bacillus subtilis* TMF-1 isolate, which has previously been proven to have several enzyme activities. *B. subtilis* TMF-1 isolate has a food grade status, making it safe for application in the food industry. Thus, the aim of this research was to examine the possibility of utilizing mentioned strain in terms of gluten-degrading enzyme production.

Tested strain was first streaked onto several agar plates in order to detect extracellular peptidase activity. Bacterial isolate has then been sequentially transferred to the same growth medium several times. Conditions varied for the submerged fermentation in 25 mL flasks were pH value of fermentation broth, concentration of gluten powder (0 - 10 g/L) in fermentation broth and concentration of peptone (0 - 1 g/L). Shaking flasks containing the fermentation broth with the bacterial strain were kept for 48 h at 37 ^{0}C.

The results obtained show that the isolate has the possibility of thriving in low acidic to neutral pH values of the fermentation broth. Varied gluten concentrations showed that even 1 g/L of gluten powder was sufficient for the bacterial strain to manifest extracellular proteolytic enzyme activity. Peptone concentrations were also varied, but even the minimal presence of peptone has proven beneficial for bacterial growth and proteolytic activity.

This research show that the *B. subtilis* TMF-1 isolate has proteolytic activity specific for wheat gluten as substrate and that it may be used in further research in order to utilize its enzymatic production abilities for lowering wheat gluten allergenicity.

***Key words**: Wheat gluten, Enzymatic hydrolysis, Submerged fermentation, Optimization, Enzyme production, Bacillus subtilis, Proteolytic enzyme.*

1. Introduction

The demand for gluten-free products has grown over the last years and many approaches have been studied regarding wheat gluten degradation and its allergenicity reduction and/or complete elimination. According to "*Codex Alimentarius*" gluten is defined as a "protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5M NaCl" [1]. Celiac disease is known as gluten-sensitive enteropathy and is known to be one of the most prevalent food intolerances worldwide [2], where only complete elimination of gluten products from the diet is considered by far the most effective therapy for Celiac patients. Gluten is a byproduct of the starch industry and is easily procurable. Gluten represents the major storage protein, accounting for approximately 80% of total proteins found in cereals and is rich in glutamine and proline [3]. Gluten serves as the main source of nitrogen and amino acids in the endosperm of the grain [4]. It contains protein components present as monomers or, if linked by disulphide bonds, as oligo- and polymers. Gluten proteins have been divided according to their solubility in 60% alcohol solutions to gliadins (soluble), and

glutenins (insoluble) [5]. High proline content causes the gluten proteins to be very resistant to digestion by proteolytic enzymes [3].

One of the approaches considering the wheat gluten allergenicity elimination is the utilization of microbial enzymes produced by bacteria. It is widely reported that bacterial enzymes have the ability to degrade wheat gluten peptides [4]. Several reports claim that *Bacillus* spp. has the ability to produce extracellular enzymes with gluten degrading ability. Stressler *et al.*, [6], have found that both *B. licheniformis* and *B. subtilis* isolates from insects conditioned in terraria have produced extracellular peptidases capable of wheat gluten hydrolysis up to 38%. Socha *et al.*, [7], have also investigated several bacteria (*B. licheniformis, B. stearothermophilus, B. thermoproteolyticus,* and *Streptomyces griseus*) for proteolytic activities. It was observed that *B. licheniformis* and *B. thermoproteolyticus* produced proteolytic enzymes active towards wheat gliadins. Utilization of enzyme technology ensures that aggressive chemicals and treatments are avoided and, thus, the processes are considered to be more environmentally friendly. Microorganisms present in nature are proven to be the biggest enzymes producers [8]. Proteases account for approximately 60% of the enzyme market. *B. subtilis* is known to produce alkaline proteases and those proteases have found applications in the detergent industry due to high thermostability and pH stability [9]. Extracellular proteases have commercial value and play a significant role in the enzyme industry. Hydrolytic enzymes, mainly the ones produced by microorganisms have a key role in the protein hydrolysis thus facilitating the absorption and utilization of the product of the hydrolysis. One of the advantages of using the microorganisms in enzyme production is their cultivation. Microorganisms suitable for protease production can be cultured in a short period of time in relatively large quantities [10]. Submerged fermentation is carried out in the presence of free water and is a preferred industrial technique for enzyme production due to easier handling, better parameter (temperature, pH, dissolved oxygen, foam formation) control and larger scale production, compared to the solid state fermentation [8]. In order to increase enzyme production, the selected strain needs to be provided with essential nutrients and optimal growth conditions. By applying a simple method like transferring a microorganism to a selective media the production of a specific enzyme can be induced.

Due to its versatility, side stream and by-product of the wheat starch industry has low cost and can be used in many different industries [11]. Aim of this research was to utilize the material rich in gluten proteins to produce enzyme specific to gluten by submerged fermentation, and one of the goals of this study was to examine if the selected bacterial strain can grow and produce proteolytic enzymes by having only wheat gluten proteins as the source of nutrients. Therefore, a screening test with the selected bacterial strain was done on several different plates containing wheat gluten powder in order to determine the presence of proteolytic enzymes which can lead to gluten hydrolysis. Next, the bacterial strain would be conditioned and the optimization of fermentation parameters would be conducted. The study was undertaken in order to investigate production of alkaline protease of the screened bacterial isolate and its characteristics by means of submerged fermentation.

2. Materials and Methods

2.1 Materials

Gluten from wheat (> 75% protein content), Folin-Ciocalteu reagent, bovine serum albumin (BSA) and Azo-casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soy peptone was procured from Organotechnie S.A.S (France). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) and Lach-Ner (Bratislava, Czech Republic).

2.2 Microorganism

The bacterial strain used was *Bacillus subtilis* TMF-1 isolate, which is a part of the Collection of cultures of Faculty of Technology and Metallurgy, University of Belgrade. This isolate was previously characterized in one of our previous papers [12].

2.3 Screening, cultivation of microorganism and protease production

The *B. subtilis* TMF-1 isolate was first transferred from the stock vial on to an agar plate (peptone I 1%; yeast extract 0.5%; NaCl 0.5%; agar 1.8%). The revived culture was then streaked onto 3 different agar plates and the fermentation process was carried out, as suggested by Stressler *et al.*, [6]. The formation of clear zones around the streaked culture indicated that the tested microorganism has potential proteolytic activity. After determining on which agar plate the microorganism had the biggest clear zone, the agar plate formulation was chosen for further experiments. The chosen agar plate formulation was then used to sequentially streak the microorganism. After several sequentiall inoculations the microorganism was then ready to be used for the submerged fermentation. The fermentation broth initially consisted of: 10 g/L wheat gluten, 1 g/L peptone, 0.6 g/L Na_2HPO_4, 0.5 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \times 7H_2O$, 2.4 g/L $CaCl_2 \times 2H_2O$, 1 g/L NaCl.

First, the microorganism was transferred form the selected agar plate to a flask containing 12.5 mL of LB medium, and was left on a rotary shaker (KS 4000i control, IKA, Staufen, Germany) for 24 h at 37 ^{0}C at 160 rpm. Then 0.25mL of bacterial culture was transferred to 100 mL Erlenmeyer flasks containing 25 mL of the fermentation broth and was kept for 48 h at 37 ^{0}C at 160 rpm on a rotary shaker. This was followed by centrifugation at 7000 x g for 20 minutes at 4 ^{0}C. The clear supernatant was collected and tested for protease activity.

2.4 Assay of protease activity

The proteolytic activity of the crude enzyme extract was measured using the method with azo-casein previously described by Salim *et al.*, [13]. 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. The specific enzyme activity was expressed as the enzyme activity per mg of protein. Protein concentration was measured by the method described by Lowry *et al.*, [14], using bovine serum albumin (BSA) as the standard.

2.5 Optimization of protease production

The optimization of the fermentation process parameters consisted of varying wheat gluten and peptone concentrations in the fermentation media and changing the pH values of the fermentation media. First, the change in wheat gluten concentration was examined, followed by change in peptone concentration. Then the pH values of the unchanged fermentation media formulation were examined.

2.5.1 Effect of different media concentration on protease production

In order to examine the effect of different concentrations of wheat gluten and peptone in the fermentation media on the protease activity, wheat gluten and peptone concentrations were varied, 0 - 10 g/L and 0 - 1 g/L respectively. The effects of varying concentrations of wheat gluten and peptone were examined independently. All other substance concentrations in the media formulation were kept constant throughout the experiments. pH of the fermentation media was set to pH 7. This value was also kept constant unless stated otherwise.

2.5.2 Effect of pH on protease production and activity

Next, the effect of pH value of the fermentation media on protease production was examined. The formulation of the fermentation media kept as originally written and was unchanged unless stated otherwise. This was done in order to examine the optimal pH value in which the microorganism has the best protease production ability.

2.6 Statistical analysis

All experiments were done in triplicate and results were expressed as mean ± standard deviation. The results were compared by one-way analysis of variance ANOVA (OriginPro 9.0 software, OriginLab Corporation, Northampton, MA, USA). Turkey test was applied for comparing differences between mean values at 95% confidence level ($p < 0.05$).

3. Results and Discussion

3.1 Screening and cultivation of microorganism

B. subtilis TMF-1 isolate was streaked onto three different agar plates, which had different formulations. The best results have been achieved on the agar plate consisting of 10 g/L wheat gluten and 15 g/L agar. The plate described is shown in Figure 1.

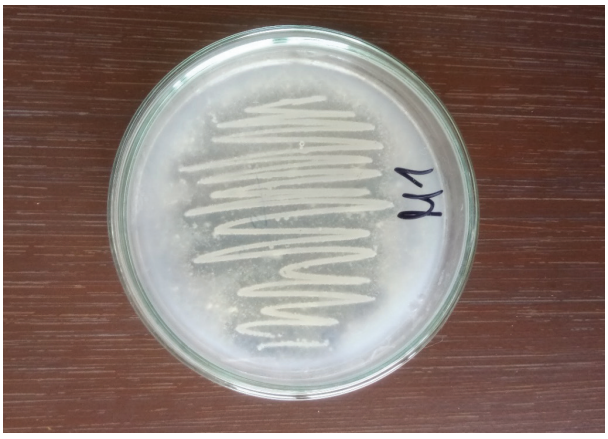

Figure 1. *B. subtilis* TMF-1 growth on plate consisting of 10 g/L wheat gluten and 15 g/L agar.

It can be observed that our isolate exhibited excellent growth just like another *B. subtilis* isolate for the same plate formulation as reported by Stressler *et al.*, [6]. Compared to the plates containing gluten and salts or gluten and glucose, the plate with only wheat gluten has proved to be a better solution. This may be due to the fact that our isolate produces gluten specific proteases. After inoculation of the LB media, the flasks were left for 24 h. This inoculation was done in duplicate. Then, 0.25 mL of the culture from the LB media was transferred to a flask with 25 mL of fermentation media, whose formulation varied as previously described. The results of these measurements are presented in the upcoming discussion.

3.2 Effect of different media on protease production

B. subtilis TMF-1 has previously shown that it is able to grow in a medium consisting only out of wheat gluten and agar. The clear zone formation indicated that protease activity was present. The culture was cultivated in LB for 24 h and then transferred to several flasks containing 0 - 10 g/L of wheat gluten, while all other component concentrations were kept constant. The pH value was then set to 7 and the flasks were left for 48 h on a rotary shaker. After 48 h the supernatants were collected and were considered as crude enzyme solutions. These crude enzyme solutions were tested for protease activity and protein content was determined. The results are shown in Table 1.

Table 1. Protease activity (U/mL) and specific activity (U/mg) of crude enzyme solutions after 48 h of incubation at 37 °C with varying wheat gluten concentration (0 - 10 g/L)

Wheat gluten, g/L	Protease activity, U/mL	Specific activity, U/mg
10	2.58 ± 0.040 ^{a}	0.85 ± 0.024 ^{a}
5	2.51 ± 0.180 ^{a}	1.25 ± 0.009 ^{a}
1.0	1.22 ± 0.126 ^{b}	1.37 ± 0.129 ^{a}
0.50	0.84 ± 0.333 ^{bc}	1.02 ± 0.385 ^{a}
0.25	0.51 ± 0.239 ^{c}	0.75 ± 0.352 ^{a}
0	0.58 ± 0.219 ^{c}	0.96 ± 0.389 ^{a}

Results are expressed as means ± standard deviation (n = 3). Means with different letters in the same column are significantly different ($p < 0.05$).

The formulation with wheat gluten concentration of 10 g/L showed the highest protease activity of 2.58 U/mL. It is also interesting to point out the presence of proteolytic enzymes in the crude enzyme solutions from the fermentation media formulations having none or minimal wheat gluten content. Wheat gluten concentrations of 0, 0.25 and 0.5 g/L showed 0.58, 0.51 and 0.84 U/mL, respectively, while the peptone concentration remained 1 g/L. Another independent set of experiments was carried out, where the wheat gluten concentration was kept constant at 10 g/L and peptone concentration was varied 0-1 g/L. The results are presented in Table 2.

Table 2. Protease activity (U/mL) and Specific activity (U/mg) of crude enzyme solutions after 48 h of incubation at 37 °C with varying peptone concentration (0 - 1 g/L)

Peptone, g/L	Protease activity, U/mL	Specific activity, U/mg
1	3.02 ± 0.090 ^{a}	0.94 ± 0.087 ^{a}
0.75	3.03 ± 0.134 ^{a}	0.98 ± 0.059 ^{a}
0.50	2.78 ± 0.060 ^{a}	0.90 ± 0.100 ^{a}
0.25	2.73 ± 0.137 ^{a}	0.88 ± 0.042 ^{a}
0	2.26 ± 0.120 ^{b}	0.73 ± 0.117 ^{a}

Results are expressed as means ± standard deviation (n = 3). Means with different letters in the same column are significantly different ($p < 0.05$).

The total protease activity measured was 3.02 U/mL and 3.03 U/mL for peptone concentrations of 1 g/L and 0.75 g/L, respectively.

The constant presence of peptone at 1 g/L throughout the whole experimental set has proven to result in higher specific activity values compared to the experimental set where the amount of peptone added varied. From the values obtained it can be concluded that 5 - 10 g/L of wheat gluten powder is sufficient for the microorganism to manifest higher protease activity. Very low protease activities were detected in the crude extracts with 0 - 0.5 g/L of wheat gluten, which is an indicator that the strain has used up all of the available carbon and nitrogen sources for its growth during the 48 h fermentation. It is interesting to point out that the highest specific activity achieved of 1.37 U/mg was in the formulation containing only 1 g/L of wheat gluten powder.

Next, the effect of varying peptone concentrations on the total protease activity showed that even minimal concentration of 0.25 g/L had beneficial effect on the protease activity. Although resulting in lower specific activity compared to the other results obtained in this experimental set, it is almost equal to the one achieved with 10 g/L wheat gluten powder and 1 g/L peptone powder. This suggests that this minimal concentration of peptone powder is beneficial to achieve satisfying results. Therefore 5 - 10 g/L of wheat gluten and 0.25 g/L of peptone powder have proven sufficient to obtain protease activities with *B. subtilis* TMF-1. This reduction of the amount of peptone added has its benefits by means of lowering the overall peptone consumption.

3.3 Effect of pH on protease production and activity

The effect of pH of the fermentation media was examined by varying the pH values from 5 to 9 with an increment of 1. This was done to investigate the pH region where the bacterial strain would show highest protease activity. The experimental set was carried out as previously described, and wheat gluten value was kept at 10 g/L and peptone at 1 g/L (Figure 2).

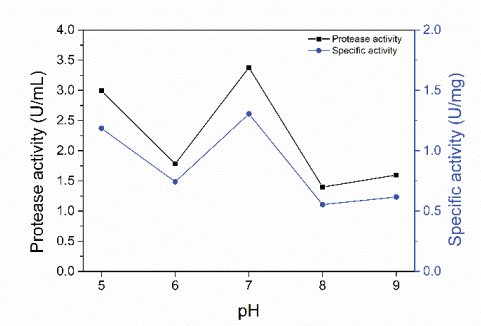

Figure 2. Protease activity (U/mL) and specific activity (U/mg) of crude enzyme solutions after 48 h of incubation at 37 °C with varying pH values (5 - 9)

The highest total protease activity of 3.37 U/mL was recorded at pH 7, followed by 2.99 U/mL at pH 5. Highest specific activity of 1.31 U/mg was recorded also for pH 7. All of the values recorded were higher than those previously measured. Specific activities recorded for all pH values were also higher than those previously recorded. pH values ranging from 5 to 7 have better effect on protease production compared to those with higher pH values. It can be concluded that the extracellular enzymes secreted by the microorganism have better access to the peptide fractions in lower pH compared to those in higher pH regions. Specific activity shows the same trend as total proteolytic activity.

Next, protease activity and specific activity were additionally tested (Figure 3 and Figure 4).

Figure 3. Protease activity (U/mL) of crude enzyme solutions after 48 h of incubation at 37 ºC with fermentation media containing 10 g/L wheat gluten or 10 g/L wheat gluten and 0.25 g/L peptone, at pH 6 and pH 7. Results are expressed as means ± standard deviation (n = 3). Means with different letters in the same column are significantly different (p < 0.05)

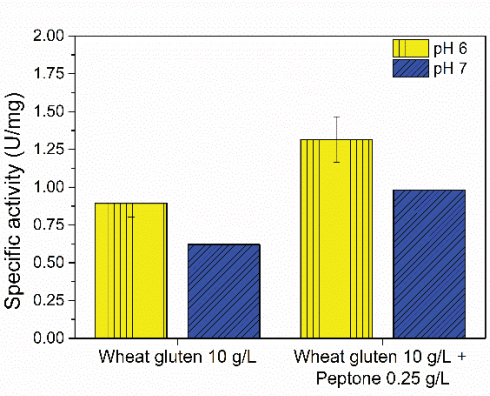

Figure 4. Specific activity (U/mg) of crude enzyme solutions after 48 h of incubation at 37 ºC with fermentation media containing 10 g/L wheat gluten or 10 g/L wheat gluten and 0.25 g/L peptone, at pH 6 and pH 7. Results are expressed as means ± standard deviation (n = 3). Means with different letters in the same column are significantly different (p < 0.05)

Two fermentation media formulations were prepared, one containing 10 g/L wheat gluten and no peptone while the other contained 10 g/L wheat gluten and 0.25 g/L peptone, while all other component concentrations were kept constant as previously described. Both media were set to pH 6 and pH 7 and the 24 h old bacterial strain was transferred and left on a rotary shaker for 48 h at 37 ^{0}C. The aim was to confirm previous findings which suggested that minimal presence of peptone had positive effect on extracellular protease production. At the same time, two pH values were examined in order to more closely determine the pH optimum of the fermentation. As shown in Figure 3 and Figure 4 the addition of 0.25 g/L peptone to the fermentation media has indeed proven beneficial for the enzyme production. In Figure 3 it is shown that the protease activity at pH 6 was higher than at pH 7. At pH 6 the protease activity recorded was 2.75 U/mL (with 10 g/L wheat gluten) and 3.90 U/mL (with 10 g/L wheat gluten and 0.25 g/L peptone). Specific activities for the same experimental set are shown in Figure 4. Specific activity of 1.31 U/mg (with 10 g/L wheat gluten and 0.25 g/L peptone) was recorded at pH 6. This recorded activity was 47.2% higher compared to the system containing no peptone. This implies that even the minimal presence of peptone ensures better protease activity. Also, it can be concluded that the bacterial strain has better extracellular protease production and shows higher protease activities when the fermentation is conducted in a mild acidic pH region. Future research will be aimed at more in depth examination of the pH region of the production, as at the time of writing this paper it was not yet examined.

According to literature data available, the addition of peptone in different concentrations affects the enzyme production in several ways. It has been reported that the addition of more peptone does not always result in increased enzyme activity [15]. On the contrary, peptone additions little above the minimum requirement can benefit the protease activity, whereas too much of peptone added decreases the protease activity [16]. Our result is in accordance with this finding.

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4. Conclusions

The aforementioned experiments have resulted in several conclusions:

- 5-10 g/L of gluten powder is sufficient to obtain protease activity, 0.25 g/L of peptone powder as supplementary carbon and nitrogen source is enough for the microorganism to produce extracellular proteolytic enzymes and the pH region for protease production, specific for wheat gluten as the main nutritional source, is in the mild acidic region, at pH 6.

- More experiments are needed to further investigate the synergistic effect of these conclusions. Bacterial isolate *B. subtilis* TMF-1 has the ability to grow in a medium with minimal presence of wheat gluten and peptone powders.

- *B. subtilis* TMF-1 isolate has manifested proteolytic activity specific for wheat gluten as the main substrate and it may be used in further research in order to utilize its enzymatic production abilities for lowering wheat gluten allergenicity. This once again proves the versatility of our isolate and its potential application in the food industry.

5. References

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