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Designing, Developing and Optimizing a Two-Stage Solid State Fermentation Process through valorization of Cereal Milling and Legume By-Products and Waste Streams for the Production of Novel Functional Protein Rich Type-Miso Nutri-Powder, Using *Aspergillus Oryzae*

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ABSTRACT

Cereal milling by-products such as wheat, corn and mavragani mixed either with chickpea or peas from third sorting (rejected from legume production line), evaluated as feedstocks to produce 'miso like powders' with high protein content *via* solid state fermentation strategies. The above by-product streams gathered from Greece and most of them from Aegean islands. Development and optimization of a novel designed process took place aiming to maximize crude protein concentration at the end-product. The effect of double stage food bioprocessing scheme studied and evaluated on its effect on the final protein content of the novel produced Greek type mixed miso like product. The effect of

1) Different temperatures (28°C, 35°C, 45°C);

2) Salt concentration (15% w/v, 18% w/v and 21% w/v) at the end- product,

3) The synthesis of the fermentation feedstock (cereal milling byproducts mixed either with rejected chickpeas or peas of legume processing line) as well as

4) Proteolytic activity to different fermentation feedstocks, studied targeting maximization of the protein content of the novel Greek type Miso powder product. The highest crude protein content of 16,95±0,84 g/100g and 17,64 ± 0,1 g/100g, achieved after 60 and 30 days of maturation at 28°C and 35°C of Greek miso like products, respectively, using *Aspergillus oryzae* 0.05 % (w/w), as microbial strain for koji preparation. The media used to produce the protein-rich Greek type miso powder, *via* the innovative developed and optimized solid state fermentation strategy designed and optimized, contained 25g/L (at a ratio 2:1:1 mavragani, wheat and corn) cereal milling by-products and waste streams mixed with 25g/L chickpeas of third sorting and salt concentration of 15% (w/v), at the end-product.

Introduction

Traditional koji is cooked wheat and/or soybean that has been inoculated with a fermentation culture or koji mold. The first step in formulating bioprocessed foods such as soy sauce, miso, sake, and others are to create the koji. During koji process the mold *Aspergillus oryzae*, produces amylases and proteases to hydrolyze or digest carbohydrates and proteins in wheat and soybean, but also other enzymes such as lipases and phytases in lower quantities and activities [1-5]. *Aspergillus oryzae* is a filamentous fungus, which could possibly secrete large amounts of a vast area of hydrolytic enzymes, under the proper cultivation conditions. It is widely used in the manufacture of traditional fermented soy sauce in Asia [6]. Taking into account the Paris Climate Accord as well as the United Nations Sustainable Development Goals [7], indicating the need of achieving multiple goals (such as zero waste and zero disposal, good health and well-being, good manufacturing processes at all stages, food for all, responsible human consumption of nutritional foods) in food production system towards the production of novel hygiene nutritional foods, (that are not only socially and financially but also land, air and water resources sustainable), exploitating alternative sources, apart from meat, such as negative valued agroindustrial by-product and waste streams derived from food sector [8-13], toward the production of protein-rich products, developing a novel strategy of solid fermentation (three phase complicated system (solid, air, water)), using *Aspergillus oryzae* (aiming to design a strategy leading to the formulation of novel miso like type powder using legumes and cereal by-product and waste streams as media), is of high academic and scientific interest.

To best of our knowledge, there are no existing publications studying and evaluating the potential usage of cereal milling by-product streams, from Greek Aegean islands (local varieties: mavragani, corn and wheat) as well as rejected legumes of food processing (chickpeas and/or peas) as media for the development of an efficient solid state bioprocessing scheme that could lead to the production of a novel type of Greek type miso, functional protein rich product. It has been noted, that red meat consumption, regarding diet, should follow guidelines owning to possible negative effects in health, associated with cardiovascular disease risks. For that reason, the World Cancer Research Fund 2007 [14], recommended that meat intake should be limited from 80g/d (the previous threshold of WCRF) to 71g/d per week, while the consumption of processed meat should be completely avoided (WCRF, 2007). Several studies using case control, cross sectional studies and cohort studies have positively correlated red meat consumption servings with cardiovascular disease (including coronary heart disease, myocardial infarction, and stroke) but still there are many contradictory data, owning to the complexity of meals containing meat [15,16]. Undisputable, red meat (beef, lamp, pork and veal) in general is a nutritional food group, especially in developed countries, since it is rich in macro and micronutrients, such as protein as well as iron, zinc and cobalamin (B12). Indeed, consumption of red meat in Western countries is greatly affected by parameters such as livestock production, socioeconomic consumer status, attitude of living and others such as sex, age, religion, body mass index and total energy intake [17].

Reported data demonstrate positive correlations regarding the portions of animal protein in diet and CHD mortality in different countries, suggesting that the quantity as well as the type of protein play a significant role in disease etiology, while highlighting the possible positive effects of isoenergetic substitution of some daily meat protein intake with plant-based protein [18]. Healthy diets and sustainable food production (Agenda, 2030), recommend higher consumption of plant-based foods than the isoenergetic animal based counterparts. Päivärinta, et al. [19] carried out a 12-week randomized clinical intervention comprised 107 women and 29 men (20-69 years), studying the effects of dietary animal proteins partial replacement with plant-based counterparts on energy yielding nutrients intake, fiber and plasma lipoproteins. In that study, it was reported that flexitarian diets could possibly offer a healthier and more sustainable dietary pattern than the predominantly animal-based diets, since polyunsaturated fatty acid and fiber intake was higher in the plant-based protein groups compared to the protein animal originated groups tested. Also, total and LDL cholesterol were lower in the plant than in the animal group ($p = 0.003$ for both). So, the designing, developing and optimization of a novel solid state fermentation process toward the production of novel Greek type miso using cereal milling by-products such as wheat, corn and mavragani mixed either with chickpea or peas from third sorting, derived from Aegean islands, (including Lemnos), despite its novelty regarding the technological innovation could possibly serve humans forming a sustainable protein rich food product that could possibly substitute some of the daily or weekly portions of meat consumption. Traditional koji fermentation process could be considered as a kind of solid-state fermentation system (SSF), [2-4], if properly designed, developed, employed and optimized. SSF is characterized as a bioprocess carried out on a solid medium with low moisture content (aw). Solid state fermentation, in fact mimics the natural habitat of microorganisms such as filamentous fungi. If SSF compared to other strategies such as liquid bioprocessing regimes, demand less sterilization energy (because of lower water activity) and is less susceptible to bacterial contamination.

Furthermore, it enables higher enzymatic productivity for many enzymes and offers several environmental advantages, since solid agro-industrial by-product streams could be used as substrate and/or energy source in their natural form, facilitating both solid waste management and minimization of wastewater production [20], while forming raw materials rich in bioactive compounds that could be used as nutrient supplements for nutritional and sustainable food production [8,9,13,21]. There are many factors affecting miso production process, such as the effect of different temperature, salt concentration as well as the fermentation feedstock. These parameters were studied in that study, targeting maximization of crude protein concentration of the end Greek Miso like products. The aim of this study was to evaluate the potential usage of local varieties of Greek agroindustrial by-product streams (the vast majority of them produced in Limnos island and in Northern Aegean region) derived from cereal milling by-products such as wheat, corn and "mavragani" mixed either with chickpea or peas (rejected from legume production line) as feedstocks for the production of 'Greek miso like powders' (like-Asian functional products), with high protein content, *via* designing and applying (at laboratory scale) a complex innovative solid state fermentation strategy, using *A. oryzae*, as a strain. Optimization of the whole solid-state bioprocessing took place aiming to maximize crude protein concentration of the end product, the novel functional Greek like-type miso powder.

Materials and Methods

Microorganism and Raw Materials

A. oryzae, were subcultured and kept in Nalgene® Cryogenic Vials (Sigma-Aldrich) of 2mL and stored in -80 °C for further use, as it has been previously described by Dimou, et al. [2]. The fungal strain maintained in slopes containing 25 g/L cereal milling by-products (at a ratio of 2:1:1), 25 g/L chickpeas and 20 g/L agar. More specifically, by-product and waste streams, used as media for strain cultivation, derived from small scale industrial agro-food flour processing sector, of Greek Aegean islands. The well-known varieties used to produce cereal by-product and waste streams derived from the well-known varieties of "Mavragani" (*Triticum durum s*), common wheat (*Triticum aestivum L*) as well as dried corn kernels (Zea Mays). Also, chickpeas (*Cicer arietinum*) of third sorting used to form the bioprocessing media. The strains growth inspected regularly to avoid contamination. The raw materials provided by Salamousas Agrifood (Lemnos-island, Greece) as well as small legume and cereal producers of Greece Aegean islands.

Chemicals

All chemicals used were of analytical grade and purchased from Sigma Aldrich. Novel two- step bioprocessing scheme (solid-state fermentation) for the production of protein rich Greek miso like products The novel-type of Greek like miso powders produced in that study was the outcome of the development and optimization of a novel two bioprocessing step. The first step included the preparation of koji-like Greek substrate to form a type of enzymatically degraded koji like powder. A oryzae novel solid-state fermentation led to the production of proteases since it was capable of producing conidiophores and dark-green brownish colored spores. Preliminary RT- PCR analysis showed that *Aspergillus oryzae* used to this study [2-4]. did not contained the aflatoxin biosynthesis gene cluster of A. flavus (aflR gene did not expressed), verifying previous research in this domain and characterization of *A. oryzae* koji strain, as GRAS (FDA 2022). At the first stage of novel solid state fermentation strategy developed and optimized 50g (at a ratio 2:1:1 mavragani, wheat and corn by-product streams) of brans derived from cereal milling byproduct streams were mixed in a blender and then autoclaved at 250°F for 30 min. Also, 50g of peas or 50g of chickpeas derived from legume processing of Northern Aegean (Salamousas AE, Lemnos island) after being autoclaved, were soaked in 2L of water for 1h, and mixed (separately) using a kitchen blender and autoclaved at 121°C for 40 min. The raw materials were left to cool down at room temperature. The final solid medium containing a total of 100g of solid material (50% cereal milling by-products, 50% peas of third sorting or 50% chickpeas of third sorting) inoculated with 0.05 % (w/w) *A. oryzae* spores [2]and incubated for 48h at 180rpm and 28°C (aerobic conditions). The internal temperature of the flasks containing the fermented solids was below 40°C. The preparation of this Greek koji like substrate finished when the culture turned down to brownish green-brown.

Then, the second stage of the solid fermentation process followed including the maturation of the novel-nutritional-Greek like protein rich miso like food product. 3 sets of experiments took place aiming at evaluating Greek agroindustrial by-product streams (the vast majority of them produced in Limnos island and in Northern Aegean region) derived from cereal milling by- products: wheat, corn and mavragani mixed either with chickpea or peas from third sorting (rejected from legume production line) as feedstocks for the production of 'miso like powders' (Asian functional products) with high protein content via solid state fermentation strategies. Optimization of the process took place evaluating the effect of different temperature, salt concentration and the synthesis of initial fermentation feedstock, aiming to maximize crude protein concentration of the end Greek-Miso like powder. The sets of experiments that took place are bellow described: 1st set: 100g of fermented mash (1st stage) containing either chickpeas or peas of the third sorting and cereal milling by-product streams were mixed with water so as to achieve final 20% humidity of the medium. The brine solution was free of salt. The mixture was left at 4°C, the initial 10 days of solid-state bioprocessing regime. Then the miso product mixed with salt solution, so as to achieve a final concentration at the end product of 15% w/v in NaCl (2M) and the temperature increased to 28°C, until the 60th day of the fermentation. (Miso Product type 1-MPt1); 2nd set: 100g of fermented mash containing either chickpeas and peas of the third sorting and cereal milling by-product streams (50:50, % w/w on db) were mixed with a salt solution, so as to achieve a final concentration at the end product of 18% w/v in NaCl. The mixture was left at 4°C, the initial 10 days of bioprocessing and then temperature increased to 28°C, until the 60th day of the bioprocessing scheme (Miso Product type 2-MPt2); 3rd set: 50g of fermented mash containing either chickpeas or peas of the third sorting and cereal milling by-product streams (50:50, % w/w on db) were mixed with a brine solution, so as to achieve a final concentration at the end product of 21 % w/w.

The mixture was maintained at 28°C, the initial 10 days of bioprocessing and then temperature maintained at the same temperature until the 60th day of the fermentation (Miso Product type 3-MPt3). These experiments took place for 30th and 60th days, as described while fermented mass was stirred twice a day. Samples were taken at random intervals. All samples were double filtered and stored at 4°C until further analyses. Experiments took place in triplicate. The highest protein content, as it can be seen in Results and Discussion, was measured to MPt1, containing cereal milling byproduct streams and chickpeas. For that reason, within the frame of optimizing solid state fermentation process, three sets of experiments (in triplicate) took place evaluating the effect of temperature on crude protein concentration of "Miso Product type-1 (chickpea based)". More specifically, 50g of fermented mash (step-1) containing chickpeas of the third sorting and cereal milling by-product streams were mixed with water so as to achieve a 15% final humidity of the medium. The brine solution was free of salt. The mixture was left at 4°C, the initial 10 days of bioprocessing. Then the miso product was mixed with salt solution so as to achieve a final concentration of 15% w/v in NaCl and the temperature increased to 28°C, 35°C, 45°C and 55°C until the 60th day of the fermentation

Analytical Methods

Protease Activity

The protease activity was assayed as previously described by Dimou, et al. [2] with slight modifications. 1g of fermented mass was diluted with a neutral buffer (0,1 M Na_2HPO_4, NaH_2PO_4, pH 7,2) or acidic buffer (0,05 M lactic acid-sodium lactate, pH 3,0), depending on the target protease. Casein (2%, w/v) mixed with 20,0 mL of 0,1M NaOH prepared in the 0,1 M neutral buffer or mixed with a few drops of concentrated lactic acid prepared in the 0.05 M acidic buffer, depending on the targeted protease at the corresponding pH. A mixture of 1,0 mL of casein solution and 1,0 mL of diluted sample incubated at 40°C for 10 min. The reaction was terminated through the addition of 2,0 mL of 0,4 M trichloroacetic acid (TCA). 1,0 mL of the filtered supernatant mixed with of 0,4 M sodium bicarbonate, before the addition of 1,0 mL of 0,4 M Folin–Ciocalteu phenol reagent (1:3 diluted in water) and incubated at 40 °C for 20 min. The absorbance at 660 nm was measured using ultraviolet spectrophotometer (Giorgio Bormac UV 10plus, Italy)). One unit (U) of protease activity was defined as the amount of enzyme that releases 1μg of tyrosine in 1ml of reaction, per minute under the assay conditions. Results were expressed as U/g of dry mass.

Protein Content

Total protein content of miso products of estimated using a nitrogen analyzer (Kjeldahl protein analyzer). Total nitrogen estimated by Kjeldahl method [22]. Nitrogen was multiplied by a factor so as to estimate total protein content. Briefly, 0.5g of grounded miso- like samples passed through 20 mm sieve. Samples transferred to a 500 mL Kjeldahl flask and 20 mL of conc. H_2SO_4 added. Mixtures left to stand for 20 minutes. Then, 5g of $Na_2S_2O_3.5H_2O$ added and and left to stand for 20 minutes. Subsequently, the catalyst mixture was added, Then, Kjeldahl flask was slowly heated until frothing and then intensively heated. The digestion further took place for 30 min. The flask was cooled down and 150 mL of water added. 130 mL of 40% w/v NaOH solution added along the flask sides as well as a few glass beads and two drops of mineral oil and immediately connected to the distillation unit. Ammonia was collected in 25mL boric acid solution containing mixed indicator. Distillation continued till 130 mL of collection of distillates. Collected ammonia was titrated against 0.1N sulphuric acid. A blank was run simultaneously with a piece of paper and other reagents excluding the sample. Percent N in the sample calculated by using following formula: Nitrogen % = ((1.4 x N x V)/W) x 100, Protein % = Nitrogen % x 6.25 where, N = Normality of H_2SO_4, V = Titre value of sample - Titre value of blank, W = Weight of the sample.

Results and Discussion

Protease Activity

Soy sauce is a liquid seasoning obtained primarily by the hydrolysis of soy proteins into peptides and free amino acids. In traditional miso soy-based products protease activity is of high significance. The primary role of koji mold for traditional soy sauce making is to break down soy proteins, so as to add umami taste to the final product. For that reason, the koji mold should have higher protease activities than amylase activities, since that balance is a key factor of quality of the final fermented product [23,24]. Indeed, in this research study, protease activity was measured to miso

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products developed through the optimization of a two-stage solid state fermentation scheme developed using cereal processing by-product streams mixed either with peas or chickpeas, since protease activity plays crucial role for the release of peptides and free aminoacids [2,8,9]. In preliminary results, it was shown that in all cases protease activities was 2 to 2,2-fold higher than amylase activities, verifying that *A. oryzae* was suitable for the production of quality protein rich Greek type miso products. According to results, Miso Product type 1, 2 and 3 neutral protease activities were higher than acidic protease, at the same time regarding the same type of fermented Greek miso like product, as it can be seen in Figure 1 and Figure 2. During the second step of maturation of Greek miso products neutral and acidic proteases plays a crucial role owning to the slightly acidic or neutral pH of the bioprocessing system. Observing Figure 1 and Figure 2 is obvious that different conditions, such as different temperature, salt concentration, the initial fermentation feedstock highly affected protease activity. The highest protease activity observed in Greek miso chickpea based like products of all types compared with Greek miso pea based like-products and more specifically in Greek Miso, chickpea based like Product Type-1 (Figure 1).

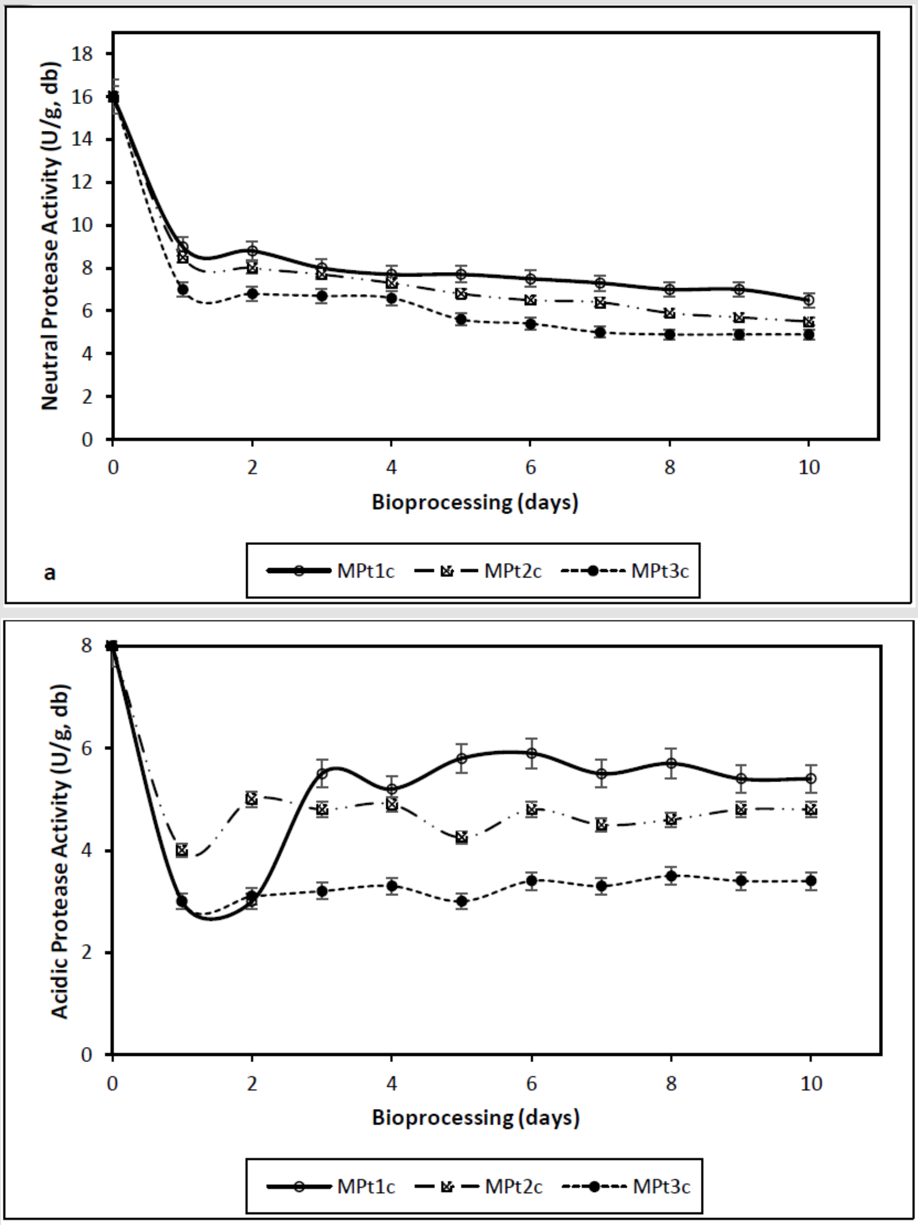

Figure 1: Neutral protease activity (a) and acidic protease activity (b) in Greek Miso, chickpea based like Products Type 1, Type 2 and Type 3 (MPt1c, MPt2c, MPt3c)*.

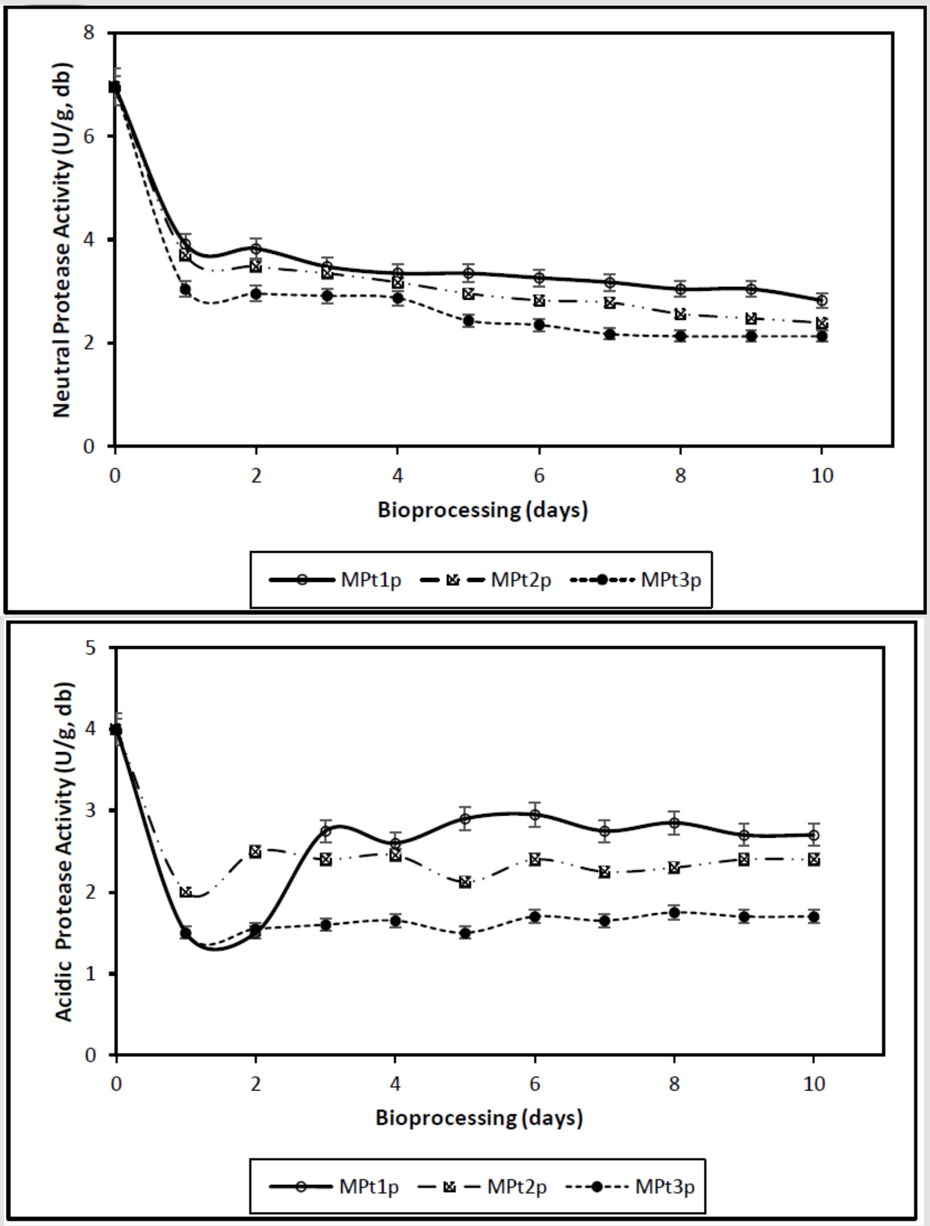

Figure 2: Neutral protease activity (a) and acidic protease activity (b) in Greek Miso, pea based like Products Type 1, Type 2 and Type 3 (MPt1c, MPt2c, MPt3c)*.

*The results are expressed as mean values of three replicates ± standard deviation

Crude Protein of Novel Greek Like Miso products

Su, et al. [25] stated that protein content is a crucial parameter regarding the quality of the end products. As shown in Table 1, the crude protein nitrogen content increased in higher rhythm the first 10 days of bioprocessing, while the rhythm decreased until the 60th day of different processed miso products. The higher crude protein content measured in miso product type-1 (chickpea based) at the 60th day and the crude protein content was equal to1 16,95±0,84 (g/100g) of Mpt1. Samples taken after the 60th day of bioprocessing revealed no further increase in crude protein content. Aiming at optimizing the final crude protein content in Greek miso like products the effect of temperature studied, as it is described to materials and methods. More specifically, three sets of experiments carried out to Miso product type-1, at different temperatures of 35°C, 45°C and 55°C for 60 days. The highest crude protein content achieved to Miso like product type-1 (chickpea based) (as it can be seen in Table 1) but at maturation stage temperature of 35°C (Table 2) and not 28°C (Table 1).

Table 1: Crude protein content*.

Crude protein content	0th day	10th day	30th day	60th day
Miso product type-1 chickpea based Crude protein (g/100g)	12,02±0,12	14,76±0,32	15,75±0,18	16,95±0,84
Miso product type-2 chickpea based (g/100g)	11,17±0,03	13,45±0,45	14,21±0,16	15,61±0,01
Miso product type-3 Chickpea based (g/100g)	11,06 ±0,22	13,45±0,11	14,35±0,45	15,72±0,07

Note: *The results are expressed as mean values of three replicates ± standard deviation

Table 2: Total nitrogen % in miso like chickpea-based products.

Total nitrogen (g/100g)	0 day	10th day	30th day	60th day
Miso product type 1-_35oC	1,58±0,32	2,34 ±0,32	2,75 ±0,07	2,81 ±0,06
Miso product type-1_45oC	1,44±0,37	1,73 ±0,11	1,96±0,04	1,96 ±0,24
Miso product type 1-55oC	1,34±0,21	1,71 ±0,37	1,71 ±0,21	1,71 ±0,06

Note: *The results are expressed as mean values of three replicates ± standard deviation.

More specifically, 50g of fermented mash (step-1) containing chickpeas of the third sorting and cereal milling by-product streams were mixed with water so as to achieve a final humidity of 15%, at the end of the optimized double staged solid state fermentation and maturation, bioprocessing. The brine solution used was free of salt. The mixture left at 4°C, the initial 10 days of bioprocessing. Then the miso product was mixed with salt solution so as to achieve a final concentration at the end product of 15% w/w (in NaCl) and then temperature increased to 35°C, 45°C and 55 until the 60th °C day of miso maturation. The highest total nitrogen percent measured the 30th day of maturation at 35°C. Comparing results of Tables 1 & 2, it seems that increasing the maturation temperature from 28°C to 35°C, did not affect the maximum crude protein production but shortened the bioprocessing time regarding total maximum crude protein measured. Higher maturation temperatures led to lower crude protein production possible attributed to reactions such as Maillard. The average crude protein content of miso products is approximately 11,8 g/100g [26]. In this research higher final crude protein production achieved using only agro-industrial by- products produced in Aegean islands. These findings are very interesting, since to the best of our knowledge this is the first time that such an investigation is carried out in Greece, developing, and optimizing a solid fermentation strategy in a double processing stage by *A.oryzae*, leading to the production of a nutritionally protein novel like miso powders using cereal milling by-product streams and legumes that have been rejected from production lines as media.

Conclusion

Production of protein rich products, such as miso like bioprocessed functional foods, via solid state fermentation using wheat milling by-product streams originated from Aegean is of high academic research regarding the fact that a new product has been generated through the development and optimization a process in a cost-efficient procedure (solid state fermentation). Setting and designing processes that require low energy consumption coupled with valorization of wheat milling by-product streams and legume processing by-product streams into a final product that would resemble Asian's miso is of high scientific, societal and industrial interest. These "newly formatted product" derived from bioprocessing technology would be a functional food, itself, containing significant amounts of protein along with other bioactive compounds, highlighting their nutritional value and possible positive effects as alternative to meat food product of vegetable origin, while being simultaneously and sustainable.

The United Nations Food and Agriculture projections in 2020 projected that the number of people affected by hunger or undernourishment, or protein "hunger" will surpass 840 million by 2030, or 10 percent of the global population [27]. COVID-19 pandemic is expected to further worsen the overall projections regarding not only security in food chain but also nutritional aspects of commercial food-products. In 2020, the number of undernourished people increased, compared to 2019 estimates, by about 118 million people compared to 2019, since COVID-19 disrupted economy cycles, job markets and supply chains, and as a consequence inflated food price. Furthermore, it has been projected the pandemic will continue to have lasting effect beyond 2020, adding approximately 30million people to the total number of undernourished, including the protein hungered, in the world in 2030 [28]. Thinking that one of the most significant reasons for malnutrition or undernutrition is protein-energy food lack, is more than obvious that the production of protein rich products from residues of agrofood processing lines, employing novel-environmentally friendly strategies, could partially solve a part of malnutrition issues. Also, transformation of low protein-energy foods such as cereal milling by-product streams, rejected legumes from agro-processing lines and vegetables to high protein products such as vegetable sauces enriched with bio-carotenoids and Greek miso-like- products might offer another alternative to produce novel superfoods and functional foods, while promoting goals of 2030 Agenda and Zero Hunger Target [29].

Author Contributions

Conceptualization: Dimou M Charalampia (CMD) and Koutelidakis E. Antonios (KEA); ii. Methodology: CMD and KEA; iii. Resources: CMD, KEA; iv. Writing-Original Draft Preparation: CMD, CD, KK, NK; v. Supervision: CMD; vi. Project Administration: CMD; vii. Funding Acquisition: CMD. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data presented in this study are available within this article.

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Conflicts of Interests

The author(s) confirm that have no conflict of interest.

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