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Effect of Glucose Concentration on the Production of Proteolytic Extract by Different Strains of *Aspergillus* under Solid-State Fermentation

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Abstract: Proteases are important enzymes because of their extended uses in several industries, such as food, beverages, pharmacy, detergents, and many others. *Aspergillus* is one of the most used fungi strains for enzyme production by solid-state fermentation (SSF). Disponibility of the carbon source is a key factor for protease production. In addition, the selection of solid support has great importance, as it must provide suitable airflow through the packed bed and nutrient diffusion inside the fermentable mass. Six *Aspergillus* strains and two inert supports (Agrolite (AL) and Polyurethane (PUF)) were tested for protease production from fish flour (FF) at different glucose concentrations (0, 5, 10, 15%) by SSF. The FF/PUF mixture at 70/30 (*w*/*w*) ratio, with 75.39% moisture, and a critical moisture point of 0.11 gH_2O/g, presented a texture that allowed heat and mass transfer and provided enough moisture to make free water available as required for microorganism growth during the fermentation process. *Aspergillus oryzae* 2095 produced higher amounts of neutral and alkaline proteases with the addition of 5% glucose to the growth medium. Kinetics studies reveal that protease production is partially associated with growth. The extracts obtained can be used in different industries, and especially to prepare fish high-value by-product hydrolysates.

Keywords: *Aspergillus*; protease production; glucose concentration; fish flour; inert support

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1. Introduction

Proteases are an important group of enzymes whose main function is to catalyze the hydrolysis of proteins into polypeptides or oligopeptides into smaller peptides and amino acids [1]. They represent 25% of the biomolecules produced for industrial applications and 60% of the global enzyme market. The proteases produced by microbes have been used to produce fertilizers, detergents, pharmaceuticals, cosmetics, textiles, leather, and food, among many other uses [2,3]. Proteases can be isolated from plants, animals, and microorganisms. Of these, microbial proteases have great potential for industrial production due to their wide biochemical diversity, susceptibility to genetic manipulation, small production spaces, and low nutritional requirements [4]. It is estimated that microbial proteases account for approximately 40% of total worldwide enzyme sales [5,6].

Although there is a wide diversity of commercial proteases, their use on an industrial scale is limited by the high costs of enzymes involved in the processes and because they are limited to a restricted range of biochemical characteristics. Therefore, it is necessary

to investigate options to lower the cost of producing microbial proteases and, at the same time, increase biochemical diverse specificity and stability [5,6].

Among the microorganisms of interest to produce fungal proteases, *Aspergillus* sp. is one of the most important fungi for producing microbial proteases, due to its ability to grow on a wide range of substrates under different environmental conditions. The fungi may secret a large number of hydrolytic enzymes, including proteases, however, only approximately 8.2% of *A. niger* proteases have been molecularly and biochemically characterized [7]. *A. oryzae* has been extensively used in the production of a variety of industrial enzymes, such as proteases [8].

Although a large percentage of microbial enzymes is produced by submerged fermentation, solid state fermentation (SSF) is a technique that allows agro-industrial by-products to be used as a substrate to produce biomolecules at lower cost and in an environmentally friendly way. In addition, fungi present a higher growth rate in solid state fermentation when compared to submerged fermentation. The physiology and morphology of filamentous fungi enable them to quickly completely colonize the substrate matrix, to nourish themselves and then secrete high levels of metabolites and enzymes [9]. Other advantages of SSF include low risk of contamination, increased yield, use of low-cost substrates, simplicity of processing, lower energy requirements, and less wastewater production, making it more attractive than submerged fermentation [10,11]. The most common substrates for SSF include food agroindustry residues, but it is also possible to use low-cost by-products of fish processing industries for different enzyme production [12].

Proteases have been used to produce fish protein hydrolysates, which have allowed the use of fish by-products as a substrate, giving the properties of the peptides such as minimizing the oxidation of lipids during the processing and storage of foods, and antioxidant, antiatherosclerosis, anticancer, anticoagulant, anti-inflammatory, antihypertensive, and antimicrobial activities. In addition to the above, some functional properties have also been evidenced, such as solubility, emulsifying, and foaming properties, which could be beneficial for industrial applications [13–15]. In addition, a high specificity for protein from aquatic sources has been proven.

This work was focused on the evaluation of glucose concentration and the use of two different inert supports for the production of proteolytic extracts by different strains of *Aspergillus* sp. under solid-state fermentation.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation

The strains of *A. niger* ANH-15 from the DIA-UAdeC strain collection, *A. niger 2088* and *A. oryzae* 2095 both from the Spanish Type Culture Collection, Valencia, Spain (STCC), A. *niger* C28B25, *A. niger* DAR2, and *A. niger* AD96-4 from the IRD-UAM collection were grown on potato–dextrose agar (BIOXON®, Monterrey, Mexico) plates. Conidiospores were harvested from a 5-day-old culture by suspending them in sterile water containing 0.1% Tween 80. After counting in a Neubauer chamber, the spore suspension was adjusted to the desired concentration.

2.2. Skimmed Milk Agar Plates

Skimmed milk agar plates were prepared by mixing 20 g L^{-1} of skimmed milk (*Svelty*®, Nestlé, Mexico City, Mexico) and 15 g L^{-1} of Bacto agar (OXOID®, Monterrey, Mexico). The skimmed milk solution was sterilized at 125 °C for 10 min while the Bacto agar solution was sterilized at 125 °C for 15 min. Solutions were left to cool and mixed (1:90 *v*/*v*) before agar solidification. The skimmed milk plates were stored at 4 °C until used.

2.3. Strain Selection

Strain selection was performed by monitoring the formation of hydrolysis halos on skimmed milk agar plates. The plates were inoculated by pitting in the center of the inverted Petri dishes and incubated in the same position at 30 °C for 72 h. The plates were observed

every 24 h to visualize the formation of hydrolysis halos, and colony and hydrolysis halos diameters were recorded to calculate the potency index (PI) using Equation (1) [16].

$$\text{Potency Index(PI)} = \frac{\text{Hydrolysis halo diameter (mm)}}{\text{Colony growth diameter (mm)}} \quad (1)$$

2.4. Inert Supports

2.4.1. Polyurethane Foam

Polyurethane foam (PUF) (Expomex®, Mexico City, Mexico) with a density of 24 kg m^3 was ground (Brandenburg® OHG 880804, Berlin, Germany) to a particle size of 0.5 mm, then washed (3X) with distilled water and then rinsed again with boiling distilled water, drained, and dried at 80 °C until a uniform dryness was achieved.

2.4.2. Agrolite

Agrolite was rinsed thoroughly with tap water, drained as much as possible, rinsed and drained again, then rinsed with boiling distilled water, drained, and dried at 80 °C until a uniform dryness was achieved, then ground (Brandendur® OHG 880804, Berlin, Germany) to a particle size of 0.5 mm.

2.5. Drying Curves and Critical Moisture Point

The kinetics of water removal during drying were obtained by setting 1 g of the sample in a thermo-balance at 120 °C to a stable moisture content (1%) [17].

The critical moisture point (CMP) was calculated after the estimation of the dry rate during the dehydration kinetics of each fish flour/inert support (FF/IS) mixture (50/50, 70/30, and 90/10; *w*/*w* %). Subsequently, drying velocities were related to the water removed with time [17,18].

2.6. Culture Medium for Solid-State Fermentation

FF, purchased from a local feed animal shop, was sieved to obtain a homogeneous particle size of 0.84 mm, then mixed with PUF previously ground to a particle size of 0.5 mm. The mixture of FF and PUF at a 70:30 (*w*/*w*) ratio was oven-dried for 12 h at 80 °C, then sterilized at 121 °C for 15 min. The raw material/support mixture was moistened with sterile 0.2 M phosphate buffer pH 7, to which the desired inoculum concentration and trace metal (to the desired water content (50%)) were added and then used as an SSF culture medium. The mineral salts composition was (g/L): $(NH_4)_2SO_4$, 12.6; K_2HPO_4, 11.8; urea, 3.0; $MgSO_4.7H_2O$, 0.2; $FeSO_4$, 0.29; $MnSO_4$, 0.001; $CuSO_4$, 0.001; $ZnCl_2$, 0.001.

2.7. Inocula Preparation

The inoculum was prepared by propagating the strain on 50 mL potato dextrose agar (PDA) in 250 mL Erlenmeyer flasks incubated at 30 °C for 7 days. The spores were harvested by adding 30 mL of a sterile solution of Tween 80 (0.1%, *v*/*v*) to each inoculum flask, and resuspended by stirring with a magnetic rod. For spore counting, a 1mL aliquot was diluted, and spores were quantified using a Neubauer chamber (Brand, Wertheim, Germany). The fermentable mass was inoculated with a spore suspension of 2×10^7 spores g^{-1}dry base (db).

2.8. Fermentation Process

SSF was carried out in packed-bed tubular (2.5 × 22 cm) reactors packed with 30 g of the culture medium described above, previously inoculated with 2×10^7 spores g^{-1}db and incubated (72 h) under static conditions at 30 °C, with an air flux set at 40 cm^3 per min. The effect of glucose concentrations (0, 5, 10% *w*/*w*) on protease production was determined at 0, 36, and 70 h of fermentation. Fungal growth was followed indirectly by online CO_2 detection [19].

2.9. Kinetics of Fermentation

The selected strains (Section 2.3) were subjected to protease production kinetics at the best FF/IS culture media mixture ratio at different glucose concentrations. The fermentation conditions were carried out at 30 °C, with an initial pH of 6, moisture content of 50%, and inoculum of 2×10^7 spores g-1dm, and the bioprocess lasted 72 h. Samples were taken at regular intervals (0, 24, 36, 40, 44, 48, 60, and 72 h). During fermentation, % M, Aw, and proteolytic activity were assayed at different pH values (3.0, 7.0, 10.0). The specific growth rate (μ) was determined by fitting the experimental data of CO_2 production to the logistic and Gompertz models (Solver MicroSoft®; Office 365).

2.10. Analytic Methods

2.10.1. pH Determination

One gram of wet fermented matter was suspended in 10 mL of distilled water, homogenized with a vortex, and filtered (Whatman® No. 41, Maidstone, UK) under vacuum, then the pH of filtrates was determined using a Conductronic® pH120 potentiometer (Puebla, Mexico). An average of three determinations was reported.

2.10.2. Determination of Moisture Content (M %) and Water Activity (A_w)

Moisture (M %) was determined by placing 2g of the fermented matter in a thermobalance (OHAUS®, Nänikon, Switzerland). at 130 °C for 15 min. Water activity (Aw) was determined in an AQUA Lab® CX-2 Hygrometer (Decagon, Pullman, WA, USA). An average of three determinations was reported.

2.10.3. Water Absorption Index (WAI)

The water absorption index (WAI) was determined using the method described by Orzua et al. [20]. The sample (1.25 g) was suspended in 15 mL of distilled water in a tared 50 mL Falcon tube, the slurry was stirred with a glass bar for 1 min at room temperature (25 °C) and centrifuged at 3000*g* for 10 min. The supernatant was discarded, and the WAI was calculated from the weight of the remaining gel in the centrifuge tube and expressed as g gel g^{-1}db.

2.10.4. Chemical and Elemental Composition of Fish Flour

Fish flour (FF) was obtained from a local feed animal shop. The nitrogen content was determined by the Micro-Kjeldahl method [21] and total sugars by the phenol-sulfuric method [22]. The elemental analyses of total carbon (C), hydrogen (H), nitrogen (N), and oxygen (O) contents were performed using an Elemental Analyzer® (Perkin Elmer Series II CHNS/O Analyzer 2400, Tucson, AZ, USA).

2.10.5. Protease Assay

Proteolytic activity was determined by the quantification of tyrosine released during the hydrolysis of peptide bonds of a protein substrate by the action of proteases present in the produced crude extract [23]. Briefly described, each sample (1.25 mL) of protease extract, previously diluted in a 1:5 ratio in distilled water, was added with 5 mL of 0.4 M Na_2CO_3, and 1 mL of Folin–Ciocalteu phenol reagent (Sigma-Aldrich®, Mexico City, Mexico). Subsequently, the tubes were stirred in a vortex and incubated at 30 °C for 30 min. Then, absorbance was read on a Perkin-Elmer Lambda25® (PerkinElmer, Inc., Waltham, MA, USA) UV-Visible spectrophotometer at 660 nm. The experiment was performed in triplicate and the average absorbances were plotted against the tyrosine concentration curve.

A proteolytic unit (U) was defined as the amount of enzyme that produces the color equivalent of 1 μmol of tyrosine in a 1 mL reaction per min at 30 °C assayed at the different pH values tested (pH buffers at 10.0, 7.0, 3.0). The enzyme activity was reported in U/mL.

2.11. Statistical Analysis

All tests were performed in triplicate and average values were analyzed and interpreted using Minitab20 Statistical Software®. Estimation of statistical parameters was realized by Analysis of Variance (ANOVA, NCSS 2020).

3. Results

3.1. Fungal Strain Selection

Protease production by the *Aspergillus* strains was evaluated using a rapid plate test on skimmed milk (Figure 1). Of the 6 strains tested, *A. niger* ANH-15 (a), *A. niger* 2088 (b), and *A. oryzae* 2095 (c) produced a translucent hydrolysis halo on the skimmed-milk agar plates, which is indicative of proteolytic activity [24]. However, *A. niger* DAR2 (d), *A. niger* C28B35 (e), and *A. niger* AD96-4 (f) showed little growth but no proteolytic activity halo was formed. Figure 1 shows typical results obtained on skimmed milk agar plates.

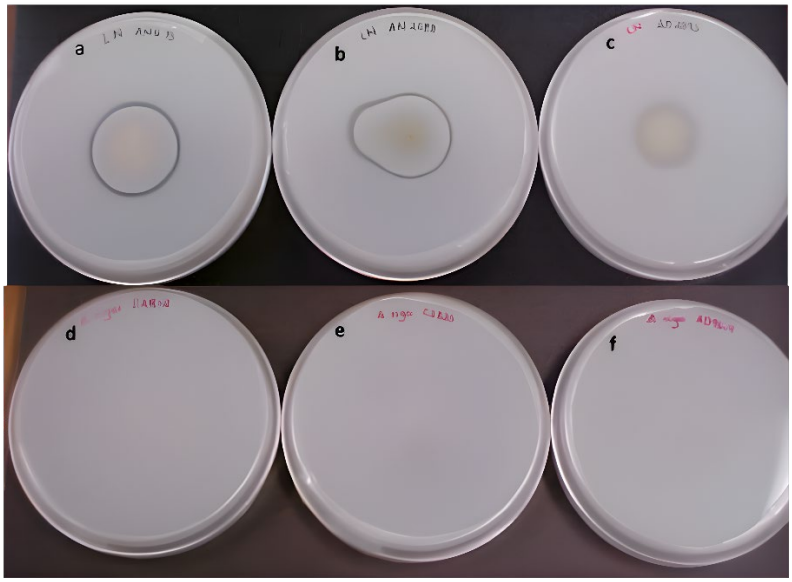

Figure 1. Growth of *Aspergillus* sp. strains on skimmed milk plates: (**a**) *A. niger* ANH-15, (**b**) *A. niger* 2088, (**c**) *A. oryzae* 2095, (**d**) *A. niger* DAR2, (**e**) *A. niger* C28B35, (**f**) *A. niger* AD96-4.

Table 1 shows the potency index (PI) of the best qualitative proteolytic activity results obtained from the skimmed-milk agar plates as determined by Equation (1). *A. oryzae* 2095, *A. niger* 2088, and *A. niger* ANH-15 are protease producers.

Table 1. Potency index (PI) on skimmed milk agar plates *.

Strain	Diameter of Strain Colony (cm)	Diameter of Hydrolysis Halo (cm)	Potency Index (PI)
A. oryzae 2095	1.1	2.1	1.9
A. niger 2088	1.9	2.9	1.5
A. niger ANH-15	2.2	3.2	1.4

* Data represents the average of triplicate measures from different experiment sets. The percentage of experimental error was below 5% for all experiments.

3.2. Fish Flour Composition

Table 2 shows the chemical composition of the FF used in this study and for protease production studies.

Table 2. Chemical composition of fish flour used for protease production *.

Component	%
Carbon	43.25
Nitrogen	10.60
Hydrogen	6.44
Glucose **	1.45
Protein	62.85

* Data represents the average of triplicate measures from different experiment sets. ** As referred from the total carbon. The percentage of experimental error was below 5% for all experiments.

3.3. Fermentation Medium

3.3.1. Selection of Fish Flour-to-Inert Support Ratio (FF/IS)

Table 3 shows the calculated results for the %M and CMP for the different FF/IS mixtures tested. For the FF/PUF mixture, the highest WAI was 6.022 gH_2O/gdm (85.76%H) and occurred in the 50/50 (*w*/*w*) mixture. In contrast, the WAI of the FF/AL at the same ratio was 3.172 gH_2O/gdm (76.03% M).

Table 3. FF/IS ratio selection parameters (agrolite and polyurethane foam) (+): slightly agglomerated, (++): agglomerated, (+++): highly agglomerated *.

Support	FF/IS	Moisture Saturation (%)	Critical Moisture Point	Texture
Agrolite 20	50/50	76.03	0.61	+
Agrolite 20	70/30	65.03	0.08	++
Agrolite 20	90/10	53.73	0.08	+++
PUF	50/50	85.76	0.13	+
PUF	70/30	75.39	0.11	++
PUF	90/10	59.88	0.09	+++

* Data represents the average of triplicate measures from different experiment sets. The percentage of experimental error was below 3% for all experiments. (+) little agglomerate; (++) agglomerate; (+++) very agglomerate.

3.3.2. Effect of Glucose Concentration on pH, A_w, and M % during Protease Production

Once the optimal substrate/support ratio (FF/PUF; 70/30 *w*/*w*) was selected, the effect of glucose concentration (0, 5, 10, and 15%) was evaluated on protease production (Table 4). The pH value increased as the fermentation time elapsed at all glucose concentrations and *Aspergillus* strains tested. The A_w value showed a slight decrease (<1%) as the glucose concentration increased; in contrast, the moisture content was slightly increased (<4%) at the end of the bioprocess.

Table 4. Effect of glucose concentration on pH, Aw, and M % during fermentation by the different *Aspergillus* strains *.

Time (h)	Glucose Concentration											
	0%			5%			10%			15%		
	pH	Aw	M%	pH	Aw	M%	pH	Aw	M%	pH	Aw	M%
	Aspergillus niger 2088											
0	5.3	0.984	44.60	5.4	0.980	50.17	5.3	0.976	48.13	5.3	0.972	47.79
36	6.8	0.989	49.68	6.9	0.989	46.81	6.5	0.988	50.64	6.0	0.983	50.20
72	8.1	0.981	51.53	7.7	0.982	51.78	7.5	0.979	51.51	7.4	0.976	53.19

Table 4. *Cont.*

Time (h)	Glucose Concentration											
	0%			5%			10%			15%		
	pH	Aw	M%	pH	Aw	M%	pH	Aw	M%	pH	Aw	M%
	***Aspergillus niger*ANH-15**											
0	5.4	0.990	48.59	5.4	0.988	48.71	5.4	0.983	48.37	5.4	0.978	47.75
36	6.4	0.991	49.21	6.0	0.991	48.78	5.3	0.990	48.97	5.3	0.985	48.20
72	7.3	0.991	51.53	7.2	0.983	51.78	7.3	0.982	51.51	7.3	0.980	53.19
	Aspergillus oryzae 2095											
0	5.5	0.992	49.22	5.5	0.985	48.71	5.5	0.980	47.62	5.4	0.975	46.48
36	7.5	0.978	49.75	7.4	0.976	49.34	6.2	0.985	47.67	5.85	0.991	54.61
72	7.7	0.959	49.74	7.7	0.960	50.81	7.8	0.966	49.99	7.7	0.964	50.82

* Data represents the average of triplicate measures from different experiment sets. The percentage of experimental error was below 3% for all experiments.

The results of the effect of glucose at different concentrations (Figure 2) show that *A. oryzae* 2095 produced the highest proteolytic activity at 36 h culture (Figure 2a) at glucose concentrations of 0 and 5% (20.0–20.5 U/mL respectively), however, enzyme activity decreased at 72 h (Figure 2b), showing a similar pattern at all glucose concentrations tested.

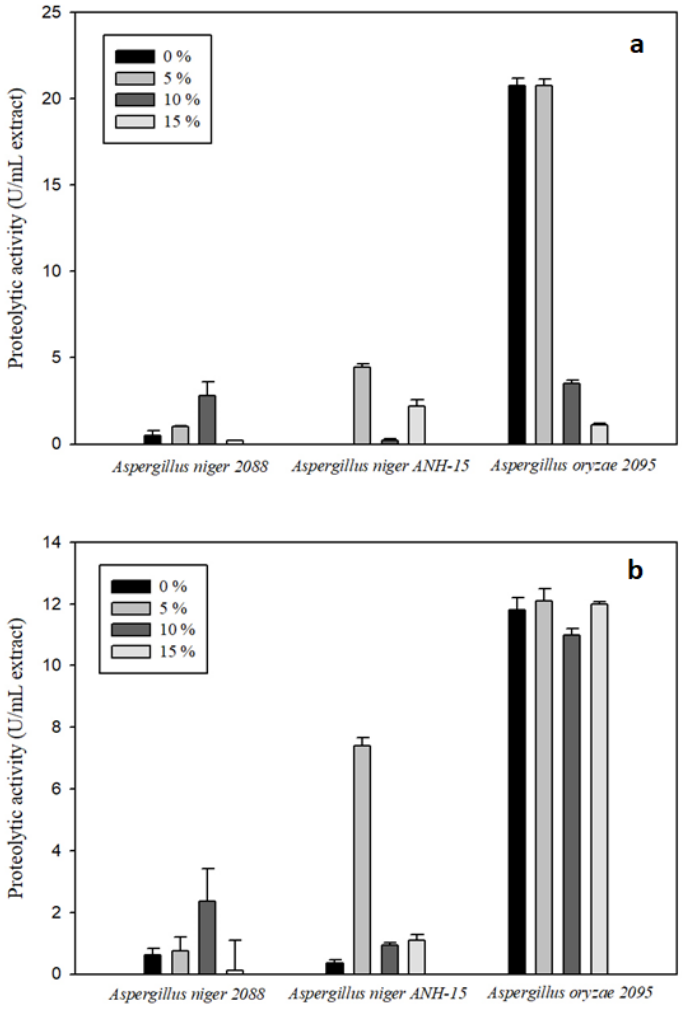

Figure 2. Effect of glucose concentration on protease production from different strains of *Aspergillus* at 36 h (**a**) and 72 h (**b**) by SSF.

According to the results, the strain *A. oryzae* 2095 had the highest production of proteolytic enzymes, both at 36 and 72 h of fermentation, with 5% glucose, therefore it was selected for further kinetic studies.

3.3.3. Protease Production Kinetics

Protease production kinetics and fungi growth as online CO_2 production are shown in Figure 3. Protease production starts at 36 h, with *A. oryzae* 2095 reaching the maximum activity at 44 h, and with alkaline activity (23 U/mL extract) being higher than neutral (17 U/mL extract), as assayed at pH 10.0 and 7.0 respectively. It was observed that protease production starts at pH values of 6.5 and 7.0 for neutral and alkaline proteases, respectively.

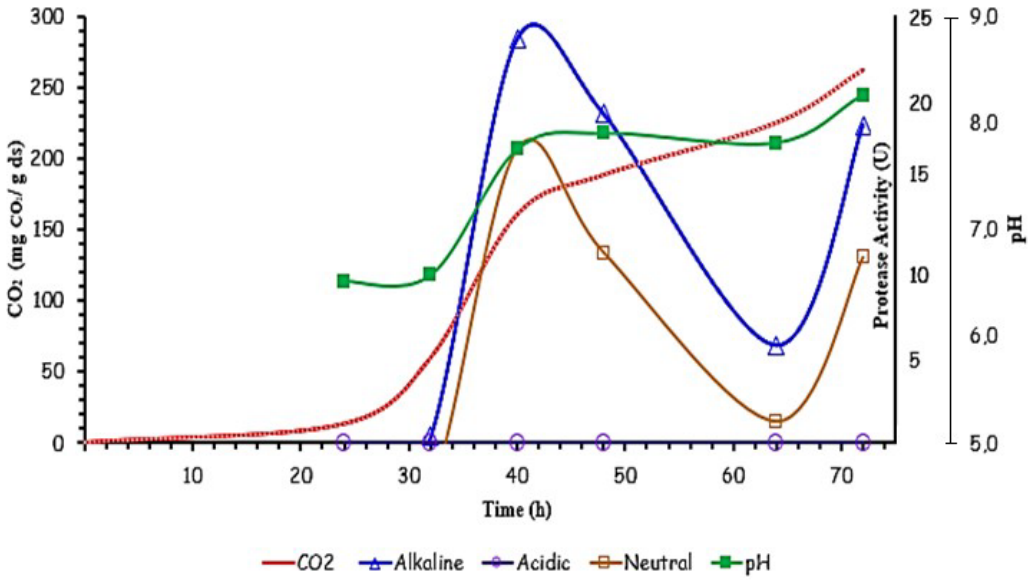

Figure 3. Kinetics of protease production (alkaline, acid, and neutral), CO_2 production, and pH during SSF by *A. oryzae* 2095. Data represents the average of triplicate measures from different experiment sets. The percentage of experimental error was below 3% for all experiments.

4. Discussion

Although the genus *Aspergillus* is reported as a protease producer, the lack of production of a proteolytic halo in 3 of the 6 strains tested may be due to these strains not having an enzymatic system (protease excretion) that allows them to use casein as the sole carbon source. It should be noted that the enzymatic tests were carried out on skimmed milk agar plates at pH 5 and 8 and that similar results were obtained at both pH values tested (Figure 1). According to the hydrolysis activity halo and PI on skimmed milk plates, *A. oryzae* 2095 is the best protease producer, so it was selected for further assays. *A. oryzae* is non-toxic and considered safe for human and animal consumption, having been classified as "Generally Recognized as Safe (GRAS)" by the FDA.

The composition of the culture medium is a key parameter in the SSF process since the source of carbon, nitrogen, and trace metals can influence growth and metabolites, enzymes, and spore production [25]. Carbon and nitrogen content are essential for microbe development and its metabolic activity. Both components are essential for the construction of macromolecules during microbial growth; therefore, the substrate must provide the basic nutrients to the microorganisms to guarantee the fermentation process [26]. Since the disponibility of the carbon source is a key factor for protease production [27,28], the effect of the addition of different glucose concentrations on the proteolytic extract production by *Aspergillus* strains was evaluated.

According to the elemental analysis, FF is composed of carbon, nitrogen, and hydrogen of 43.25, 10.60 and 6.44%, respectively; however, only 1.45% of the total carbon corresponded to glucose, as determined by the phenol-sulfuric method. The rest of the

carbon content is found as a structural part of other macromolecules present in the FF [29]. In comparison with the protein content of the FF used in the present study (62.85%), Ellouz et al. [26] reported values from 45.50 to 80% protein content in the fish section used. Likewise, it was found that the carbohydrate content of the FF was 1.45%, while that reported in the literature is null [26,27], which may indicate that the FF used in this study may have some by-products of other aquatic species.

Most microorganisms prefer glucose as their primary source of carbon. However, if the glucose in the medium is depleted, the microorganisms will start consuming other compounds due to their metabolic activity. This can involve the activation of other metabolic pathways and the synthesis of different enzymes to utilize other sources of carbon, such as starch, cellulose, proteins, and so on. We measured C_{total}/N_{total} and CHO/protein ratios of 4.0 and 0.023, respectively, in contrast to the ratio of 6.6 of CHO/protein reported by Battaglinio et al. [30] for *A. oryzae* for proteases formed in solid state fermentation. This shows the need to add another source of carbon to meet the initial nutritional and energy requirements to ensure the adequate growth of *Aspergillus* prior to the initiation of the enzymatic machinery that releases carbon molecules as a result of the proteolytic activity generated by the microorganism itself when a carbon restriction is detected.

FF is an excellent raw material for SSF as it contains all the nutrients for microbial activity [31,32]. However, FF tends to agglomerate when its moisture content increases so the addition of structural support is required; thus, the use of AL and PUF as individual IS was tested (Table 3). The values in Table 3 show that the highest WAI (6.022 gH_2O/gdm; 85.76% M) occurred at 50/50 (*w*/*w*) with the FF/PUF mixture. In contrast, the WAI of the FF/AL at the same ratio was 3.172 gH_2O/gdm (76.03% M). These values are higher than those reported (0.43 gH_2O/gdm; 43%M) by Tahli et al. [28] who used oat flour and other cereals as raw material for protease production. For the selection of the best IS and the appropriate FF/IS ratio for optimal growth of the microorganism, drying curves of both IS were constructed [33]. Likewise, texture, critical moisture point (CMP), and water absorption index (WAI) of each FF/IS were evaluated, since these variables directly influence heat and mass transfer phenomena during the SSF processes and, therefore, affect the aeration and nutrient diffusion inside the fermentable mass [34].

To determine the CMP, the rate of water loss was calculated at the constant drying phase, and then the CMP was obtained from the intersection in the change of slopes of the kinetics drying curves [33] obtained for each FF/IS tested [17]. Texture was also considered in the selection of the FF/IS ratio, which improves as the proportion of IS increases in the mixtures, as FF tends to agglomerate at higher moisture content. Likewise, the saturation moisture was calculated, which increases as the proportion of IS increases, especially with PUF due to the WAI of this material.

The FF/PUF mixture ratio at 70/30 (*w*/*w*) with a saturation humidity of 75.39% and a CMP of 0.11 gH_2O/gss was selected (Table 3). This FF/IS ratio presented a texture that may allow heat and mass transfer and provide enough moisture to make free water available as required for microorganism growth during the fermentation process [20,35]. In addition, the selected ratio provides a higher amount of FF and still has adequate moisture and texture to allow fungi growth, aeration, and nutrient diffusion inside the fermentable mass. As per the studies conducted by Raimbault [35] and Orzua et al. [20], to create a fermentable culture media, the substrate/IS and the IS used must fulfill certain prerequisites. These include: (i) being a solid porous matrix; (ii) having the ability to absorb more water weight than its dry weight; (iii) allowing the flow of gases through the matrix; (iv) providing a conducive habitat at the solid/gas interface for the rapid development of microbes; (v) possessing good mechanical properties for compression or gentle stirring; and (vi) being free from possible contaminants or microbe inhibitors and having the capacity to absorb the necessary nutrients for microbial growth.

The effect of glucose concentration on pH, A_w, and M% during fermentation is shown in Table 4. It can be seen that pH values increase as fermentation time elapses at all glucose concentrations in the three *Aspergillus* strains selected for their proteolytic activity. This

effect is explained by the increase of ammoniacal nitrogen as a result of protein hydrolysis, which causes an increase in pH [36]. Similar results are reported by Huang et al. [37] and Yang et al. [38] using Sofu and Tofu, two fermented soybean products. The slight decrease in A_w (<1%) as glucose concentration increased is explained on the basis that glucose is a polar compound that hydrates in the presence of water through the formation of hydrogen bonds [39], resulting in less water availability for physiological microbial activities. In general, the reduction in initial moisture content during fermentation has been reported in SSF. This is explained as the result of water evaporation and metabolic heat generation, however, this problem has been solved by the addition of saturated air [40]. Nevertheless, in this work, the moisture content was slightly increased (<4%) at the end of the bioprocess, possibly caused by the water generated from metabolic activity or because of the condensed water from the saturated air, which is rapidly absorbed by the PUF [40].

The results of the effect of glucose at different concentrations in protease production showed that *A. oryzae* 2095 produced the highest proteolytic activity at 36 h culture (Figure 2a) with added glucose concentrations of 0 and 5% (20.0–20.5 U/mL respectively). However, enzyme activity decreased at 72 h (Figure 2b), showing a similar pattern at all glucose concentrations tested. Activity decrease is explained as the result of protease self-hydrolysis, instability of the protease's activity, or a decrease or even lack of nutrients in the culture medium [37. A similar pattern is observed in all the strains studied, however, *A. oryzae* 2095 produces 4 and 17 times higher proteolytic activity than *A. niger* ANH15 and *A. niger* 2088, respectively, at 5% glucose and 36 h fermentation.

The lower protease activity produced at 10 and 15% glucose (Figure 2a,b) is explained by the preference of the microorganism for easier to assimilate carbon sources, therefore, reducing the production of proteases, as there is no need for carbon source release from protein hydrolysis. Similar results were found by Mamo et al. [41], who evaluated the effect of the addition of different carbon and nitrogen sources on the production of proteases by SSF with *A. oryzae*, finding that supplementing the culture medium with glucose at a concentration of 5% allows the highest production of proteases, while higher concentrations have the opposite effect; their results are well correlated with our findings. Then, *A. oryzae* 2095 was used in further kinetic studies.

Enzyme activity was assayed at pH values of 3.0, 7.0, and 10 for acid, neutral, and alkaline proteases (Figure 3). Protease production starts at 36 h, reaching the maximum activity at 44 h, and exhibits higher alkaline (23 U/mL extract) than neutral (17 U/mL extract) activity as assayed at pH 10.0 and 7.0, respectively. Protease production starts at pH values of 6.5 and 7.0 for neutral and alkaline proteases, respectively. *A. oryzae* has been reported as a producer of acid, neutral, and alkaline proteases [30], however under our study conditions, acid proteolytic activity (pH 3.0) was not detected. This is because the initial pH (5.0) of the fermentation medium favors the production of neutral and alkaline proteolytic extracts [30].

The specific growth rate (μ) of *A. oryzae* 2095 was determined by fitting the experimental data of CO_2 production to the logistic (0.229 h^{-1}, R^2 = 0.999) and Gompertz (0.144 h^{-1}, R^2 = 0.997) models to calculate the specific rate of CO_2 production (μCO_2 = 0.151 h^{-1}, R^2 = 0.998). From the plot (Figure 3), it is observed that the best fit was obtained with the logistic model, however, the values of the specific growth rate obtained by the Gompertz model and μCO_2 are very close to each other. This is explained by Koutinas et al. and Villegas et al. [19,42], who found that fungal growth in heterogeneous media is directly related to the production of CO_2. For this reason, the fit with the Gompertz model is closer to the real process. On the other hand, the value of the specific growth rate (μ = 0.144 h^{-1}) is lower than that obtained by Acevedo [43], who reported a value of μ=0.216 when producing proteases using a mixture of FF and wheat bran (60/40 *w*/*w*). This could be due to the FF/wheat bran mixture (70/30 *w*/*w*) used by Acevedo [43] preventing air diffusion during fermentation by agglutination of the culture medium, since it is known that oxygen and nutrient diffusion influence both growth and enzyme production [31].

On the other hand, by performing an elemental balance (Equation (2)) as reported by Nagel et al. [44], yields can be estimated.

$$Y_{S/CO2}CH_{1.79}O_{0.66}N_{0.21} + Y_{O2/CO2}O_2 \rightarrow Y_{X/CO2}CH_{1.72}O_{0.55}N_{0.17} + CO_2 + Y_{W/CO2}H_2O + Y_{N/CO2}NH_3 \quad (2)$$

For the fermentative system of this work, the reaction described in Equation (3) was proposed, where the terms on the left side of the equation correspond to the elemental composition of the FF, while the terms on the right side indicate the elemental composition of the biomass [4], the production of CO_2, water, and ammonia, with the latter being the element that causes the alkalization of the fermented medium [45]. The coefficients are obtained by performing an elemental balance on both members of the reaction, and subsequently, a matrix is solved [46]:

$$1.96\ CH_{1.79}O_{0.66}N_{0.21} + 0.88\ O_2 \rightarrow 0.96\ CH_{1.72}O_{0.55}N_{0.17} + CO_2 + 0.54\ H_2O + 0.25\ NH_3 \quad (3)$$

From this elemental balance, Y_S/CO_2 (1.96) and Y_X/CO_2 (0.96) were obtained, and by applying the Luedeking-Piret model (Equation (4)), which describes product formation as a function of biomass [47], it was determined that protease production is partially associated with growth because the term of the equation associated with growth is 0.17 and the term independent of growth is 0.06.

$$\frac{dP}{dt} = 0.17\mu X + 0.06X \quad (4)$$

In summary, in the production of proteases, the glucose concentration (Figure 2) of the culture medium had an important effect on the growth of the fungus and therefore, on the production of extracts with proteolytic activity. Comparing the obtained results with those reported in the literature, it was found that the proteolytic activity of the extracts obtained (U/mL) was 2 and 4 times higher than those reported by Malathi et al. [48] and Couri et al. [49], respectively. However, reports were also found in which the proteolytic activity was 2.77, 4.42, and 2.29 times lower than the proteolytic activity of the extracts obtained in other works [50–52] (Table 5).

Table 5. Protease production from agroindustry residues using different fungi strains.

Protease	Substrate	Fungi Strain	Proteolytic Activity	Reference
Alkaline	Fish Flour	*A. oryzae* 2095	21.77 U/mL 62.2 U/g SS	* Present study
Neutral	Fish Flour	*A. oryzae* 2095	17.21 U/mL 51 U/g SS	* Present study
Alkaline	Wheat bran	*A. flavus*	10.47 U/mL	[48]
Acid	Wheat bran	*A. niger*	52 U/g SS	[42]
Alkaline	Wheat bran	*R. oryzae*	341 U/g SS	[53]
Alkaline	Wheat bran	*R. oryzae*	60 U/mL or 290 U/g SS	[50]
Alkaline	Wheat bran	*R. oryzae*	275 U/g SS	[51,52]
Acid	Wheat bran and mango peel	*A. niger*	5.27 U/mL	[49]
Alkaline	Wheat bran	*R. oryzae*	50 U/mL	[52]

* SS = solid substrate.

5. Conclusions

A. oryzae 2095 produced the highest proteolytic activity both qualitatively and quantitatively. The best mixture of FF/IS for protease production was the FF/PUF at a 70/30 (*w*/*w*) ratio. In addition, a low concentration (5%) of glucose favors protease production. *A. oryzae* 2095 produces neutral and alkaline proteases associated with growth in a short time

(44 h). The obtained extracts are suitable for use in the preparation of high-quality fish by-product hydrolysates.

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