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Temperature and pH Optimization for Protease Production Fermented by *Yarrowia lipolytica* from Agro-Industrial Waste

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Abstract: The use of yeasts for the production of proteases has increased in demand in recent years. *Y. lipolytica* has been reported as a strain with high yields of protease production. This work aimed to evaluate the impact of pH and temperature on the production of proteases using *Y. lipolytica* in solid-state fermentation (SSF). Soybean, canola meal, cottonseed meal, and sesame meal wastes were used as nutrient sources at seven pH levels (4, 5, 6, 7, 8, 9, 10) and five temperatures (25, 30, 35, 40, 45 °C). The waste source and optimal conditions for maximum enzyme production (EP) were obtained by Box–Benken design. The results revealed that at pH of 7, temperature of 30 °C, and for 48 h cultivation period, canola meal showed the best EP with 188.75 U/L, followed by soybean with 117.07 U/L, cottonseed meal with 66.71 U/L, and sesame with the lowest production, reaching 88.5 U/L up to 35 °C. The temperature factor exhibited the greatest effect on protease production. The biotechnological and economic potential of canola meal in the production of enzymes is highlighted.

Keywords: proteases; enzyme production; SSF; canola meal; soybean; cottonseed meal; sesame

1. Introduction

Proteolytic enzymes have a wide range of applications in industrial processes such as food, detergent, proteolytic hydrolysis, leather, textiles, cosmetics, and pharmaceuticals [1]. With the rising demands and applications, researchers are exploring various approaches to discover, redesign, or artificially synthesize enzymes with better applicability in industrial processes. These enzymes offer a sustainable and environmentally safer option besides possessing economic and commercial value [2]. Biotechnological processes have been implemented for their production, which is cheap and environmentally friendly, offering the possibility of using natural substrates for the production of these types of enzymes. Since the emergence of enzymology, microbial proteases have been the most studied and have gained interest not only for their vital role in metabolic activities but also for their extensive use in industries [3,4].

Microbial proteases stand out compared to animal and plant proteases because they possess all the suitable characteristics for industrial applications [5,6]. Proteases are classified based on optimal pH of activity (acidic, basic, or neutral), type of reaction catalyzed, and chemical nature of the catalytic site. These differ widely in their substrate specificity, and combinations of different proteolytic enzymes may be used to increase the degree of hydrolysis of a protein [7]. For this reason, scientists are constantly working in the search for cost-effective and environmentally friendly substrates. This is especially important due to the enormous volume of enzyme demand, as currently, approximately two-quarters of the production cost is allocated to the substrate [8,9].
Therefore, the waste resulting from agro-industrial processes presents a great opportunity to be utilized through the implementation of specialized technologies. Among these wastes, those from the brewing, food, sugar, and cereal industries stand out, such as corn, wheat, rice, and oils [10]. These wastes can be utilized and valorized through protein enrichment, for which simple procedures mediated by organisms recognized as generally recognized as safe (GRAS) by worldwide food safety organizations are used [11], such as Y. lipolytica. Considered a non-conventional yeast, it is a microbial model suitable for producing large amounts of lipases [12] and proteases, mainly due to the process’s metabolic characteristics and operational conditions that corroborate its viability. In addition, it can be produced through solid-state fermentation that offers high volumetric productivity, target-concentrated compounds, tolerance of high substrate concentration, and less wastewater generation. Another advantage that supports the use of Y. lipolytica for protease production is the possibility of using agro-industrial residues as a substrate since these residues are generated in large quantities during the processing of raw materials, making it extremely necessary to adopt strategies for the integral use of residues [13,14] or, even, for conversion into higher value-added products. Due to their ability to act on insoluble substrates in aqueous and non-aqueous media, lipases and proteases produced by Y. lipolytica have versatility for potential applications [15] in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [16].

As for the available technologies for the processing and valorization of these wastes, they include submerged fermentation and solid-state fermentation [17,18].

Solid-state fermentation (SSF) is a simple process used to enhance solid waste and safely and environmentally friendly add nutritional value to them. According to Soccol et al. [18], this approach offers several advantages, low cost, higher production yields, easier purification, and higher concentrations of the products, simplicity, use of inexpensive and widely available agricultural residues as substrates, low water content, minimal microbial contamination, tolerance to high substrate concentration and less wastewater generation, higher product stability, less protein breakdown, less catabolic repression, low operating trouble, better oxygen circulation and less effort in post-processing [19,20]. The technical and economic feasibility is directly related to the choice of essential parameters, including the nature of the solid substrate, the chemical compositions, and the physical state of the substrate (particle size), which affect the microbial physiology and, consequently, the productivity of the substrate process [16,18,20]. However, certain operational limitations of SSF, such as difficulty in controlling the moisture level of the substrate and avoiding heat buildup, have limited its industrial application. Characterization of each microorganism, in terms of the influence of temperature and substrate moisture content on the kinetics of growth and product formation, is essential for SSF process scale-up [21,22].

Furthermore, Aggelopoulos et al. [23] point out that the most commonly used microorganisms in the bioconversion of agro-industrial waste into value-added products are yeasts, algae, fungi, and bacteria. Through this process, various useful products have been obtained in both the laboratory and industry, such as enzymes, organic acids, antibiotics, biofuels, and aromatic compounds, among others [24].

The pH and temperature are important control parameters within the fermentative process for maximum microbial growth and consequent enzymatic production [25,26]. The pH of the culture affects the enzymatic processes and the transport of several components by the cell membrane. It is possible that, within the optimum pH, the relative metabolic efficiency is high since the proton motive force in chemiosmosis is affected by the medium pH value [27]. Variations in pH values during fermentation can provide information on the start and end of the protease production period [25].

The temperature of the process is a parameter that influences cellular metabolism [28] and the kinetics of molecules such as proteins. According to Elias et al. [29] the reaction rates and collisions, the strength of molecular interactions, and other physico-chemical characteristics of proteins are also affected.
In addition to pH and temperature, protease production is greatly influenced by physicochemical and nutritional factors, such as substrate, nitrogen, carbon, moisture, aeration, agitation, dissolved oxygen, and inorganic salts [2,30]. There is no defined culture condition and medium for microbial protease production, and every microbial strain has its own particular nutritional and physicochemical requirements to reach maximum EP [31].

In Mexico, the oil industry annually processes thousands of tons of oil seeds such as canola, soybean, cottonseed, sesames, and sunflower, which generate a significant amount of waste traditionally known as protein-rich and carbohydrate-rich residues. However, only a percentage of these residues are used as concentrate in animal feed. These wastes contain sugars, fibers, proteins, minerals, and fats, which can be used as a substitute in biotechnological processes using microorganisms capable of taking advantage of these nutrients. The wastes used in this work are briefly described below.

Canola (Brassica napus): Currently, canola is the second most produced oilseed worldwide, after soybeans. The main commercial use of canola is to obtain vegetable oil, where a protein paste or cake is obtained as the main by-product; its physical presentation is solid powder and dark brown, which is composed of proteins that have a good balance of amino acids and functional properties, as well as non-protein compounds such as dietary fiber, phenolics and glucosinolates [32-34]. Their nutritional profile consists of protein 35%, carbohydrates 34.5%, oil 1.5%, fiber 12%, and moisture 12%. Its main application is in cattle, pig [35], and poultry feed and, in recent years, in biotechnological applications.

Soybean (Glycine max): It is the most consumed oilseed in Mexico, with an import volume of 95%. Soybean meal is a by-product obtained from the crushing of dehulled soybeans after most of the oil has been extracted with hexane. Mexico processes more than 20,000 tons of this by-product annually, which is exported to Caribbean countries as cattle feed. Its nutritional profile consists of protein 46%, fat 0.5%, crude fiber 7%, ash 6%, and moisture 12% [36,37].

Cotton (Gossypium hirsutum L.): The by-product, also known as cottonseed meal, is the product of milling the cottonseed after most of the oil has been extracted by mechanical or chemical means. It is light to dark yellow in appearance, with brown or black particles coming from the seed hull and not from overheating. It is an excellent source of animal protein used in feeding dairy cows. Its nutritional profile is composed of 41% protein, 3% fat, 13% fiber, 12% moisture, 8% ash, and 20% carbohydrates [38].

Sesame (Sesamum indicum): Sesame waste is the product of mechanical oil extraction. The physical characteristics of the product are as follows: it is dark and has an intense or strong odor and flavor because the sesame seed is roasted to achieve a very intense color to obtain an oil with these characteristics. Its nutritional profile consists of 38% protein, 6.5% fat, 12% moisture, 17% crude fiber, and 12% ash [20].

This study aimed to evaluate the impact of pH and temperature of the substrate on the production of proteolytic enzymes using a non-conventional yeast strain of Y. lipolytica CDBB-L-232, using canola meal, soybean meal, cottonseed meal, and sesame wastes as substrate-support and protein sources.

2. Materials and Methods

2.1. Conditioning of the Microorganism

In this study, the Y. lipolytica strain CDBB-L-232 was used, which was preserved in 30% glycerol as a cryoprotective agent at a temperature of −20 °C. This strain is part of the Microbial Culture Collection of CINVESTAV, belonging to the National Polytechnic Institute. The strain was propagated in Erlenmeyer flasks with a capacity of 500 mL; 100 µL were added to each flask, which contained 30 mL of sterile PDA, and incubated at 30 °C for 48 h. After this period, the spores were recovered using 10 mL of a sterile 0.01% (v/v) Tween 80 solution. Subsequently, cryopreservation was performed using a mixture of 8.5% skim milk and 10% glycerol. The strain was stored in 2 mL Eppendorf tubes until its use in SSF and preserved at −20 °C.
2.2. Physical–Chemical Characterization and Preparation of Substrates

The characterization of the residues was determined by the standard method of the AOAC [39]. The apparent density (AD) was determined by the test tube method described by Ansorena [40]. The water absorption index (WAI) was determined by the method of Sharma et al. (27). The pH was measured by potentiometry, and the critical humidity point was measured by the drying method in a thermobalance [41]. The water activity (Aw) of the residues was determined by placing 0.5 g of sample in an Aqualab Series 3B hygrometer and total sugars by the Dubois method [42].

The canola meal, soybean meal, cottonseed meal (harinoline), and sesame meal residues used in this study were obtained from oil processing industries located in the state of Guanajuato, Mexico. Each sample was dehydrated at a temperature of 65–70 °C for 24 h to remove the original moisture. Subsequently, the particle size was reduced to obtain a homogeneous sample in the reactors. Physical tests were performed to determine the Aw, WAI, CHP, and AD of the samples to determine their potential as substrates.

To prepare the fermentation conditions in the solid substrate, 10 g of dehydrated and sterilized samples were placed in Erlenmeyer flasks of 500 mL, sealed with aluminum caps to avoid contamination. The moisture level was adjusted to 60% [6] using a mineral substrate composed of the following components in grams per liter (g/L): KH$_2$PO$_4$·1.0; MgSO$_4$·7 H$_2$O (0.5); KCl (0.5). The pH values of the mineral substrate were adjusted to 4, 5, 6, 7, 8, 9, and 10 using a 0.1 M buffering solution. A microbial inoculum concentration of $2 \times 10^7$ spores/g DM (51, 52) [6,20] was used. The incubation temperature was maintained at 25, 30, 35, 40, and 45 °C for 96 h in a forced-air convection oven (Yamato DKN 302C). Samples of enzymatic production were taken every 12 h.

2.3. Enzymatic Extraction of Fermented Sample

The crude enzyme extract was obtained using the technique described by Abraham et al. [43,44]. For this, the fermented solid material was thoroughly mixed with 10 mL of 50 mM HCl-Tris buffer (tris(hydroxymethyl)aminomethane) at a pH of 8 for 45 min in agitation. Subsequently, the extract was separated by centrifugation (10,000 × g/20 min/4 °C). The enzymatic extract was filtered using Whatman paper with a pore size of 0.45 mm, and the pH of the resulting extract was measured.

2.4. Enzyme Activity Test

The protease activity was determined using the Kembhavi method described by Johnvesly et al. [45]. An amount of 100 µL of the enzymatic extract was added to 900 µL of 1% (w/v) hydrolyzed tyrosine, dissolved in a 0.05 M phosphate buffer at pH 7, and pre-incubated at 40 °C for 10 min. The enzymatic reaction took place for 10 min at a temperature of 40 °C, and it was then stopped by adding 1 mL of 5% (w/v) trichloroacetic acid (TCA). The sample was centrifuged at 4000 × g for 10 min at 4 °C and filtered using Whatman paper No. 1. The soluble peptides present in the filtrate were determined using the Lowry method or the colorimetric method for quantitative protein estimation at 660 nm, using a tyrosine standard curve as a reference. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the conditions of this test.

The quantification of enzymatic activity in unit per liter (U/L) was obtained using the following equation:

$$U/L = \left(\frac{g}{L}\right) \times \left(\frac{Vol.Test}{Vol.E.E.}\right) \times \left(\frac{1 \text{ mol tyrosine}}{181.21 \text{ g/mol}}\right) \times \left(\frac{10^6 \mu \text{mol tyrosine}}{1 \text{ mol tyrosine}}\right) \times \left(\frac{1}{\text{Time}}\right) \times \text{[Dilution factor]}$$

where E.E. = enzyme extract.
2.5. Effect of pH on Enzyme Production

The assays were carried out using a 1% tyrosine solution with a pH of 4, 5, 6, 7, 8, 9, and 10. Buffer solutions were prepared for each pH value using citrates (50 mM) for pH values of 4 and 5, phosphates for pH values of 6 and 7, TRIS-HCl for pH values of 8 and 9, and glycine–NaOH for a pH of 10 [46]. The enzymatic extract was diluted at a ratio of 1:10 in the corresponding buffer solution and incubated for 1 h at 32 °C.

2.6. Effect of Temperature on Enzyme Production

The experiment was conducted by varying the incubation temperature of the enzyme–substrate reaction mixture. The enzymatic extract was pre-incubated at a temperature of either 25, 30, 35, 40 and 45 °C for 1 h. During the incubation period, aliquots were taken every 15 min.

2.7. Statistical Analysis

To assess the effects of pH and temperature on enzymatic production, a Box–Benkhen design was employed. The data were analyzed using ANOVA with a significance level of \( p \leq 0.05 \), utilizing the statistical software SAS 9.4 Institute Inc. (Cary, NC, USA) [47].

3. Results

3.1. Characterization of Agro-Industrial Wastes

Table 1 shows the physicochemical composition of various agro-industrial wastes. All wastes have a moisture level close to 10%, which is the minimum requirement for their use in the oil industry. The crude fiber content should be between 4% and 12%, except in the case of sesame paste, which has a high value of 19.65%. As for crude protein, it varies within a range of 30% to 40%, soybean meal (40.30 ± 0.15%), canola meal (38.76 ± 1.5%), cottonseed meal (31.47 ± 1.4%), and sesame (32.92 ± 1.0%) are the main nitrogen sources for microorganisms in SSF. Crude protein is essential for the synthesis of proteins, nucleotides, and secondary metabolites, which are crucial for the metabolism and growth of microorganisms in EP [35]. The crude protein content is variable in each waste. For example, during dry seasons, canola has a low oil content and a high level of protein [31,48]. Additionally, the quality of canola meal is affected by the type of oil extraction process [31]. The expeller-pressed paste contains more residual oils than solvent-extracted waste [32,49].

<table>
<thead>
<tr>
<th>Component</th>
<th>Soybean Meal</th>
<th>Canola Meal</th>
<th>Cottonseed Meal</th>
<th>Sesame Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dry matter (TDM)</td>
<td>90 ± 1.3</td>
<td>90 ± 1.5</td>
<td>92 ± 3.9</td>
<td>97 ± 0.6</td>
</tr>
<tr>
<td>Humidity</td>
<td>9.9 ± 0.01</td>
<td>9.8 ± 0.03</td>
<td>9.4 ± 0.02</td>
<td>9.1 ± 1.0</td>
</tr>
<tr>
<td>Crude protein</td>
<td>40.30 ± 1.8</td>
<td>38.76 ± 1.5</td>
<td>31.47 ± 1.4</td>
<td>32.92 ± 1.0</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>3.31 ± 1.8</td>
<td>12.42 ± 0.8</td>
<td>13.64 ± 0.5</td>
<td>19.65 ± 0.1</td>
</tr>
<tr>
<td>Ether extract</td>
<td>1.12 ± 0.3</td>
<td>1.59 ± 0.2</td>
<td>2.25 ± 0.5</td>
<td>13.84 ± 1.6</td>
</tr>
<tr>
<td>Nitrogen-free extract</td>
<td>38.5 ± 0.3</td>
<td>36.9 ± 0.8</td>
<td>28.9 ± 0.4</td>
<td>31.4 ± 0.7</td>
</tr>
<tr>
<td>Neutral detergent fiber (NDF)</td>
<td>13.50 ± 3.2</td>
<td>25.3 ± 0.50</td>
<td>39 ± 0.1</td>
<td>22.6 ± 0.4</td>
</tr>
<tr>
<td>Ash</td>
<td>8.02 ± 0.2</td>
<td>9.45 ± 0.6</td>
<td>8.81 ± 0.3</td>
<td>8.35 ± 0.2</td>
</tr>
<tr>
<td>Total sugars</td>
<td>0.93 ± 0.01</td>
<td>0.84 ± 0.2</td>
<td>0.73 ± 0.03</td>
<td>0.83 ± 0.1</td>
</tr>
<tr>
<td>Water absorption index (WAI) (gel/g DM)</td>
<td>4.35 ± 0.1</td>
<td>4.24 ± 1.0</td>
<td>3.14 ± 1.0</td>
<td>3.05 ± 0.5</td>
</tr>
<tr>
<td>Critical humidity point (CHP)</td>
<td>17.5 ± 0.5</td>
<td>23.3 ± 0.8</td>
<td>16.6 ± 0.6</td>
<td>13.6 ± 0.5</td>
</tr>
<tr>
<td>Water activity (Aw)</td>
<td>0.46 ± 0.02</td>
<td>0.34 ± 0.03</td>
<td>0.487 ± 0.1</td>
<td>0.310 ± 0.3</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ± 0.15</td>
<td>5.9 ± 1.42</td>
<td>6.9 ± 0.31</td>
<td>6.5 ± 0.23</td>
</tr>
<tr>
<td>Apparent density, AD (g/mL)</td>
<td>0.64 ± 0.0</td>
<td>0.50 ± 0.6</td>
<td>0.60 ± 0.1</td>
<td>0.43 ± 0.2</td>
</tr>
</tbody>
</table>

Data correspond to the mean values ± standard deviation. The means with different lowercase letters among columns differ significantly (\( p \leq 0.05 \).
Sesame waste recorded the highest value of ether extract, at 13.84 ± 1.6, while soybean meal, canola meal, and cottonseed meal had very similar values of 1.12 ± 0.3, 1.59 ± 0.2, and 2.25 ± 0.5, respectively. This component plays a fundamental role in substrates, providing the necessary energy and carbon source for the cellular functioning and development of microorganisms. Regarding the NDF value, cottonseed meal had the highest value, at 39 ± 0.1%, while soybean had the lowest value, at 13.50 ± 3.2%. Canola meal and sesame meal registered values of 25.3 ± 0.50% and 22.6 ± 0.4%, respectively. The higher this value, the lower the degradability and organic matter consumption by microorganisms, as this fraction represents the cell wall composed of cellulose, hemicellulose, and lignin, which are generally difficult for microbes to break down to access nutrients.

The fiber content has a significant impact on the WAI, according to Wanasundara and Shahidi [33]. They stated that solvent-extracted oilseed pastes have higher water adsorption, possibly due to the presence of polysaccharides in the husk. Ye et al. [50] mentioned that this index is a measure of the volume occupied by starch after swelling due to excess water. Pryor and Chang [51] conducted an evaluation of this index in canola and soybean wastes, finding that the water absorption capacity in both wastes exceeded 200%. Their results are consistent with the values reported for various varieties of oilseed wastes, ranging from 209% to 380% [52,53]. Important factors can be considered from these results: when the particle size exceeds a particular value, the enzyme production can be affected owing to the deduction of the contact surface between the substrate’s particles and fungus. However, small particle sizes affect the air supplied in terms of aeration, which can hinder microbial growth [54].

3.2. Growth of Y. lipolytica in the Substrates

The results obtained demonstrate that the Y. lipolytica strain exhibits the ability to efficiently utilize the nutrients present in soybean meal, canola meal, cottonseed meal, and sesame wastes, at different rates for each substrate (see Figure 1). Furthermore, it was observed that the optimal time for maximum enzyme synthesis was 48 h under initial conditions of 50% humidity, an inoculum concentration of $2 \times 10^7$ spores per gram of DM, and an incubation temperature of 40 °C.

![Figure 1. Agro-industrial waste used in SSF: canola meal, cottonseed meal (Harinoline), sesame, soybean. Source: by the author.](image-url)
It can be observed that the substrate that showed the best performance was canola, with an enzymatic production of 46.28 U/L. It was followed by soybean with 40.54 U/L, cottonseed meal with 20.21 U/L at 72 h, and sesame with 31.47 U/L up to 84 h. Figure 2 shows that both canola and cottonseed meal exhibited rapid strain adaptation to the substrate during the initial hours, while soybean and sesame showed microbial growth up to 12 h. Therefore, the adaptation and consumption of components were slower in the latter substrates. These data indicate the strain’s ability to develop in all of the evaluated wastes.

![Enzyme Production](image)

**Figure 2.** Kinetics of initial SSF of *Y. lipolytica* strain to synthesize proteolytic enzymes on agro-industrial wastes, humidity 50%, inoculum $2 \times 10^7$ spores/g DM, temperature 40 °C at 96 h.

### 3.3. Effect of pH on Enzyme Production

The microorganism adapted uniformly to each waste, as shown in Figure 3. A high cell concentration was observed in the canola meal and soybean wastes, while the concentration was lower in cottonseed meal and sesame. The optimal pH for catalysis was determined to be 7, with EP ranging from 4 to 10. The ideal conditions for EP included a modified humidity of 60% and a temperature of 30 °C.

![Microorganism Adaptation](image)

**Figure 3.** Cont.
The synthesis of acid and alkaline proteases showed the same trend at pH values of 4–6 and 8–10, as observed in Figure 4. This allowed for doubling the catalytic activity of the enzyme compared to the levels obtained in the preliminary assay. Therefore, pH plays a crucial role in solid substrates, as it can either promote the production of bioactive compounds of interest or inhibit their formation, depending on specific needs.

In terms of the recorded values, maximum EP levels of 98.45 U/L were obtained for canola 85.48 U/L, for soybean 78.72 U/L, for sesame and 52.12 U/L for cottonseed meal. The synthesis of acid and alkaline proteases showed the same trend at pH values of 4–6 and 8–10, as observed in Figure 4. This allowed for doubling the catalytic activity of the enzyme compared to the levels obtained in the preliminary assay. Therefore, pH plays a crucial role in solid substrates, as it can either promote the production of bioactive compounds of interest or inhibit their formation, depending on specific needs.

**Figure 3.** Growth of *Y. lipolytica* CDBB-L-232 in SSF on agro-industrial wastes: (a) soybean, (b) canola meal, (c) cottonseed meal, (d) sesame. Humidity of 60% and temperature of 30 °C at 48 h.

**Figure 4.** Effect of pH on EP at 48 h of SSF (60% moisture, inoculum 2 × 10⁷ spores/g DM) on agro-industrial wastes by *Y. lipolytica*.

### 3.4. Effect of Temperature on Enzyme Production

The increase in temperature of the substrate leads to an increase in the reaction rate, eventually reaching the optimum temperature. However, once this point is surpassed, the production begins to decline. Figure 5 represents the behavior of the microorganism when modifying the temperature of the substrate, and it can be observed that the ideal...
temperature for the synthesis of neutral proteases is 30 °C. Figure 5 shows the behavior of protease enzyme synthesis in the substrates used. After 48 h of fermentation, the best enzymatic production response was observed at 30 °C for soybean meal, canola meal, and cottonseed meal by-products, while sesame showed the best response at 35 °C. In all cases, there was evidence of proteolytic production. Enzymatic production reached its maximum in the following order: canola meal showed the best result with 188.75 U/L, followed by soybean meal with 117.07 U/L, cottonseed meal with 66.71 U/L, and finally, sesame with 85.51 U/L.

Figure 5. Effect of temperature on EP at 48 h of SSF (60% humidity, inoculum 2 × 10⁷ spores/g DM) on agro-industrial wastes using Y. lipolytica.

At this temperature (30 °C), the microorganism was able to break down the structure and access the substrate’s nutrients more effectively. The role of temperature in the performance of the microbial strain on agro-industrial wastes increased enzyme productivity by 48% for canola, 27% for soybean, 22% for cottonseed meal, and 8% for sesame, for the values obtained for the effect of pH on EP.

4. Discussion

In the oil industry, it is common for waste or by-products to be generated that cannot be reused as raw materials in the production chain [55]. In the agro-industrial sector, this is no different, as each subsector produces specific wastes. According to Saval’s definition [20], these wastes are solid or liquid materials that are obtained from the direct consumption of primary products or their industrial processing. Although they are no longer useful for the process that generated them, there is still the possibility of utilizing or transforming them to obtain other products of economic, commercial, or social value.

It has been established that Y. lipolytica is a strictly aerobic species that tends to grow as a contaminant in substrates that are rich in lipids and proteins, such as cheese, yogurt, sausages, and meats. Its presence has also been observed in other substrates, such as alcohols and acetates. This species possesses the remarkable ability to store lipids as well as degrade hydrophobic substrates such as fish oil and tallow. Additionally, it is notable for its ability to secrete proteins, among other characteristics [56].

4.1. Characterization of Agro-Industrial Wastes

All components present in a solid-state biological process are of vital importance and have diverse effects on the microorganism’s performance in the production of target metabolites. Therefore, it is essential to have precise knowledge of the proportions of these
components before conducting the experiment or incorporating them appropriately. The characteristics of CHP, WAI, AD, and Aw fall within ranges that allow optimal performance of the microorganism in SSF, as can be seen in Table 1. Furthermore, it is important to note that the initial pH of each substrate should be maintained in the range of 5.9–7.0 [18]. Slight variations in pH were observed after fermentation. Wastes derived from the industrial processing of agricultural products are an abundant source of minerals, sugars, proteins, fats, and fibers. However, the quantification results of these components can vary depending on the analysis method used, as well as the handling of harvest conditions and environmental conditions during crop growth [57–59].

In an SSF, controlling the moisture content in the substrate is a crucial factor to consider, as it affects the efficiency of processes [60]. It is important to maintain a minimum amount of water in this type of process, as excess moisture can limit the space between the pores of the material, thus preventing the presence of necessary oxygen [61]. At the end of an SSF, a significant loss of water commonly occurs, which varies depending on the characteristics of the reactor used. The optimal moisture content for microbial growth is typically between 40% and 70%, depending on the microorganism and substrate employed [22,62].

4.2. Effect of pH, Temperature, and Fermentation Time on Enzymatic Production

During the enzyme recovery process, it is crucial to identify the most favorable conditions for extraction, as this can have a significant impact on the outcome. Among the variables to consider are the type of solvent, pH, solid–liquid ratio, temperature, agitation type, and contact time [22,63,64]. As shown in Figure 4, all wastes exhibited satisfactory EP. The pH in the substrate has an impact on proton concentration, which can alter the structure and growth rate of the enzyme. At alkaline or acidic pH, the enzyme can denature and become inactive [36]. Additionally, previous research by Germano et al. [65] and Hernández et al. [66] emphasizes the importance of rigorously controlling pH during the enzyme recovery process, as it can affect its stability. According to the study by Morcelle del Valle et al. [67], proteases can function in a wide pH range, and this parameter plays a crucial role in their biological activity. It is proposed that the active sites of proteolytic enzymes are composed of ionizable groups that must be in the proper ionic form to maintain the conformation of the active site, thereby allowing substrate binding and catalysis of the reaction. Additionally, James [68] states that aspartic peptidases are primarily active at acidic pH, although a percentage also show activity under neutral conditions, while carboxypeptidases exhibit catalytic activity within a pH range from 6 to 9 [69,70].

It was observed that the highest levels of EP for each waste were obtained at a pH of 7. López-Flores et al. [15] reported similar results in the production of neutral enzymes by Y. lipolytica using soybean residue and found that at pH 7, a temperature of 45 °C and 24 h of culture, the maximum production of protease was 105 U/mL, similar to the value found in the present work (117.07 U/L) at a temperature of 30 °C and 48 h of culture. The difference in temperature and time of maximum production could have been due to the strain used, particle size of the substrate, nutrient composition, humidity, oxygen concentration, and agitation. Instead, Farias et al. [38] reported that soybean meal and cottonseed meal showed promising results for protease production by achieving 139 ± 4 U/g and 102 ± 6 U/g, respectively, at 48 h of solid fermentation, using Y. lipolytica without the use of any supplement in the culture medium, while Carvalho et al. [35] reported that a mixture of soybean residue and andiroba (Carapa guianensis) (50:50 ratio) produced an amount of 82.52 U/g of proteases using Y. lipolytica IMUFRJ50682 in 24 h of fermentation at 28 °C. The literature reports that most yeasts have a metabolic activity of maximum enzyme production at temperatures of 24–48 °C; above these temperatures, stability in enzyme production is lost. Most of the proteases synthesized by Y. lipolytica have an affinity for slightly acidic and alkaline media [63]. It has been reported that Y. lipolytica possesses recombinant genes capable of generating three types of proteases: acid, neutral and alkaline. Therefore, it can be deduced that the proteases produced under the conditions of this study are mostly neutral, depending on the initial pH of the experiment [71,72].
Figure 4 shows how the change in pH increases the production of enzymes, obtaining that at a pH of 7, the maximum quantity (98.45 U/L) was produced, which reflects the mesophilic nature of the microorganism. On the other hand, at a pH lower than 7 and higher than this value, acid, and alkaline enzymes were synthesized, in which the values were similar. The initial pH of the samples is acid due to the presence of free fatty acids present in the oily matrix, and this condition favors the growth of *Yarrowia lipolytica* since this yeast needs a slightly acid medium for good growth [35]. A significant change in pH in the culture medium can generate a significant increase or decrease in the production of proteases.

The use of filamentous fungi for the synthesis of proteases also becomes important in SSF. De Castro and Sato [36] reported the production of proteases in soybean meal (147.81 U/g) and cottonseed meal (186.81 U/g) at a temperature of 40 °C during 48 h of fermentation, using a strain of *Aspergillus niger*. According to this, the production of proteases shows that substrates with high protein content induce the production of this enzyme during the first 48 h of fermentation [36,37]. Freitas et al. [60] have reported on the production of neutral proteases using canola meal, achieving 64 U/g of proteases from *A. oryzae* CCBP 001 and 25 U/g using *A. niger* IOC 4220 in the same period under different conditions. In a similar study, Sandhya et al. [3] reported similar EP values using *Penicillium* and *A. oryzae* NRRL 1808 in SSF and SmF, with the latter capable of synthesizing the enzyme in high quantities. *R. oligosporum* cultivated on rice bran, an increase in its proteolytic activity was observed at pH 7, but this activity decreased by up to 16% at pH 9, according to the study conducted by Ikasary and Mitchell [34,73]. In the present study, the EP titers obtained exceed those reported by the previously mentioned authors under similar conditions.

The substrates used in this research are mainly oilseeds composed of proteins and oils remaining after processing. These compounds can be significantly different from other agro-industrial substrates such as fruit and vegetable peels, starch, molasses, husks, or chemical substrates used in solid-state fermentations commonly used for the synthesis of enzymes or biological compounds such as organic acids, aromatic compounds, biofuels, antibiotics, among others. Oilseed residues are mainly used for the synthesis of hydrolytic enzymes and lipases, according to the literature. In SSF processes, the function of enzyme inducers stands out; in the case of canola and sesame, the main inducer of proteases are their respective oils. High levels of oils accelerate the synthesis of enzymes, contrary to if the levels are low, while in soybean and cottonseed meal, reports suggest that the main inducers of protease and lipase enzymes come from carbon/nitrogen sources in the culture medium. According to what has been reported in the literature, conventional yeasts are good producers of proteolytic enzymes. On the other hand, in the production of enzymes using various strains of *Y. lipolytica*, titers have been determined up to three times higher than those obtained by conventional strains modeling similar culture conditions. For this reason, *Y. lipolytica* is considered a strain with excellent yields in the secretion of hydrolytic and lipolytic enzymes in the biotechnological treatment of hydrophobic substrates.

In this study, temperature had the greatest effect on the production of the proteolytic enzyme, as shown in Figure 5. The five temperatures evaluated produced enzymatic activity. However, at 25 °C, it was noted that the microorganism does not have enough energy to colonize the culture medium, which reflects a poor enzyme profile. At 30 °C, the maximum production of the protease is reached. As the temperature increases, there is an exponential drop in production because the microorganism begins to lose stability due to the heat generated in the culture medium. The culture medium limits the energy transfer phenomena, causing overheating and, therefore, a loss of moisture from the culture medium, which causes a limitation in the availability of nutrients for the growth of the microorganism [60,65], so it is not convenient to use temperatures above 45 °C in solid-state fermentation using microorganisms of the *Yarrowia* genus.

Microbial growth rates increase by approximately 4% to 8% per degree Celsius as temperature increases. However, at high temperatures, protein denaturation can decrease
product formation. To make a comparison with other temperatures, it is important to consider the temperature coefficient, which is around 6% per degree Celsius [64,74,75], as a relevant approximation. According to Dos Santos and Sato [30], enzymes are very sensitive to temperature. At low temperatures, most enzymes show little activity because there is not enough energy for the catalyzed reaction to take place. At higher temperatures, enzyme activity increases as the reactant molecules move faster to generate more collisions with the enzymes. At temperatures above 50 °C, the tertiary structure, and thus the shape of most proteins, is destroyed, causing a loss of enzymatic activity. Temperature is the parameter that produced the best results in this study concerning pH since it was possible to increase the production of proteases.

5. Conclusions

The four substrates investigated show great potential as raw materials for the production of hydrolytic enzymes. The capacity to synthesize acidic, basic, and neutral enzymes has been observed using these wastes, although in lesser amounts for acidic and basic enzymes compared to neutral ones. Despite this, it is important to highlight that appropriate methodologies can be developed to obtain acidic or basic enzymes according to specific needs.

The optimal temperature for the growth of the microorganism used in this study has been determined to be 30 °C, while the ideal pH for the Y. lipolytica strain is 7. These findings support the feasibility and profitability of using protein-rich agro-industrial wastes for the production of proteolytic enzymes. The physical properties of these substrates facilitate the proper implementation of a solid-state process.

The results of this study highlight the competitive potential of the Y. lipolytica CDBB-L-232 strain in the production of proteases compared to the major producers of proteases in the Aspergillus, Penillium, S. cereviceae, and R. oligosporum, as reported in the scientific literature using different agro-industrial wastes.

Temperature is the factor that exerts the greatest influence on the synthesis of neutral proteases than pH and the source of nutrients.

It is essential to continue exploring and optimizing these methodologies to fully harness the potential of these substrates and advance the development of high-quality and efficient hydrolytic enzymes.


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References


35. Carvalho, A.S.; Sales, J.C.; Nascimento, F.V.; Ribeiro, B.D.; Souza, C.E.; Lemes, A.C.; Coelho, M.A. Lipase Production by *Yarrowia lipolytica* in Solid-State Fermentation Using Amazon Fruit By-Products and Soybean Meal as Substrate. **Catalysts** 2023, 13, 289. [CrossRef]


41. AOAC. Protein (Crude) in Animal Feed, Forage (Plant Tissue), Grain, and Oilseeds. **Block Digestion Method Using Copper Catalyst and Steam Distillation into Boric Acid; AOAC Official Method; AOAC: Washington, DC, USA, 2001**; p. 3.


50.표현 기술 제고를 위한 캐나다 쓰레기 생산공정의 향상: 2007년 연합 가공화학자 협회 발표자료, 29. [CrossRef]

51. Pryor, S.W.; Chang, S.K.C. Separation and Evaluation of Canola Meal and Protein for Industrial Bioproducts; An ASABE Section Meeting Presentation Paper Number: RRV-07116; American Society of Agricultural and Biological Engineers: St. Joseph, MI, USA, 2007; pp. 2–11. [CrossRef]

52. Aider, M.; Barbana, C. Canola proteins: Composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity—A practical and critical review. **Trends Food Sci. Technol.** 2011, 22, 21–39. [CrossRef]


55. Rosas, D. Revalorización de algunos residuos agroindustriales y su potencial de aplicación a suelos agrícolas. **Agro Product.** 2018, 9, 18–23.

56. Guo, X.; Tomonaga, T.; Yanagihara, Y.; Ota, Y. Screening for yeasts incorporating the exogenous eicosapentaenoic and docosahexaenoic acids from crude fish oil. **J. Biosci. Bioeng.** 1999, 87, 184–188. [CrossRef]


60. Freitas, A.C.; Farinas, C.S.; Castro, R.J.; Fontenele, M.A.; Pinto, G.A.; Egitto, A.S. Canola Cake as a Potential Substrate for Proteolytic Enzymes Production by a Selected Strain of *Aspergillus oryzae*: Selection of Process Conditions and Product Characterization. **ISRN Microbiol.** 2013, 8, 369082. [CrossRef]


63. Coelho, M.A.; Amaral, P.F.; Belo, I. *Yarrowia lipolytica: An Industrial Workhorse; Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*; Formatex Research Center: Badajoz, Spain, 2010; Volume 2, pp. 930–944.


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