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# Gel Electrophoresis for Investigating Enzymes with Biotechnological Application

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## 1. Introduction

Enzymes are vital biological catalysts with essential action in the metabolism of all living beings. Moreover, enzymes have a very significant role in various industrial sectors, including baking, brewery, detergents, textile, pharmaceutical, animal feed, cellulose pulp biobleaching, biofuels and others (Polizeli et al., 2005; 2009; 2011). Industrial enzymes are especially produced from microorganisms, such as bacteria, yeasts and filamentous fungi.

Here, we describe some basic knowledge about microbial enzymes with potential application in industry, their properties and some biochemical methods for the detection of amylase, pectinase, proteases, and xylanase activities on polyacrylamide gel electrophoresis. These methods are more qualitative procedures than quantitative, once they may be used to confirm the electrophoretic homogeneity of purified enzymes through chromatographic process. In addition, we described the principle of each method approached in this chapter, which grants a better understanding of each procedure.

### 1.1 Overall considerations about industrial enzymes

Enzymes are optimum biological catalysts present in all living beings, and they, under adequate conditions, catalyze in their active sites the natural substrates from the metabolic route reactions. Quite often, the metabolic enzymes act in a sequence, and the product generated in a reaction becomes the substrate for the following phase, as diagramed:



Some properties of the enzymes make them excellent competitors against traditional chemical catalysts, due to their great catalytic efficacy (kcat), considering that the main

objective of any biological transformation is to obtain a short-term high conversion of substrate into product. Besides this characteristic, enzymes present a high specificity and selectivity, according to their metabolic role, acting in optimum conditions of pH and temperature. Also, they do not pose damage to the environment because they are biodegradable.

Applied biological catalysis had its origins in ancient China and Japan, in the manufacturing of foods and alcoholic beverages in which amylases and proteases of vegetal and microbial origins were employed. It dates back to the end of the XIX century with the introduction of standardized preparations of rennilases in the production of cheese. After this period, the implementation of new industrial applications of enzymes was slow, arising intensively in the last 40 years.

The main reasons for the current importance of enzymes in the industrial scenario are due to the development of application processes of proteases in detergents, pectinases in juices and glucoamylase in the production of glucose from starch. Additionally, the employment of recombinant DNA techniques allowed the obtainment of high productivity and the most suitable design of enzymes.

Following, we present the application of enzymes in several technical sectors justifying the development of methods for the visualization of enzymatic activities in electrophoresis gels.

## 1.2 Some industrial enzymes and their applications

Proteases, amylases, cellulases and lipases are used in the preparation of detergents and that is the greatest industrial market. Enzymes are added to increase wash efficiency. For so, they require lower usage temperatures, reduce wash periods and agitation costs. They act on both proteic and starchy residuals as well as on fats; they enhance clothes softness and restore color brightness. They require, in general, suitable thermostability and activity in alkaline pH.

Animal feed – the addition of cellulases, xylanases, proteases, lipases, ligninases and phytases in ruminants and monogastrics foods leads to the digestibility of grass and forage, reduces pollutants, decreases the release of carbon dioxide, soluble carbon hydrates and phosphorus.  $\beta$ -glucans increase food viscosity decreasing starch digestibility, but the addition of  $\beta$ -glucanases increases food assimilation, resulting in weight gain (Facchini et al., 2011a, 2011b).

Swine and poultry feed needs the addition of phytase, a phosphatase that acts on acid or alkaline media and dephosphorylates phytic acid releasing phosphorus to the environment (Haefner et al., 2005). That can damage the environments where the soil contains plenty of phosphate, as it is observed in Europe.

Xylanases and ligninases may also take part in the biobleaching of the cellulose pulp for the manufacturing of paper, whereas cellulases are used for the modification of the textile fiber properties, giving them the pre-wash effect. Those three enzymatic systems participate in the sugar cane hydrolysis for the bioethanol manufacturing. Such procedure has been widely adopted in many countries like Brazil, which has a number of flexpower vehicles (Betini et al., 2009; Michelin et al., 2009, 2011).

Proteases and lipases also have a role in the dairy industry, acting in the production of cheese (Gupta et al., 2002). Chemokine, extracted from the stomach of calves, acts in the Milk coagulation, leading to the formation of cheese. Lipases give the aroma and hot flavor in cheese (Hasan et al., 2006). Still in the food industry, pectinases and cellulases increase the extraction of oils through pressing (coconut, sunflower, soybean, olive, etc.).

One sector that has been widely economically explored is the application of amylases in processes of starch saccharification (Silva et al., 2009a, 2009b). For so, there is the need of several enzymes such as  $\alpha$ -amylase, which forms maltooligosaccharides; glucoamylases and  $\beta$ -amylases, which hydrolyze starch to glucose and maltose and the glucosyltransferases with the production of cyclodextrins. With synergistic action of the amylolytic system, there is the production of maltose syrup used in breweries, as well as the glucose syrup which is preferably converted by glucose isomerase to fructose syrup, due to the high sweetening power. Such compounds may be used in the manufacturing of sauce, child feeding, gums, candies, ice-cream, pharmaceutical products, canned products etc. Amylases also act in the textile industry to remove starch added to cotton to increase resistance (Gupta et al., 2003).

In the pharmaceutical industry, the use of enzymes is increasingly growing, reflecting the *in vivo* catalysis potential. We can highlight the use of pancreatin, obtained from the swine pancreas, which is used as adjuvant in the digestive process of people who have genetic disorders leading to digestive problems or who, due to surgical removal of the pancreas or precocious aging, present digestive problems. Many enzymes are used as therapeutical agents, such as asparaginase and glutaminase, collagenase, hyaluronidase, ribonuclease, streptokinase, uricase and uroquinase (Prakashan, 2008).

Semi synthetic penicillins (ampicillin and amoxicillin) were launched in the market to replace penicillin, given the acquired resistance of some microorganisms. All kinds of penicillin have the same basic structure: 6-aminopenicillanic acid (6-APA), a thiazolidine ring bound to a beta lactam that takes a free amino group. In the synthesis of semi synthetic penicillin there is the enzymatic hydrolysis of G penicillin with the penicillin G acylase. After purified and concentrated, the 6-APA released in the hydrolysis is used as an intermediary in the synthesis of amoxicillin or ampicillin (Cabral et al., 2003).

The enzymes can be used in the cosmetics industry in creams against skin aging and acne, buffing cream, oral hygiene and hair dying.

Enzymes may be used in analytical applications, due to their high specificity, identifying substances in complex mixtures such as blood, urine and other biological fluids. They participate in tests for glucose (glucose oxidase), urea, amino acids, proteins, ethanol, etc (Godfrey & West, 1996).

In fine chemistry, the list of compounds produced by enzymatic biocatalysis is huge, and we highlight, as an example, vitamin C and several L-amino acids. Acrylamide is used as a monomer in the production of polyacrylamide, widely used as flocculating polymer. Acrylamide was initially produced chemically, but the technological disadvantages such as the formation of toxic residuals and the costs in the purification process made the enzymatic way a viable process.

## 2. Some industrial enzymes and their applications

### 2.1 Preparation of polyacrylamide gel electrophoresis for activity enzymatic

The detection of enzymatic activities for industrial use, in electrophoresis gel, happens when PAGE (polyacrylamide gel electrophoresis) is employed, which is an electrophoresis performed in non-denaturing conditions. The Figure 1 illustrates some steps used to preparation of the electrophoresis gel.

In this kind of procedure, there is not preferably the addition of the sodium dodecyl sulfate – SDS detergent,  $\beta$ -mercaptoethanol or another reducing agent, such as dithiothreitol - DTT or urea. Also, the protein samples in its native form (not denaturated) are not boiled before their application in the gel because enzymes will lose its activities, if denaturated. The enzymatic activity may also be detected in gels of the SDS-PAGE type, if the samples were not boiled or added by any reducing agent that denatures the protein. Sodium dodecyl sulfate (SDS) is an anionic surfactant whose role is to bestow the proteins with uniform load density. SDS presents a high negative load and a hydrophobic tail that interacts with the polypeptidic chains in an approximated ratio of 1.4 g of SDS for each gram of protein, making them negatively loaded. In the lack of SDS, the proteins with equal mass may migrate differently in the pores of the gel due to the load differential of their tridimensional structures.

PAGE may be performed in a pH 4.5 or 8.9, depending on the isoelectric point - pI, of the sample under study. In order to accomplished zymograms, it is performed SDS-PAGE; however, the samples generally correspond to a crude extract or a partial purified extract which are either not boiled or added by  $\beta$ -mercaptoethanol, DTT or urea.

### 2.2 Preparation of the sample for application in electrophoresis

The preparation of proteic solutions for the application in electrophoresis is an important phase. It is important to highlight once again that most enzymes used industrially have microbial origin (fungi, yeasts or bacteria) and also that normally, their synthesis is followed by the elimination of a number of primary and secondary metabolites produced by the very microorganisms, as well as other compounds present in the cultivation medium, such as vitamins, salts, carbohydrates, amino acids and peptides.

In order to avoid the interference of such factors in the electrophoresis, especially when there is the application of enzymatic extracts without previous purification, it is necessary to pay close attention to the type of sample that is being prepared.

Below are some measures and precautions that must be adopted:

- i. Dialysis: This procedure aims at the removal of substances with smaller molar mass, such as salts, carbohydrates and amino acids, which may interfere in the electrophoresis quality;
- ii. Attention to the concentration of the sample applied in the electrophoresis. In general lines, around 10  $\mu$ g of proteins is necessary for a good visualization in the electrophoresis gel after the dying phase. For the detection of the enzymatic activity, considerable enzymatic levels are necessary. If the protein solution presents a lower concentration, the application of any procedure for the concentration of proteins is

necessary, such as lyophilization, use of filtering membranes with defined molar mass, ammonium sulfate precipitation and even the use of solvents (ice acetone or ethanol). We must bear in mind that those two last processes need an additional dialysis for the removal of the ammonium sulfate or carbohydrates when the precipitation happens through the action of solvents.

- iii. For the detection of activity in electrophoresis gel, the run must take place in low temperatures, such as a refrigerator or a cold chamber.
- iv. Attention must also be paid to the native load of proteins and the separation must depend only on its molar mass. For so, proteins may be mixed with SDS, becoming negatively loaded, as it has already been described.

## 2.3 Electrophoresis separation techniques

### 2.3.1 Electrophoresis in non-denaturing conditions (PAGE)

Electrophoresis is going to be performed in pH (4.5 or 8.9), in a polyacrylamide gel that may range from 5 to 15%, depending on the size and the load of the protein under study. For proteins loaded negatively, the running buffer will consist of Tris-HCl and glycine, pH 8.9. For proteins loaded positively, there is going to be a buffer with  $\beta$ -alanine and glacial acetic acid, pH 4.5. Both procedures must be performed at 4°C. Table 1 indicates necessary volumes to obtain PAGE gels with different concentrations.

#### 2.3.1.1 PAGE for acid proteins (-), (Davis, 1964)

##### Solution A

Tris-HCl	9.75 g
HCl (1 M)	12 mL
TEMED	0.05 mL
Distilled water	25 mL
pH adjusted for pH 8.7	

##### Solution C

Acrylamide	9.6 g
Bis-acrylamide	0.32 g
Distilled water	20 mL
pH adjusted for pH 8.9	

##### Solution G

Ammonium persulfate	0.007 g
Distilled water	5 mL

##### Dye

Bromophenol blue  
Glycerol

##### Running buffer

Tris-HCl 50mM  
glycine 36mM, pH 8.9

Preparation of the samples and markers:

- In a sterile microtube, put 18  $\mu\text{L}$  of proteic sample and 2  $\mu\text{L}$  of running buffer Tris-HCl 50mM and glycine 36mM, pH 8.9;
- Add 2  $\mu\text{L}$  of the dying solution Bromophenol blue 0.1% and 4  $\mu\text{L}$  of glycerol.

### 2.3.1.2 PAGE alkaline proteins (+), Reisfeld et al. (1962)

<b>Solution A</b>	
KOH (1 M)	48 mL
Glacial acetic acid	17.2 mL
TEMED	4 mL
Complete with distilled H <sub>2</sub> O	100 mL
pH adjusted for 4.3	

<b>Solution C</b>	
Acrylamide	19.2 g
Bis-acrylamide	0.54 g
Complete with distilled H <sub>2</sub> O	40 mL
pH adjusted for 4.5	

<b>Solution G</b>	
ammonium persulfate	0.28 g
Complete with distilled H <sub>2</sub> O	100 mL

**Dye**  
Methyl green  
Glycerol

Sample preparation:

- In a sterile microtube put 18  $\mu\text{L}$  of sample and 2  $\mu\text{L}$  of running buffer consisting of 31.2 g of  $\beta$ -alanine, 8 mL of glacial acetic acid and an amount of distilled water sufficient to reach the volume of 1000 mL;
- Add 2  $\mu\text{L}$  of the solution Methyl green 0.1% and 4  $\mu\text{L}$  of glycerol.

Solution	Polyacrylamide concentration (%)							
	4	5	6	7	8	10	12	14
A (mL)	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05
C (mL)	0.71	0.876	1.05	1.22	1.49	1.77	2.10	2.45
H <sub>2</sub> O (mL)	6.65	6.47	6.30	6.13	5.97	5.60	5.25	4.95

Table 1. Preparation of 8.4 mL of PAGE in different concentrations.

- Set up the electrophoresis bowl;
- In a Becker, add the solutions A, C and H<sub>2</sub>O. Mix;

- Add 50  $\mu\text{L}$  of ammonium persulfate 10% and quickly put the mixture in the electrophoresis bowl;
- Place the comb;
- Wait until the gel solidifies and apply the samples (up to 30  $\mu\text{L}$ ) in different lanes;
- Connect the energy cables in the respective jacks of the energy source adjusted to 70 mAmps and 120 Volts;
- Turn on the source and wait for the samples to run throughout the gel before turning it off;
- Remove the gel carefully and process it.

### 2.3.2 Electrophoresis gel SDS-PAGE, Laemmli (1970)

The electrophoresis must be performed in pH 8.9 and in the presence of SDS (sodium dodecyl sulfate), with the gel concentration ranging from 5 to 15%. Table 2 indicates necessary volumes to obtain SDS-PAGE gels with different concentrations.

#### Solution A

Tris-HCl	36.5 g
TEMED	230 $\mu\text{L}$
Distilled water	9 mL

Adjust the pH with HCl concentrated for pH 8.9 and store it at 4-6°C.

#### Solution C

Acrylamide	28 g
Bis-acrylamide	0.74 g
Distilled water	100 mL

Store at 4-6°C in a glass flask with Amberlite resin due to the degradation of acrylamide in acid and ammonia.

#### Solution E

SDS	0.21 g
Distilled H <sub>2</sub> O	100 mL

Store at room temperature.

#### Sample buffer

Tris-HCl	0.755 g
Glycerol	1 mL

Dissolve with 17.5 mL of distilled H<sub>2</sub>O and adjust the pH to 6.75 with concentrated HCl and add:

SDS	2 g
Bromophenol blue	0.001 g
Distilled water	100 mL

Freeze aliquots for a further use.

#### Run Buffer

Tris	3.025 g
glycine	14.4 g
SDS	1.0 g

Dissolve in an amount of distilled water sufficient for 1000mL, pH 8.9.

Preparation of samples and markers:

- In a sterile microtube, place a sample buffer and a molar weight marker in the ration of 1:1;
- In a sterile microtube, place the buffer sample and the sample in the ratio of 1:3.

Solution/Reagent	Gel concentration (%)					
	5	6	7	8	10	12
A (mL)	1	1	1	1	1	1
C (mL)	1.4	1.7	2	2.3	2.85	3.45
E (mL)	3.8	3.8	3.8	3.8	3.8	3.8
H <sub>2</sub> O (mL)	1.8	1.5	1.2	0.9	0.35	-
Ammonium persulfate(g)	0.00425	0.00425	0.0057	0.006	0.007	0.00708

Table 2. Preparation of SDS-PAGE

- Set up the electrophoresis bowl;
- In a Becker, add the solutions A, C, E and H<sub>2</sub>O and mix;
- Add the ammonium persulfate and quickly place the solution in the electrophoresis bowl; Place the comb;
- Wait until the gel solidifies and apply the patterns (0.5-5  $\mu$ L) and the samples (up to 30  $\mu$ L) in different lanes;
- Connect the energy cables in the respective jacks of the source regulated for 70 mAmps and 120 Volts;
- Connect the source and wait until the samples run throughout the gel before turning it off;
- Remove the gel carefully and process it.

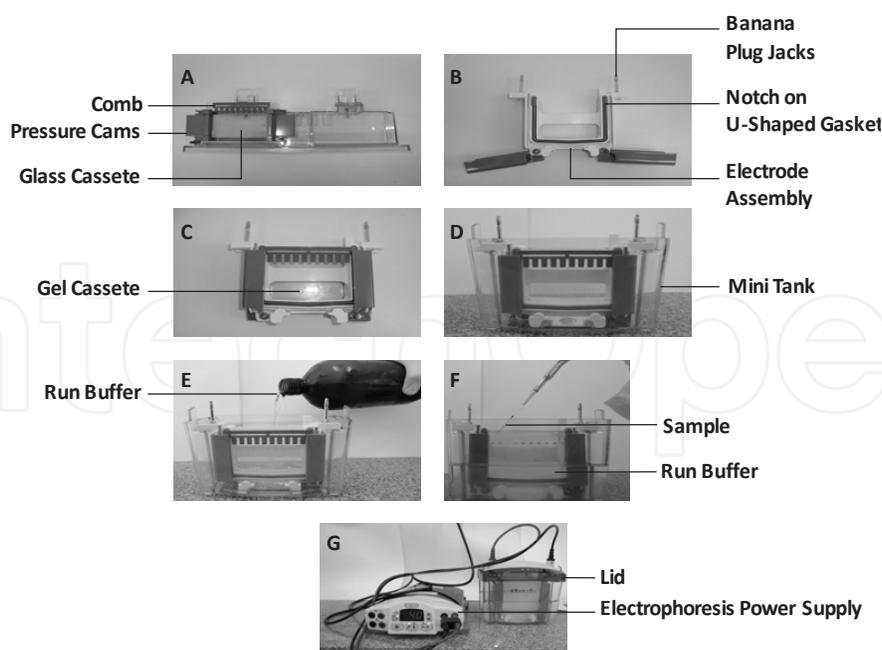


Fig. 1. Preparation and development of vertical polyacrylamide electrophoresis gel using Bio-Rad™ system. (A) and (B) accessories for stacking gel. (C) and (D) preparation of the electrophoresis gel; (E) addition of run buffer; (F) application of the samples; (G) development of the electrophoresis.

### 3. Methods for specific enzymes detection in electrophoresis gel

#### 3.1 Amylase

The method consists of the conduction of an electrophoresis in polyacrylamide gel polymerized with starch 0.5%. The electrophoretic run is performed in the pH adequate to the amylase isoelectric point. After the end of the run, the gel must be immersed in the suitable temperature and buffer during at least one hour. The gel is going to be revealed with a solution of iodine ( $I_2$  10 mM) and potassium iodide (KI 14 mM) until the appearance of activity bands. Fig. 2A illustrates the activity of  $\alpha$ -glucosidase, one of the enzymes of the amylolytic system, which leads to the formation of glucose as end product (Aquino et al., 2001; 2003; Silva et al., 2009a, 2009b).

#### 3.2 Pectinase

Method I - After conducting a PAGE 4.5 or 8.9 (depending on the enzyme pI), the gel containing the enzyme must be incubated with the substrate - a solution containing citric pectin or sodium polypectate 1% in the suitable buffer of the enzyme under study. In Fig. 2B there was the use of 1% of sodium polypectate in a sodium acetate buffer 100mM, pH 4.0 and incubation at 50°C (enzyme optimum temperature), for 2 hours for the dyeing with 0.02% Ruthenium red  $[(Ru_3O_2(NH_3)_{14}C_{16}.4H_2O)]$ , a dye capable of interacting with the pectic substates (Sterling, 1970). Thus, in the region where the protein migrated to and hydrolyzed the substrate, there is a halo with a whitened coloration that contrasts against the rest of the red-colored gel.

Method II - The citric pectin must be dissolved in gel buffer with the aid of a magnetic agitator, followed by the addition of acrylamide, bis-acrylamide and TEMED solutions. Crystals of ammonium persulfate are added immediately before the plate gel is overflowed. After the run, incubate the gel for 1-2 hours with 100 mL of malic acid 0.1M, at 4°C, in order to cause a gradual change to pH 3.0. Such period allows the enzyme to interact with the pectin polymerized in the acrylamide gel in its suitable pH range. Wash with distilled water and color in Ruthenium red 0.02%, during 30 to 120 min. Wash with distilled water.

Result: against a redish gel, it is possible to notice the polygalacturonase activity due to the formation of clear, opaque or colorless areas.

#### 3.3 Xylanase

In order to detect the xylanase activity in gel, the polyacrylamide must be polymerized with 0.5% xylan dissolved in the buffer of the electrophoresis to be performed (PAGE 4.5 or 8.9, depending on the isoelectric point of the enzyme under study). After the electrophoresis run the gel must be incubated in the temperature and in the reaction buffer which is mostly suitable for the xylanase under study for at least 1 hour. After this period, the gel is going to be stained with 1% Congo red ( $C_32H_{22}N_6Na_2O_6S_2$ ) a sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid. Thus, in the region where the protein migrated to and hydrolyzed the substrate, there is a halo with a whitened coloration that contrasts against the rest of the red-colored gel. (Fig. 2C) (Sandrim et al., 2005; Damásio et al., 2011).

Xylanases may also be observed through zymograms (Fig. 2D). For so, the samples must be applied in SDS-PAGE without the addition of  $\beta$ -mercaptoethanol, any other reducing agent

and without boiling. The advantage of the activity detection through zymograms is that effectively all active isoforms present there may be detected, once that regardless on the isoelectric point, all proteins will migrate in the gel, because of their molar mass.

In this case, the SDS gel must be performed with the running buffer commonly adopted in the protocols and, after the electrophoresis run, the proteins will have to be transferred to another gel composed by agarose and xylan, a substrate that is specific to the activity to be detected. Such transferring happens when there is a kind of “sandwich” with the polyacrylamide gel and the agarose + substrate gel. The transferring must happen overnight. After this period, the agarose + substrate gel, now also with the proteins to be analyzed, will have to be incubated in the buffer that is suitable for the isoforms under study, during one hour, following with the coloration suitable for the activity detection of the enzyme analyzed.

### 3.4 Proteases

The zymography may also be applied for proteases. It is a simple, quantitative and functional technique to analyze the activity of proteases (Leber & Balkwill, 1997). It consists basically of two stages, the separation through electrophoresis, followed by the activity detection of the enzyme in polyacrylamide gel, in non-reducing conditions (without treatment with DTT or  $\beta$ -mercaptoethanol) (Dong-Min et al., 2011). This technique has been used to evaluate the level of proteases in tissues or biological fluids, and it bears the advantage of distinguishing different kinds of enzymes due to the characteristic of mobility that each enzyme presents (Raser et al., 1995). The protease activity in zymography is observed as a clear band, indicating the substrate proteolysis after colored with Coomassie Brilliant Blue (Kim et al., 1998).

This methodology is widely employed for the detection of Matrix metalloproteinases (MMPs). However, it can also be employed for other types of proteases, with the need of adjustments in the methodology, such as the substitution of the substrate, generally gelatin for casein. Unfortunately, the zymography with casein is very little sensitive, when compared to the zymography with gelatin. Besides, casein migrates in the gel during the electrophoresis due to its relative low molar mass. That results in two clearly defined areas in the gel: the upper part, which still contains excess casein and the lower part, with less casein (Beurden and Von denHoff, 2005).

For the detection of proteases (Fig. 2E), the sample must be diluted in the sample buffer (5x) of the gel (0.4M Tris-HCl, pH 6.8; 5% SDS; 20% glycerol 0.03% Bromophenol blue). The samples cannot be boiled, because this process denatures the enzyme and it will no longer present activity (Kleiner & Stetler-Stevenson, 1994). The electrophoresis of the samples containing the protease must be performed according to Laemmli (1970). The gel concentration must be prepared according to the molar mass of its protease. The electrophoresis may be performed in constant 100V for 1-2 hours at 4°C.

For the development of the proteolytic activity, the gel must be incubated with 70 mL of buffer with the appropriate reaction pH, for 5 min., 4°C, 100 rpm. Following, the buffer must be removed and the gel must be incubated with 70 mL of Triton X-100 2.5% prepared in the reaction buffer. The gel must be kept at 100 rpm, for 30 min, 4°C. This step is for the removal of the SDS and the activation of the protease. Afterwards, the excess Triton X-100 must be

removed. For so, add 70 mL of buffer with the appropriate reaction pH, incubate for 30 min., at 4°C, 100rpm.

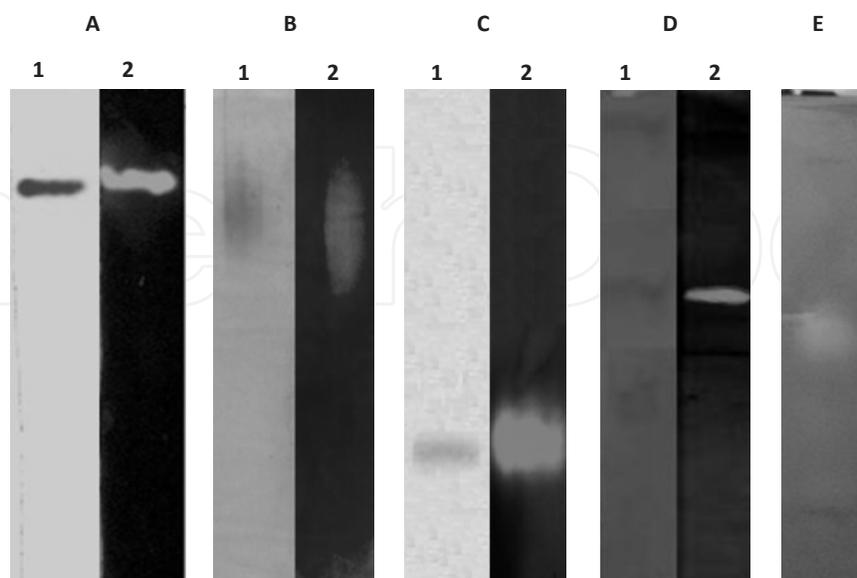


Fig. 2. Activity gels for different enzymes in SDS-PAGE 10%. (A1)  $\alpha$ -glucosidase (a type of amylase) revealed with Coomassie Blue; (A2)  $\alpha$ -glucosidase revealed with 10 mM Iodine solution and 14 mM potassium iodide; (B1) polygalacturonase revealed with silver solution; (B2) polygalacturonase activity revealed with 0.02% ruthenium red; (C1) xylanase revealed with silver solution; (C2) xylanase activity revealed with Congo red; (D1) Zymogram for xylanase revealed with silver solution and (D2) Congo red; (E) protease activity revealed with Coomassie Blue.

Following, remove the buffer and add the casein solution at 3%, prepared in a buffer with the enzyme reaction pH. The gel must be incubated for 30 min, at 4°C, for the diffusion of the casein to the gel. After that, the gel must be bathed at the enzyme reaction temperature for the period of 1-2 hours, so that the enzymatic reaction occurs (such period may be adjusted according to each enzyme and concentration).

The excess casein must be removed by bathing the gel for 5 times with distilled water at room temperature (García-Carreño et al., 1993 and Kleiner & Stetler-Stevenson, 1994) with modifications.

For the coloration, the gel must be stained with a solution containing 40% ethanol, 10% acetic acid and 0.1% Coomassie brilliant blue R-250 (García-Carreño et al., 1993). In this stage, the gel shows a blue bottom and where the hydrolysis took place, there will be a white halo. The gel needs to have the excess Coomassie brilliant blue removed; hence, it will have to be discolored with a discoloring solution composed by 40% ethanol and 10% acetic acid (García-Carreño et al., 1993). The clear zones over the blue bottom indicate the protease activity.

Some enzymes need activators, such as  $\text{Ca}^{2+}$ , DTT and EDTA to show their activity. Those can be added together with the substrate. Other substrates, such as hemoglobin, bovine serum albumin, gelatin and collagen, may have their coloration improved through the use of other dyes, as for example, Amide black (García-Carreño et al., 1993).

#### 4. Conclusion

The biological catalyzers present several advantages over their chemical similar, particularly, the regio and stereoselectivity that lead to the formation of products which are enantiomerically pure and in conformity with the norms established for the food, pharmaceutical and agriculture industry. The enzymes are efficient under the energetic point of view, operating in controlled pH, temperature and pressure. The development of the recombinant DNA technology enabling the expression of enzymes in different hosts has resulted in the production of more efficient biological catalyzers.

Several methods are attributed to enzymatic determinations. The most widely used are the colorimetric ones, where the reactions occur with specific substrates, generally leading to the formation of colored products, which can be easily quantified in spectrophotometers or through acrylamide gel electrophoresis, non-denaturing conditions. The detection of enzymatic activity through PAGE involves the migration potential of the enzyme in gel, which is influenced by the molar mass of the protein and its loads in specific pH. Thus, the visualization of the enzymatic activity in gels is seen as an advantaging condition, once that it is possible to consider that hardly did that enzyme migrate together with interferents or contaminating substances that could be in a crude extract and that could lead to errors in the enzymatic levels. PAGE for enzymatic activities can be considered as an elegant method that has been increasingly employed in researches.

The zymogram technique demands low enzyme concentrations, which can reach the order of nanograms. Several adaptations of substrates may be made in this technique, because the majority of substrates used are low-cost and yield good results. With this technique, we can infer how many types or isoforms of enzymes of a same class are present in the crude extract, for example, what the molar mass is and even in some cases, the quantification.

Hence, the proposal for the optimization of stages of enzymatic activity detection in electrophoresis must be conducted for each specific extract to be studied, yielding in this way high sensitivity and precision.

#### 5. Acknowledgment

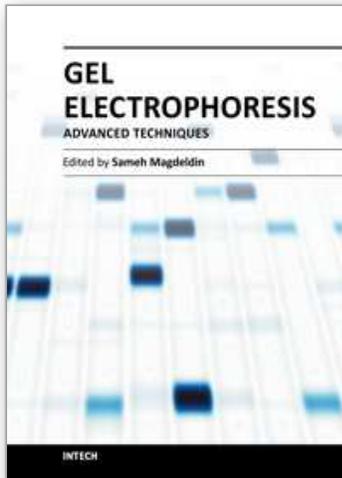
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#### 6. References

- Aquino, A.C.M.M., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2001). Thermostable glucose-tolerant glucoamylase produced by the thermophilic fungus *Scytalidium thermophilum* 15.1. *Folia Microbiologica*, Vol. 45 (6), pp. 11-16.
- Aquino, A.C.M.M., Jorge, J.A., Terenzi, H. F. & Polizeli, M.L.T.M. (2003). Studies on a thermostable alpha-amylase from the thermophilic fungus *Scytalidium thermophilum*. *Applied Microbiology and Biotechnology*, Vol. 61, pp. 323-328.

- Betini, J.H.A., Michelin, M., Peixoto-Nogueira, S.C., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2009). Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. *Bioprocess and Biosystems Engineering*, Vol. 32, pp. 819-824.
- Beurden Sanoek-van, P.A.M. & Von den Hoff, J.W. (2005). Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *BioTechniques*, Vol. 38, pp. 73-82.
- Cabral, J.M.S., Aires-Barros, M.R. & Gama, M. (2003). *Engenharia Enzimática*, Lidel edições Técnicas, 972-757-272-3, Lousã.
- Damásio, A.R.L., Silva, T.M., Almeida, F.B.R., Squina, F., Ribeiro, D.A. R., Leme, A.F.P., Segato, F., Prade, R.A., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2011). Heterologous expression of an *Aspergillus niveus* xylanase GH11 in *Aspergillus nidulans* and its characterization and application. *Process Biochemistry* Vol. 46, pp. 1236-1242.
- Davis, B.J. (1964). Disc Electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*. Vol.28, 121, pp. 404-427.
- Dong-Min, C., Kim, K.E., Ahn, K.H., Park, C.S., Kim, D.H., Koh, H.B., Chun, H.K., Yoon, B.D., Kim, H.J., Kim, M.S. & Choi, N.S. (2011). Silver-stained fibrin zymography: separation of proteases and activity detection using a single substrate-containing gel. *Biotechnology Letters*, Vol. 33, pp. 1663-1666.
- Facchini, F.D., Vici, A.C., Reis, V.R., Jorge, J.A., Terenzi, H.F., Reis, R.A. & Polizeli, M.L.T.M. (2011a). Production of fibrolytic enzymes by *Aspergillus japonicus* C03 using agro-industrial residues with potential application as additives in animal feed. *Bioprocess and Biosystems Engineering*, Vol. 34 (3), pp. 347-55.
- Facchini, F.D.A., Vici, A.C., Benassi, V. M., Freitas, L.A.P., Reis, R.A., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2011b). Optimization of fibrolytic enzyme production by *Aspergillus japonicus* C03 with potential application in ruminant feed and their effects on tropical forages hydrolysis. *Bioprocess and Biosystems Engineering*, Vol. 34, pp. 1027-1038.
- García-Carreño, F.L.; Dimes, L.E. & Haard, N.F. (1993). Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Analytical Biochemistry* Vol. 214, pp. 65-69.
- Godfrey, T & West, S. (1996). *Industrial Enzymology* (2<sup>o</sup> ed.), Stokton Press, 1561591637 0333594649, New York.
- Gupta, R., Beg, Q.K. & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, Vol. 59, (April 2002), pp. 15-32, 0175-7598.
- Gupta R, Gigras, P., Mohapatra, H., Goswami V.K. & Chauhan, B. (2003). Microbial  $\alpha$ -amylases: a biotechnological perspective. *Process Biochemistry*, Vol 38, (june 2003), pp. (1599-616), 1359-5113.
- Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M. & Zelder, O. (2005). Biotechnological production and applications of phytases. *Applied Microbiology and Biotechnology*. Vol. 68, (5), (September 2005), pp. 588-597, 1432-0614.
- Hasan, F., Shah, A.A.A. & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*. Vol. 39, 2, (june 2006), pp. 235-251, 0141-0229.
- Kim, SH., Choi, N.S. & Lee, W.Y. (1998). Fibrin zymography: a direct analysis of fibrinolytic enzymes on gels. *Analytical Biochemistry*, Vol. 263, pp. 115-116.

- Kleiner, D.E. & Stetler-Stevenson, W.G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Analytical Biochemistry*, Vol. 218, pp. 325-329.
- Laemmli, U.K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, Vol. 227, pp. 680-685.
- Leber, T.M. & Balkwill, F.R. (1997). Zymography: a single-step staining method for quantification of proteolytic activity on substrate gels. *Analytical Biochemistry*, Vol. 249, pp. 24-28.
- Michelin, M., Peixoto-Nogueira, S.C., Betini, J.H.A., Silva, T.M., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2009). Production and properties of xylanases from *Aspergillus terricola* Marchal and *Aspergillus ochraceus* and their use in cellulose pulp bleaching. *Bioprocess and Biosystems Engineering*, Vol. 33, (7) (December 2009), pp. 813-821, 1615-7591.
- Michelin, M., Silva, D. P., Ruzene, D. S., Vicente, A. A., Jorge, J. A., Terenzi, H. F., & Polizeli, M. L. T. M. (2011). Production of xylanolytic enzymes by *Aspergillus terricola* in stirred tank and airlift tower loop bioreactors. *Journal of Industrial Microbiology & Biotechnology*, DOI 10.1007/s10295-011-0987-7.
- Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R.; Terenzi, H.F., Jorge, J.A. & Amorim, D.S. Xylanases from fungi: properties and industrial applications. Review. (2005). *Applied Microbiology and Biotechnology*, Vol 67, pp. 577-591.
- Polizeli, M.L.T.M. (2009). Properties and commercial applications of xylanases from fungi. *In: Mycotechnology: Current Trends and Future Prospects*, M.K.Rai (Editor). I.K. International Publisher, New Delhi., chapter 4, pp. 82-101.
- Polizeli, M. L. T. M., Corrêa, E. C., Polizeli, A. M., & Jorge, J. A. (2011). Hydrolases from microorganisms used for degradation of plant cell wall and bioenergy production. *In: Routes to Cellulosic Ethanol*. Editors: Marcos S. Buckeridge & Gustavo H. Goldman. Springer Verlag, chapter 8, pp. 115-134.
- Prakashan, N. (2008). *Biochemistry Basics and Applied* (3<sup>o</sup> edition), Rachana Enterprises, 81-85790-52-3.
- Raser, K.J., Posner, A. & Wang, K.K.W. (1995). Casein Zymography: A method to study  $\mu$ -Calpain, m-Calpain, and their inhibitory agents. *Archives of Biochemistry and Biophysics*, n<sup>o</sup> 1, v. 319, pp. 211-216.
- Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962). Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* Jul. Vol. 21 (195), pp. 281-283.
- Sandrim, V.C., Rizzatti, A.C.S., Terenzi, H.F., Jorge, J.A., Milagres, A.M.F. & Polizeli, M.L.T.M. (2005). Purification and biochemical characterization of two xylanases produced by *Aspergillus caespitosus* and their potential for the bleaching of kraft pulp. *Process Biochemistry* Vol. 40(5), pp. 1823-1828.
- Silva, T.M., Maller, A., Damásio, A.R.L., Michelin, M., Ward, R.J., Hirata, I.Y., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2009). Properties of a purified thermostable glucoamylase from *Aspergillus niveus*. *Journal of Industrial Microbiology and Biotechnology*, Vol. 36, (august 2009a) pp. 1439-1446, 1367-5435.
- Silva, T.M., Michelin, M., Damásio, A.R.L., Maller, A., Almeida, F.B.D.R., Ruller, R., Ward, R.J., Rosa, J.C., Jorge, J.A., Terenzi, H.F. & Polizeli, M.L.T.M. (2009b). Purification and biochemical characterization of a novel alpha-glucosidase from *Aspergillus niveus*. *Antonie van Leeuwenhoek*, Vol. 96, pp. 569-578.
- Sterling, C. Crystal-structure of ruthenium red and stereochemistry of its pectic stain. (1970). *American Journal of Botany*, Vol. 57, pp. 172-175.



## **Gel Electrophoresis - Advanced Techniques**

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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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