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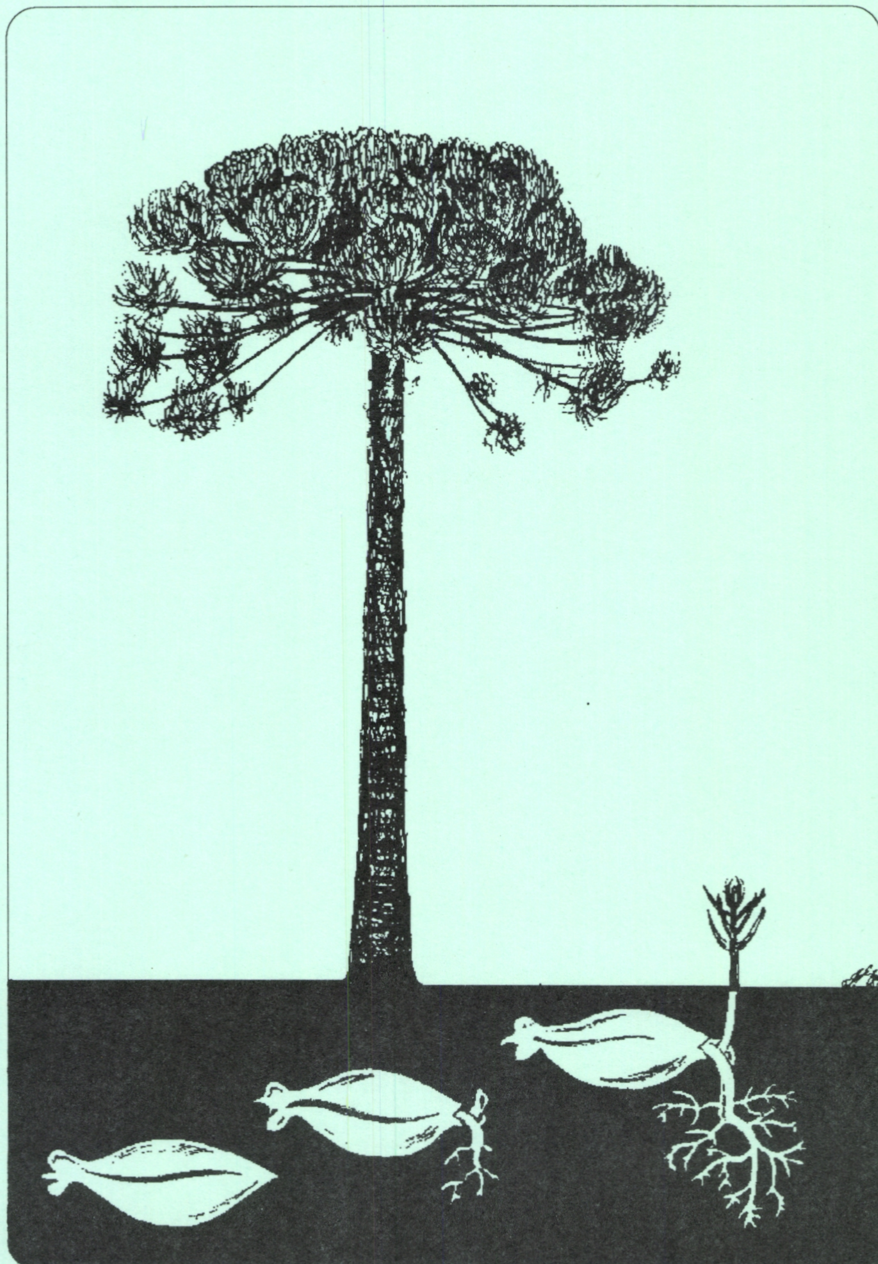

Adson Ramos, Jose G.A. Garneiro and Gilberto B. de Souza

Methods for the determination of amylase, invertase and cellulase in seeds of *Araucaria angustifolia*

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METHODS FOR THE DETERMINATION OF AMYLASE, INVERTASE AND CELLULASE IN SEEDS OF ARAUCARIA ANGUSTIFOLIA

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CONTENTS

1. INTRODUCTION ....
2. MATERIALS AND METHODS ....
3. RESULTS AND DISCUSSION ....
4. CONCLUSIONS ....
REFERENCES ....

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Federal University of Parana (Curitiba, Brazil) have carried out the cooperation programme in forestry research. The aim of the programme is to promote research of mutual interest and to increase the exchange of forestry information in general. This publication belongs to the scientifical themes of the cooperation.

Abstract

RAMOS, A., CARNEIRO, J.G.A. & DE SOUZA, G.B. 1989. Methods for the determination of amylase, invertase and cellulase in seeds of Araucaria angustifolia.

Seeds of Araucaria angustifolia (Bert.) O. Ktze collected in Tres Barras, State of Santa Catarina, were analysed to observe the enzymatic activity of amylase, invertase and cellulase. The reaction conditions were established considering the buffer, pH, temperature, incubation time and dilution of the material. The results indicated that the best reaction conditions for the amylase and invertase were a pH 5.0 buffer, a temperature of 35 ^{o}C and 90-min incubation time. The best incubation time for cellulase was 24 hours at 50 ^{o}C using the same buffer and pH. The adequate proportion of substrate + enzyme (original material) was 10:1 in all three cases.

Key words: Araucaria angustifolia, amylase, cellulase, invertase, enzymatic activity, pH.

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1. INTRODUCTION

Araucaria angustifolia (Bert.) O. Ktze. - the Parana pine - is a commercially highly valued species but the exports of its timber have been going down due to a decrease in production resulting from inadequate harvesting procedures (Santos and Costa 1985). The species propagates mainly through seeds. However, these seeds are only viable for a six-month period after they mature (Alves 1965, Bandel 1966, Prange 1964, Suiter Filho 1966, Cozzo 1961, Jankauskis 1970) and very little is known about their physiological and biochemical properties.

A cell must be able to perform a number of chemical transformations in order to live, grow and reproduce. It must modify environmental nutrients before and after they enter it. Part of the material assimilated may be synthetised into components which will form the cellular structure while the rest will be degradated to supply the energy required for these transformations. The enzymes, substances found in small quantities in the cells, perform these highly complex modifications and are also able to make other changes associated to vital processes. The enzymes can be regarded as the executive unit of the cell. Any damage to them will provoke alterations or even the death of the cell. There is no life without enzymes (Pelcar et al. 1980, Lehninger 1984). This is the reason why the comprehension of phenomena associated with vital processes requires a knowledge of the nature of enzymes and of enzymatic reactions.

Among the facts which affect enzymatic activities some like concentration, temperature and incubation time, the pH and substrate concentration (Anderson 1973, Pelcar et al. 1980, Villela et al. 1973, Lehninger 1984, Cantarow and Schepartz 1968, Ting 1982) can be emphasized.

This paper aims at defining methods to study the activities of amylase, invertase and cellulase, enzymes which act in the metabolism of carbohydrates, the main components of seeds of Araucaria angustifolia.

2. MATERIALS AND METHODS

100-gram samples of chopped materials from 20 seeds chosen at random from a lot collected at Tres Barras National Forest, State of Santa Catarina, were used. An equivalent volume of phosphate buffer was added to this material before it was ground for 4 minutes with the use of a hand mixer. The resulting material was then sieved through a 0.5 mm-mesh sieve. 32 ml aliquots were taken form the material sieved and 28 ml of glycerol were added, the product being then put into plastic containers and kept in deep freezers at temperatures below -15 ^{o}C for future analysis.

1st phase: Amylase, invertase and cellulase activities as a function of pH.

The following buffers were prepared according to Villela et al. (1973) with the use of a "Digimed" potentiometer: pH 3.0, pH 4.0 and pH 5.0 citrate buffer, pH 5.0, pH 6.0 and pH 7.0 acetate buffer, pH 7.0 and pH 8.0 phosphate buffer and pH 8.0 and pH 9.0 boric-borax buffer.

The material was controlled in two different ways: with de-ionized water + substrate and with de-ionized water + enzyme.

The substrates used were saccharose (1 g/50 ml of buffer), starch (0.277 g/50 ml of buffer) to determine the activity of invertase, amylase and cellulase, respectively.

The temperature was 37 ^{o}C, the enzymatic concentration of the original material was 1:51 and incubation times in "Fisher" water-bath were 24, 48, 72 and 96 hours.

The Somogy-Nelson (Somogy 1945) method was used to measure to total activity of reducing sugars and a "Spectronic - 20" spectrophotometer was used in 540 nm to interpret the results.

Statistical studies were not done. The graphic interpretation of results shown in micrograms/ml/min of glucose was based on the mean of three repetitions.

2nd phase: The effect of concentration, temperature and incubation time.

A pH 5.0 acetate buffer, which proved to be ideal at the first phase of the experiment, was used. The substrate for invertase was 1 g of saccharose / 50 ml of buffer and for amylase 0.277 g of amid / 50 ml according to the results of a previous work done to adapt methodology. This concentration is equivalent to 3 times the maximum speed of this enzyme. The temperature were 25, 35, 45, 55 and 65 ^{o}C for incubation times of 45 min, 90 min, 180 min and 360 min and the material was diluted in 1:5, 1:10, 1:20 and 1:40 dilutions.

For the activities of cellulase, the carboxymethol cellulose was the substrate in a proportion of 0.5 % (weight/volume) of pH 5.0 acetate buffer, which was also defined at the first phase. Temperatures used were 35, 45, 50, 55 and 65 ^{o}C. Incubation times were 45 min, 90 min, 180 min, 360 min, 22 hours and 24 hours and the rates of dilution were the same used for invertase and amylase.

Results obtained were calculated in micrograms/ml/min of glucose and submitted to linear regression analysis.

3. RESULTS AND DISCUSSION

1st phase: Activities of amylase, cellulase and invertase as a function of pH.

For amylase (Figure 1) the results obtained for the acetate buffer and for pH 5.0 have coincided with those found by Monerri, Garcia-Luis and Guardiola (1986) when working with cotyledons of Pisum sativum L., with those found by Revilla & Fernandez-Tarrago (1986) in cotyledons of Lens culinaris, and also with Ballou and Luck (1941). Bernfeld (1955) verified the activity of this enzyme in sweet potato using pH between 4.0 and 5.0 in acetate buffer. Paleg (1960) used a pH 4.6 acetate buffer for his studies with soya beans.

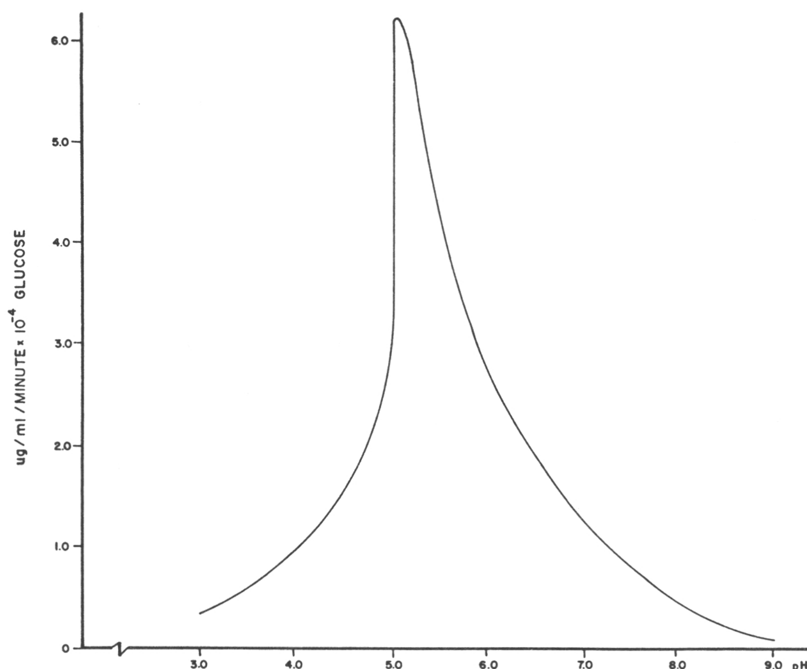

Figure 1. Variation of the activity of amylase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of pH.

For invertase activites (Figure 2), the pH 5.0 acetate buffer obtained for this enzyme was similar to results achieved with human blood (Villela et al. 1973). The pH value coincides with the indication for fungi preparations (Bacon 1955).

The pH 5.0 acetate buffer obtained for cellulase (Figure 3) coincided with the one used by Dekker and Willis (1983) when working with sugar cane bagasse and also with the indication made by Kristiansson (1950) for soya beans.

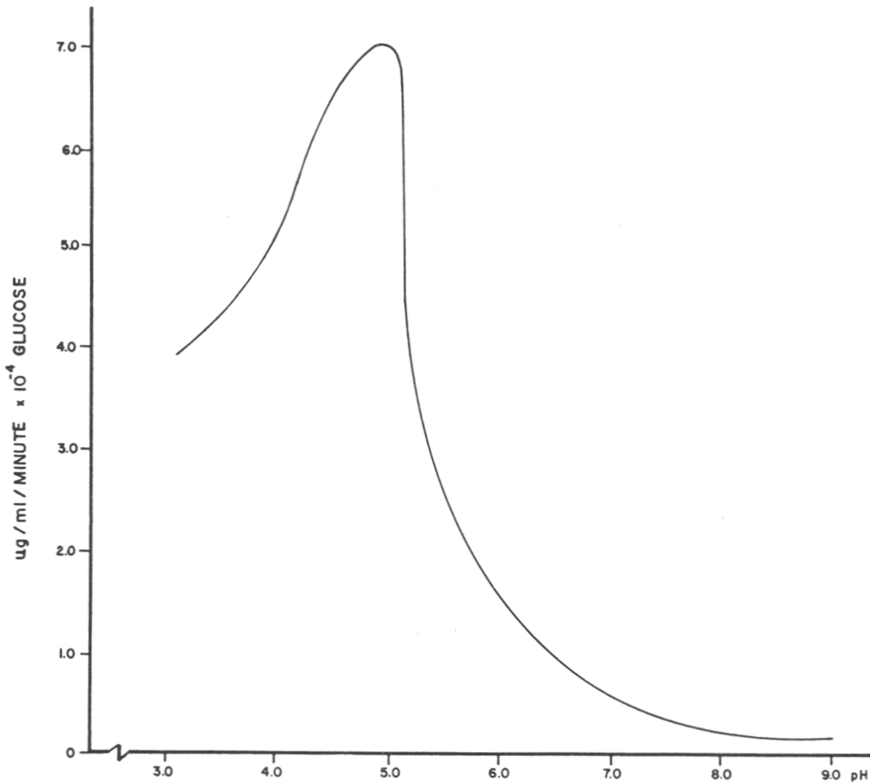

Figure 2. Variation of the activity of invertase in seeds of *Araucaria angustifolia* (Bert.) O. Ktze. as a function of pH.

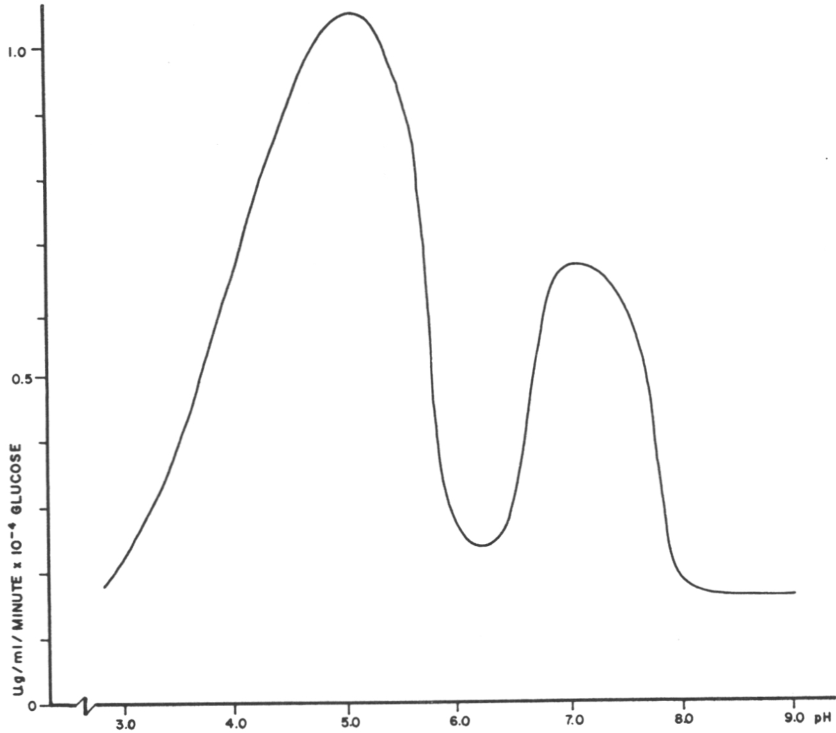

Figure 3. Variation of the activity of cellulase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of pH.

The use of the pH 5.0 buffer resulted in more activity for the three enzymes. The level of activity decreased below or above this pH, which confirms the results reported by Lehninger (1984), Kramer and Kozlowski (1972) and Ting (1982). Therefore, these are the best buffer and pH to determine the activity of amylase, cellulase and invertase in seeds of Araucaria angustifolia.

<u>2nd phase:</u> The effects of concentration, temperature and incubation time.

Amylase

The increase in incubation temperature from 25 to 35 ^{o}C, as shown in Figure 4, resulted in increased enzymatic activity for all the dilutions, which happens with most chemical reactions, whether catalysed or not (Cantarow & Schepartz 1968, Crueger and Crueger 1984, Lehninger 1984). Results presented in Table 1 in micrograms/ml/min x 10^{-4} of glucose show this growth in speed of action.

The results shown as a percentage of maximum activity in Figure 5 demonstrate that 35 ^{o}C is the temperature at which 1:10, 1:20 and 1:40 dilutions present more activity and increased speed (324.169, 321.067 and 294.598 micrograms/ml/-min 10^{-4} of glucose, respectively, according to Table 1. This temperature is similar to what Revilla & Fernandez-Tarrago (1986) used for their work with cotyledons of Lens culinaris.

The temperature of 45 ^{o}C and a 1:5 dilution, corresponding to 1 ml of the original material + 4 ml of buffer + substrate, resulted in an increased percentage of maximum activity with 290.050 micrograms/ml/min x 10^{-4} of glucose (Table 1). The other dilutions resulted in decreases in the speed of action.

The other temperatures analysed, 55 ^{o}C and 65 ^{o}C had a negative effect on the activity of amylase causing marked reductions in the speed of action. The descending part of the curves shown in Figure 5 illustrates the thermal denaturation reported by various researchers (Pelcar et al. 1980, Villela et al. 1973, Cantarow and Schepartz 1968, Ting 1982).

Figure 6 shows the effect of the dilutions studied on the speed of action of amylase. Although the linearity required for obtaining the optimum speed for enzymatic action (Villela et al. 1973) has not been established, it was ob served that from the dilutions tested the most adequate one should be similar to the 1:10 used.

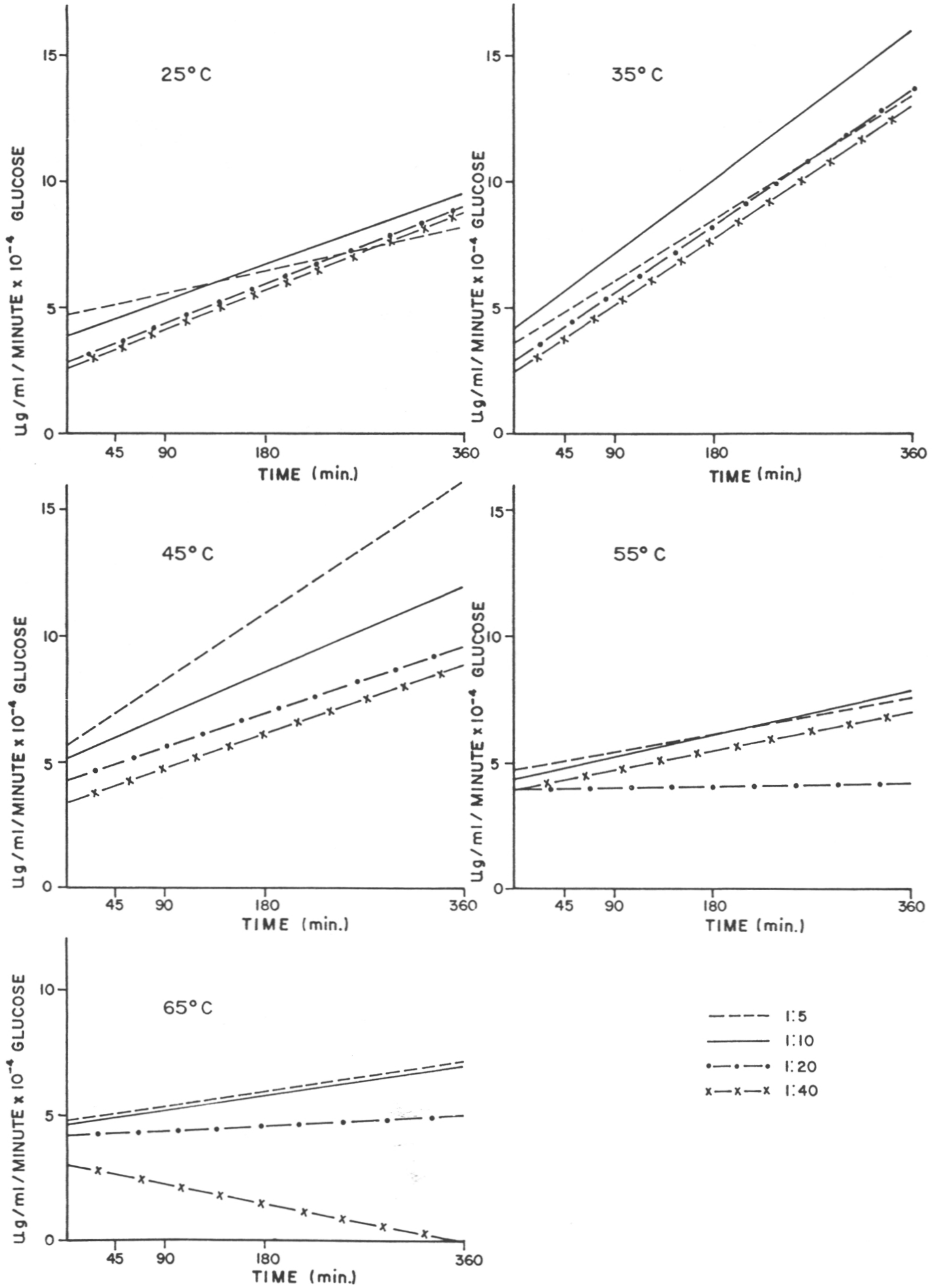

Figure 4. Variation of the activity of amylase in seeds of *Araucaria angustifolia* (Bert.) O. Ktze. as a function of temperature and time of dilution and incubation.

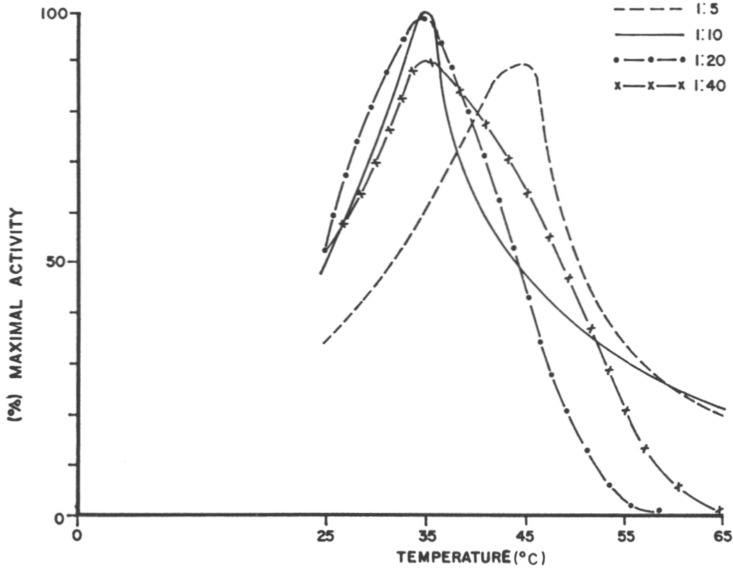

Figure 5. Percentage of the maximum activity of amylase in seeds of Araucaria angustifolia (Bert.) O. Ktze. in different dilutions as a function of temperature.

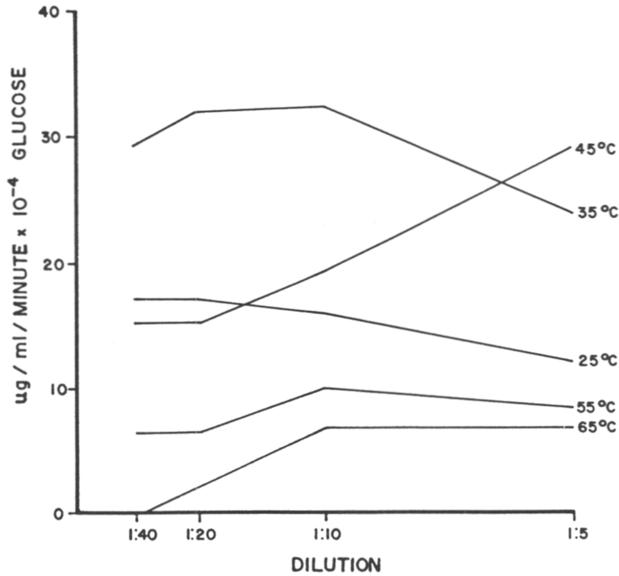

Figure 6. Effects of dilution on the action of amylase in seeds of Araucaria angustifolia (Bert.) O. Ktze. As a function of temperature.

Table 1. Speed of the amylase enzyme action in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature and four different dilutions (migrograms/ml/min x 10^{-4} of glucose).

Temperature	Dilutions			
(°C)	1:5	1:10	1:20	1:40
25	124,283	158,413	173,439	169,654
35	239,257	324,169	321,067	294,598
45	290,057	191,746	156,009	153,257
55	83,007	100,098	75,315	74,042
65	69,022	68,938	23,335	0,000

Table 2. Speed of the action of cellulase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature and four different dilutions (micrograms/ml/min x 10^{-4} of glucose).

Temperature	Dilutions			
(°C)	1:5	1:10	1:20	1:40
25	18,262	12,494	13,010	8,612
35	20,715	48,229	38,810	19,533
45	24,977	49,995	38,957	35,629
50	67,452	117,258	24,657	46,910
55	9,486	8,690	8,380	0,000

Cellulase

The highest level of activity (Figure 7) as well as the highest percentage of maximum activity (Figure 8), corresponding to the fastest speeds of action (Table 2) for cellulase, was obtained at 50 °C during 24 hours for 1:5, 1:10 and 1:40 dilutions with 67 452, 11 258 and 46 910 micrograms/ml/min x 10^{-4} of glucose, respectively. This temperature and incubation time were also used by Dekker & Willis (1983) and Fontana et al. (1984) with sorghum and sugar cane bagasse.

The fastest speed of action for the 1:20 dilution was obtained at 45 °C with a 24-hour incubation time (Figure 7 and 8).

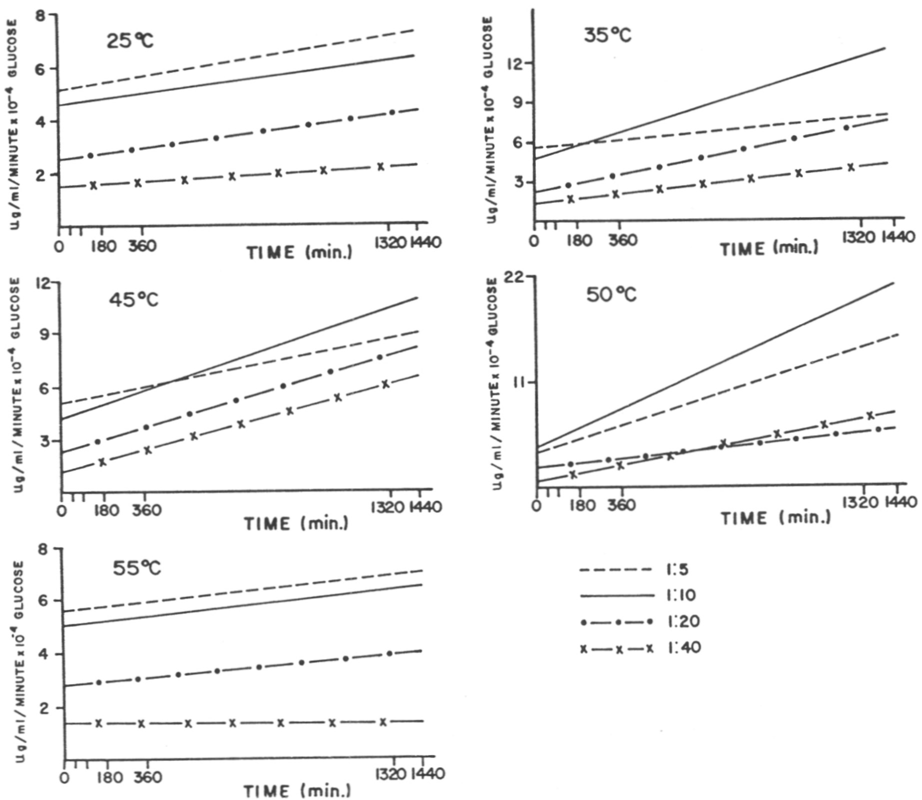

Figure 7. Variation of the activity of cellulase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature and of dilution and incubation times.

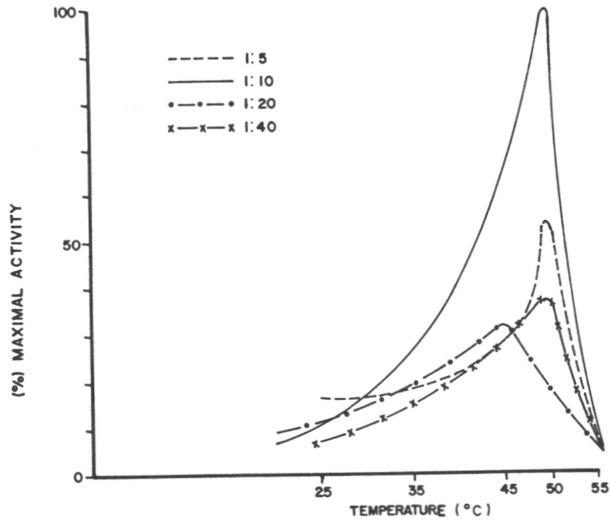

Figure 8. Percentage of the maximum activity of cellulase in seeds of Araucaria angustifolia (Bert.) O. Ktze. in different dilutions as a function of temperature.

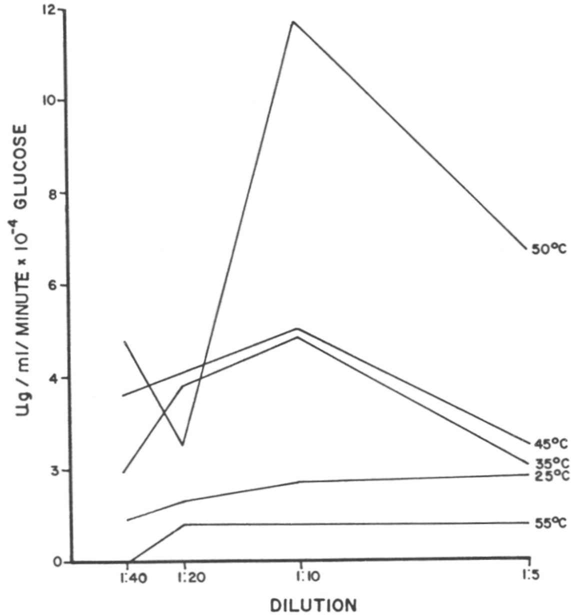

Figure 9. Effect of dilution on the action of cellulase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature.

Increasing the temperature to 55 ^{o}C, a very marked decrease in the activity of cellulase was noticed (Figure 8), which demostrates the optimum temperature, 50 ^{o}C, is near the point of thermal denaturation (Pelcar et al. 1980, Villela et al. 1973, Cantarrow & Schepartz 1968).

The speed of enzymatic action as a function of temperature (Figure 9) shows that, among the dilutions tested, the 1:10 is the most adequate for obtaining optimum speed (Villela et al. 1973).

Invertase

Figures 10 and 11 show that for all dilutions 35 ^{o}C was the temperature at which most activity was obtained for the determination of invertase.

A decrease in the speed of action of all dilutions was noticed at 45 ^{o}C. At 55 ^{o}C the speed was null for the less concetrated dilutions, 1:20 and 1:40. The enzymes were not active in any dilution at 65 ^{o}C. At 35 ^{o}C the buffer + substrate control presented a decrease in the production of glucose measured from an incubation time of 180 min onwards. This interference proves that 35 ^{o}C and 90 min incubation time is the safe limit for the determination of invertase in seeds of Araucaria angustifolia.

Similary to amylase and cellulase, the 1:10 invertase dilution (Figure 12) should also be near that at which the necessary linearity and consequent optimum speed for enzymatic action would be obtained (Villela et al. 1973).

The increase in temperature from 25 to 35 ^{o}C provoked a marked increase in the speed of action (Table 3) resulting in 409 661, 573 311, 540 819 and 553 278 micrograms/ml/min x 10^{-4} for dilutions of 1:5, 1:10, 1:20 and 1:40 respectively.

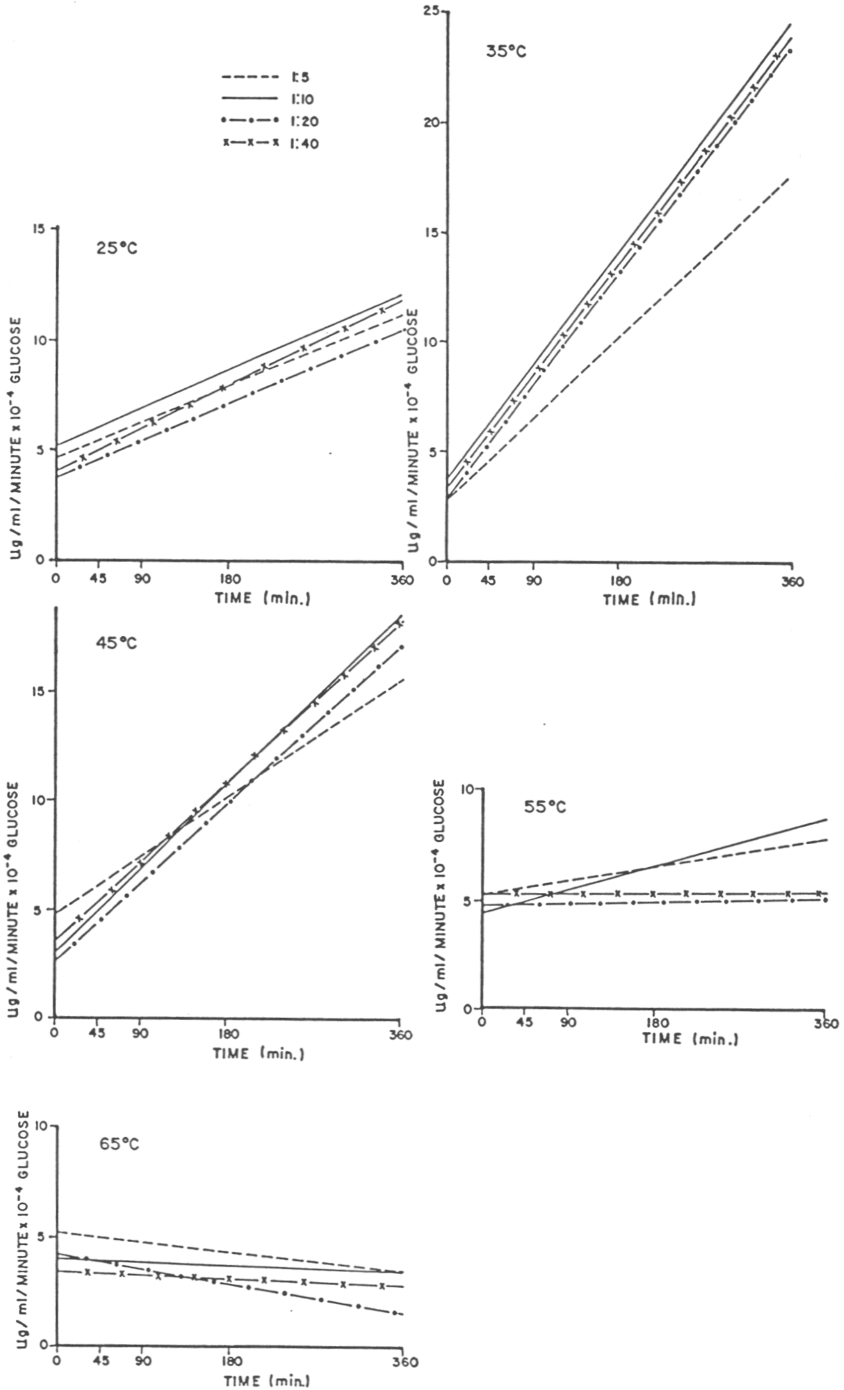

Figure 10. Variation of the activity of invertase in seeds of of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature and of dilution and incubation times.

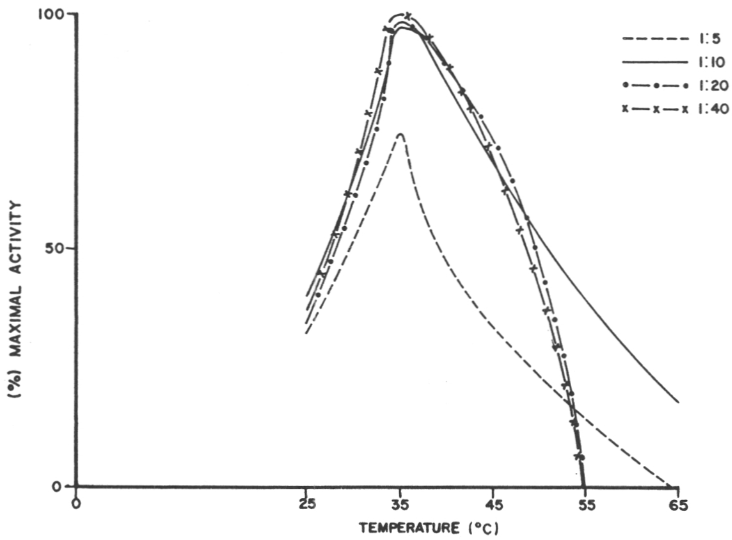

Figure 11. Percentage of the maximum activity of invertase different dilutions as a function of temperature.

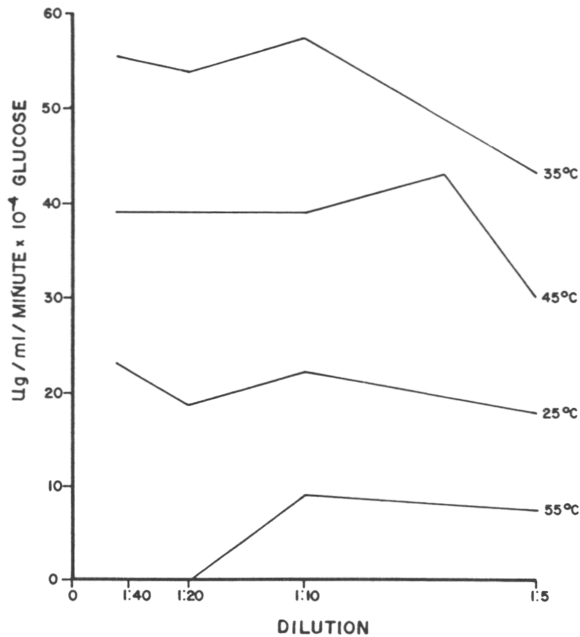

Figure 12. Effects of dilution on the action of invertase in seeds of Araucaria angustifolia (Bert.) O. Ktze. as a function of temperature.

Table 3. Speed of action of invertase action in seeds of *Araucaria angustifolia* (Bert.) O. Ktze. as a function of temperature in four different dilutions (micrograms/ml/min x 10^{-4} of glucose).

Temperature	Dilutions			
(°C)	1:5	1:10	1:20	1:40
25	176,333	221,791	185,861	180,195
35	409,661	573,311	540,819	553,278
45	302,819	432,967	397,209	390,915
50	75,433	95,885	0,000	0,000
55	0,000	0,000	0,000	0,000

4. CONCLUSIONS

1. Amylase, cellulase and invertase were more active in pH 5.0 acetate buffer.

2. 35 °C and 90 min incubation time in a 1:10 dilution were the adequate conditions for the determination of amylase and invertase activities.

3. 50 °C and 24-hour incubtion time in a 1:10 dilution result in maximum activity for cellulase.

4. Temperature above 35 °C and incubation times over 180 minutes interfere with the method used for the determination of invertase.

5. An increase of 5 °C, from 50 to 55 °C provoked a very marked reduction in the activity of cellulase.

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