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YEAST CULTIVATION ON NATURAL STARCHES

BY

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ABSTRACT

This research project is concerned with the use of an amylolytic yeast, Endomycopsis sp., for simultaneous production of yeast protein and crude amylase preparations from natural starch materials. The Endomycopsis yeasts were cultivated alone and in combination with other yeasts which are unable to attack starch directly. The propagations were carried out in the presence of urea and phosphate, under aerobic conditions, with vigorous agitation, at pH 5.0 and 28°C. At daily intervals, the cultures were analyzed for protein yield, cell density, and amylase activity.

The cell crop harvested after propagation of Endomycopsis yeasts on 6.0% potato media contained 19% protein and the culture filtrate obtained after biomass separation had an activity of 1.5 units. Variations in activity and protein content were observed, depending on the starch substrate used, the concentration of urea added, and apparently, the amount of oxygen supplied.

Mixed preparations using Candida utilis as ancillary yeast, gave higher protein yields and amylase activities compared to single propagations of Endomycopsis sp. and mixed propagations with Saccharomyces cerevisiae.

Purple yam and cassava tubers were examined for protein enrichment and amylase production. It was observed

that the protein content of the cell crops obtained from these substrates could be increased about ten-fold but that the amylase activities of the culture filtrates were very low.

ACKNOWLEDGEMENTS

The author expresses gratitude to Dr. P.M. Townsley, under whose supervision this project was undertaken, for support, his advice, helpful criticisms and unfailing enthusiasm.

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INTRODUCTION

Today, more than half of the world population is undernourished, often in regions where raw materials are available for microbial utilization. The importance of microorganisms as potential nutritional sources stems from the finding that microbial cell matter is especially rich in most B-vitamins and in proteins containing essential amino acids. Therefore, microbial cells constitute potential enrichment for deficient diets.

It is agreed, currently, that yeasts have the most favorable characteristics for use as a major food source. Not only are yeasts one of the richest sources of B-vitamins but also, they contain large proportions of high quality protein, carbohydrate, and lipid. Yeast's ability to utilize cheap raw materials and maintain a rapid rate of growth, palatability and lack of pathogenicity are additional advantages. All of the essential amino acids except methionine are present in adequate quantities in yeast protein (Bhattacharjee, 1970).

Vast quantities of cheap, fermentable substrates, which can readily be converted into yeast proteins and vitamins are available in countries with an acute food shortage. The Philippines is one of several countries where severe protein deficiency exists. In these countries, low production is undesirable, the number of skilled agricultural workers is inadequate, arable land is scarce and capital

investment for intensive marine and agricultural production is unavailable. Hence, yeast protein conversion from abundant, cheap carbohydrate materials requires investigation.

Starchy tubers and root crops are the second most important sources of carbohydrates in the Philippines. On the average, 120 grams of tubers and root crops are available per person daily, but only 4.2 grams are actually consumed. Of the total root crops available, sweet potatoes, cassava, yam and taro are most abundant (Molinyawe, 1968). In view of the relative abundance of starchy raw materials in the Philippines and the severe deficiency of proteins and B-vitamins in the Pilipino diet, (Pascual, 1964), the Biological Research Center of the Philippine National Institute of Science and Technology has directed much of its efforts toward the use of local materials as substrates for high protein yeast production. Large scale propagations of Rhodotorula pilimanae (Wickerham) have been carried out on coconut water giving a product containing about 50% protein and appreciable amounts of B-vitamins. Studies conducted by the Food and Nutrition Research Center of the institute have shown the product to be highly acceptable when used as a protein supplement in the preparation of cookies. However, so far, none of the root crops have been utilized (Baens-Arcega, 1968).

Patented and workable processes are known for the utilization of starchy substrates for yeast production but

these are based on the principle of acid hydrolysis prior to yeast propagation since yeasts are generally considered unable to assimilate starch (Jarl, 1969; Hattori, 1961). By using amylase-synthesizing yeasts, which will convert starch without costly preliminary hydrolysis, a process might be devised for an efficient conversion of inexpensive raw materials, surplus production, and waste organic matter into edible products.

It is well known that some of the wastes from pea processing, corn starch manufacturing, potato processing, etc. consist of high concentrations of starch and therefore constitute potential sources of carbon and energy for growth and enzyme synthesis of amylase producing yeasts. Waste disposal might be made economically more attractive by combining it with a process for producing amylolytic enzymes. The starch degradation products may further serve as substrates for the production of yeast biomass that may be used as food or fodder. Alternatively, they may serve as substrates for the growth of selected microorganisms for the manufacture of amino acids, vitamins, flavor enhancers, and other compounds of nutritional or medical value.

This study is mainly concerned with the application of an amylase producing yeast specie, Endomycopsis, for simultaneous production of yeast biomass and crude amylolytic enzyme preparations. The experimental plan includes a study of the utilization of potato waste and of protein enrichment of root crops widely cultivated in the Philippines.

LITERATURE REVIEW

Yeasts are generally considered unable to utilize starch and few reports are available regarding yeast fermentation of starchy substrates. According to Ebertova (1966), only some representatives of the genera Endomycopsis and Endomyces and rarely Candida (Ebertova, 1968) have amylolytic properties.

Pioneering work on extracellularly produced Endomycopsis amylases was performed by Wickerham, et al.(1944). A high ratio of α- to β-amylase was found in crude preparations of the enzyme system. The composition and properties of the Endomycopsis amylolytic complex were investigated more thoroughly by Marroquin and Fitch (1946). Marroquin and Soloranzo (1947), and Marroquin and Gavarron (1947, 1948). Marroquin and Fitch (1946) showed, as Wickerham did, that the enzyme complex produced by Endomycopsis fibuliger was principally dextrinizing. While investigating the amylolytic and fermentative activities of mixed cultures of Endomycopsis fibuliger and Saccharomyces carbajali under varying conditions of pH, temperature, nature and concentration of substrate, and proportions of inocula, Marroquin and Soloranzo (1946) showed that cell growth occurred most rapidly at 37°C and pH 5.0. Amylolytic and fermentative activities were highest in 10% premalted wheat mash with a 2% inoculum consisting of 5 parts Endomycopsis fibuliger to

3 parts Saccharomyces carbajali. The optimum temperatures for amylase activity was 65°C for α-, and 40°C for β-amylase. The optimum pH range was 5.5 - 6.5 for α-, and 4.5 - 6.0 for β-amylase.

Subsequently, Fukumoto et al. (1960) and Hattori and Takeuchi (1961) examined the production of Endomycopsis and Endomyces amylolytic enzyme systems on various starch media and the properties of purified amylases obtained. Ammonium sulfate and rivanol treated preparations were studied with respect to pH and stability to heat and ethylene-diaminetetraacetate (EDTA). The optimum ranges for α- and β-amylase were 5.5 - 6.0 and 4.5 - 5.5 respectively, in close agreement with the values reported earlier by Marroquin and Gavarron (1946). α-Amylase was stable in the pH range 5.0 - 7.5 and β-amylase in the range 5.0 - 9.0. Instability to heat and EDTA was noted.

Systemmatic studies by Ebertova (1966) on the production of amylase using different carbon sources revealed that an enzyme exhibiting maltase and transglucosidase activities was released by Endomycopsis capsularis at the beginning of the logarithmic growth phase whereas starch hydrolyzing enzymes were released at the end. Glucoamylase was produced at pH 4.0 and α-amylase at pH 6.0.

Sadova et al. (1969) found pentoses, phosphodextrins and starch to be the best carbon sources for the synthesis of

amylolytic enzymes by Endomycopsis sp. 20-9.

Recent reports on the production, separation, and properties of extracellular Endomycopsis amylases include those of Hostinova and Zelinka (1969), Gracheva et al. (1969) and Koltsova and Sadova (1970). Hostinova and Zelinka (1969) observed maximum amylase synthesis by Endomycopsis fibuliger during the logarithmic phase of cultivation. Koltsova and Sadova (1970) precipitated 64% of the total glucoamylase and 53.5% of the total α-amylase at 60% saturation with ammonium sulfate, whereas all of the glucoamylase, 73% of the α-amylase, and 28% of the maltase were precipitated at 90% saturation with ammonium sulfate.

Kuehner's work (1953) is believed to be the first attempt at using amylolytic yeasts deliberately for the production of a nutritional material. Using a mixed culture consisting of Endomycopsis fibuliger or Endomycopsis chodati and Candida utilis grown on cooked wheat, corn, rice, or potatoes, a 60% yield was obtained containing 20 - 30% protein, independent of the ratio of organisms used. A 22" X 4" propagator equipped with baffles, a ring sparger and propeller blades was operated under the following conditions: 650 RPM agitation rate, 2 volumes of air per volume of mash per minute, 28°C and pH 5 - 6.

As a result of Kuehner's studies, Wickerham and Kuehner (1956) proposed a process for yeast protein produc-

tion using Endomycopsis fibuliger and Endomycopsis chodati but the process has remained undeveloped to date.

Yeast amylolytic properties have also been applied in a Swedish process by Tveit (1967) and Jarl (1969). Designed to reduce the biological oxygen demand of starchy effluents, Endomycopsis fibuliger and Candida utilis were grown symbiotically on starch media at pH 5.0 and 28°C. The only additions made were phosphate and urea. Most of the studies done by Tveit and Jarl were carried out in 100 - 1000 liter fermenters equipped with a single orifice sparger, impeller, and baffles. The harvested products were screened and concentrated. Subsequently, the separated biomass was dried for use as animal feed supplement.

At about the same time, in Czechoslovakia, Polivka and Zelinka (1969) produced yeast protein on a much smaller scale. Using corn starch, corn steep liquor and ammonium sulfate, Polivka and Zelinka (1969) obtained Endomycopsis fibuliger biomass containing more than 40% proteins. Subsequently, they explored the possibilities of using waste products of starch plants such as potato starch effluents.

There appears to be no process designed to produce yeast protein from starchy materials while providing the amylolytic enzyme complex as a by-product. However, α-amylase has been prepared from Endomycopsis fibuliger (Davies, 1963). Amylolytic enzyme products have been obtained by

cultivation of Endomycopsis fibuliger, Endomycopsis lindneri, Endomycopsis javaensis and Endomycopsis hordei in nutrient media containing 0.1 - 15.0% by weight of at least one higher fatty acid, higher fatty acid ester or natural fat or oil (Matsutani Chem. Co. Ltd., 1965). Rutloff (1968) has described a process of preparing glucoamylase from Endomycopsis yeasts.

MATERIALS AND METHODS

I. Microorganisms

The amylolytic yeast used in this study was isolated by Browne, (1973) from Lao-chao, a Chinese fermented rice product. Lao-chao was made by mixing glutinous rice and a locally available commercial starter called chiu-yueh or peh-yueh. Wang and Hesseltine (1970) showed that Lao-chao culture contained Mucorangeous fungi and a yeast specie Endomycopsis.

The Endomycopsis yeasts were maintained on slants consisting of 0.2% soluble starch, 0.5% peptone, 0.3% yeast extract and 1.5% agar. Sterilization was effected by autoclaving capped tubes for 15 minutes at 15 lbs pressure. After inoculation, slants were incubated at 37°C for 48 hours and stored at 4°C.

The non-amylolytic yeasts used were Candida utilis and Saccharomyces cerevisiae. Pure cultures were obtained from the University of British Columbia, Department of Microbiology. The Candida utilis culture originated from the American Type Culture Collection (ATCC 9256) and the Saccharomyces cerevisiae from the University of California (Berkeley). The organisms were maintained on slants consisting of 0.3% yeast extract, 0.5% peptone and 1.5% agar. Inoculated slants were incubated at 30°C for 48 hours and stored at 4°C.

II. Inocula

A. Shaken flask propagation

From the slants, 100 ml of sterile media containing 0.2% soluble starch, 0.3% yeast extract, and 0.5% peptone in 250 ml Erlenmeyer flasks were inoculated with Endomycopsis sp. using standard techniques in an Envirazone module inoculating hood. The cultures were incubated for 36 hours on a New Brunswick gyratory shaker operating at a speed of 196 RPM in an incubator maintained at 28°C and 85% relative humidity. Ten milliliters of these cultures, aseptically pipetted, were used to inoculate the shaken flask propagators. Typically, such cultures contained about 10^7 cells/ml.

For mixed fermentation, 100 ml of sterile media containing 0.3% yeast extract and 0.5% peptone in 250 ml Erlenmeyer flasks were prepared. A set of media was inoculated with Endomycopsis sp. and another set with Candida utilis or Saccharomyces cerevisiae. The cultures were incubated as described previously. After 36 hours, separate counts were made of the amylolytic and non-amylolytic yeasts. Appropriate amounts of each were mixed and used to inoculate the shaken flask propagators.

B. Six liter propagations

The entire contents of three flasks containing 100 ml each of the 36-hour yeast cultures were aseptically transferred into a Vir Tis fermenter assembly containing six liters of sterile media.

III. Media

A. Shaken flask propagations

Washed, unpeeled potatoes were weighed and homogenized in a Waring blender with some distilled water. The volume of the resulting slurry needed to prepare a medium containing the desired concentration of potato solids was ascertained by determining the solids content of the slurry. The solids content of the prepared potato slurry was measured using an Ohaus moisture determination balance. Five-gram samples were dried under an infrared lamp at 60 watts for 25 minutes. Media so prepared were dispensed in 100 ml portions into 250 ml flasks, cotton plugged, and autoclaved for 15 minutes at 15 lbs pressure. After overnight refrigeration at 4°C, and before inoculation, pH was adjusted from 7.5 to 5.0 using autoclaved 1N HCl.

B. Six liter propagations

Six liters of media similarly prepared were poured into a 12 liter Vir Tis fermenter flask with a lid, all ports of which were cotton plugged. Sterilization was effected by autoclaving for 45 minutes at 15 lbs pressure. After cooling overnight in the fermenter, thermostatically controlled at 10°C, pH was adjusted using autoclaved 3N HCl.

IV. Propagations

A. Shaken flask propagations

Cotton-plugged 250 ml Erlenmeyer flasks containing

100 ml of culture were placed on a New Brunswick gyratory shaker operating at a speed of 196 RPM in an incubator kept at 28°C and 85% relative humidity. Periodically, samples were aseptically withdrawn for analyses.

B. Six liter propagations

A three-station Vir Tis fermenter with 12-liter fermenter flasks was used in this study. The fermenter was equipped with spargers, adjustable baffles, magnetic impellers as well as foam, pH, and temperature controls. Media were autoclaved for 45 minutes at 15 lbs pressure in a 12 liter fermenter flask. After cooling, its lid was replaced with an ethylene oxide gas-sterilized fermenter head with dissolved oxygen, foam, and pH probes. A 12-hour exposure to ethylene oxide gas (cryoxide) was used to ensure sterilization of the fermenter heads and probes.

Cultivations were carried out at 28°C. Five to nine parts per million of dissolved oxygen was maintained in the medium which was agitated at the highest rate compatible with foam control. Generally, the magnetic impellers were operated at 650 - 750 RPM unless foaming was extensive.

V. Analyses

Routine analyses included:

A. Cell count

Cells were counted with the aid of a Haemacytometer with Neubauer ruling, according to the method devised by the

American Society of Brewing Chemists (34).

B. Enzymatic activity

Amylase activity was measured following the method used by Gracheva et al. (1969) on Endomycopsis sp. amylase and by Fukumoto (1955) and Hattori (1960) on Endomyces amylases. The procedure consisted of a 30-minute hydrolysis of a 2.0% soluble starch (Lintner's) solution buffered at pH 5.6. Reducing sugars were spectrophotometrically determined before and after hydrolysis at 45°C by the Nelson-Somogyi method (1945) employing a low-alkalinity copper reagent and arsenomolybdate. Amylase activity was expressed as grams of glucose released in 100 ml of culture in one hour.

C. Protein

Proteins were extracted from centrifuged cells and residual potato solids by soaking in 2N NaOH as described by Mitsuda (1970). After 100 hours, the spectrophotometric method of Lowry (1951) was used for quantitative protein analysis.

D. Yield

Yields were measured gravimetrically using the method recommended by the Institute of Brewing (20) for analysis of yeast concentration. Yields were expressed as grams of dry centrifuged cells obtained from 100 ml of culture.

E. Starch

Residual starch in the culture fluid was measured following the procedure outlined by Gilbert and Spragg (1968) after perchloric acid treatment as described by McReady et al. (1950).

RESULTS

Optimum Levels of Nitrogen and Phosphorus

A preliminary study was conducted in order to establish the levels of assimilable nitrogen and phosphate most suitable for growth and amylolytic enzyme synthesis by the yeast Endomycopsis sp. In media containing 0.5% soluble starch and 0.4% ammonium hydrogen phosphate, it was observed that over a period of four days, the pH of the culture dropped gradually from 5.0 to 3.4. Figure 1 shows the time course of the propagation. It is known that the amylases are inactivated at pH values less than 4.0 (Tsuchiya et al., 1950; Marroquin, 1947) and there is evidence in the literature indicating better pH control and higher protein accumulation by using urea instead of ammonium salts in propagations of this type (Keuhner, 1953). Therefore, it was decided that urea would be used with a phosphate source, ie. KH_2PO_4, in subsequent experiments. Unlike urea, ammonium salts gave residual acid as the nitrogen-containing portions of the molecules were utilized.

Various amounts of KH_2PO_4 were tried and it was inferred from the results of these experiments, shown in Table I, that the phosphate level did not exert a significant effect on either growth rate or starch hydrolysis.

Several propagations in which the amount of urea used was varied showed a remarkable dependence of rate of yeast

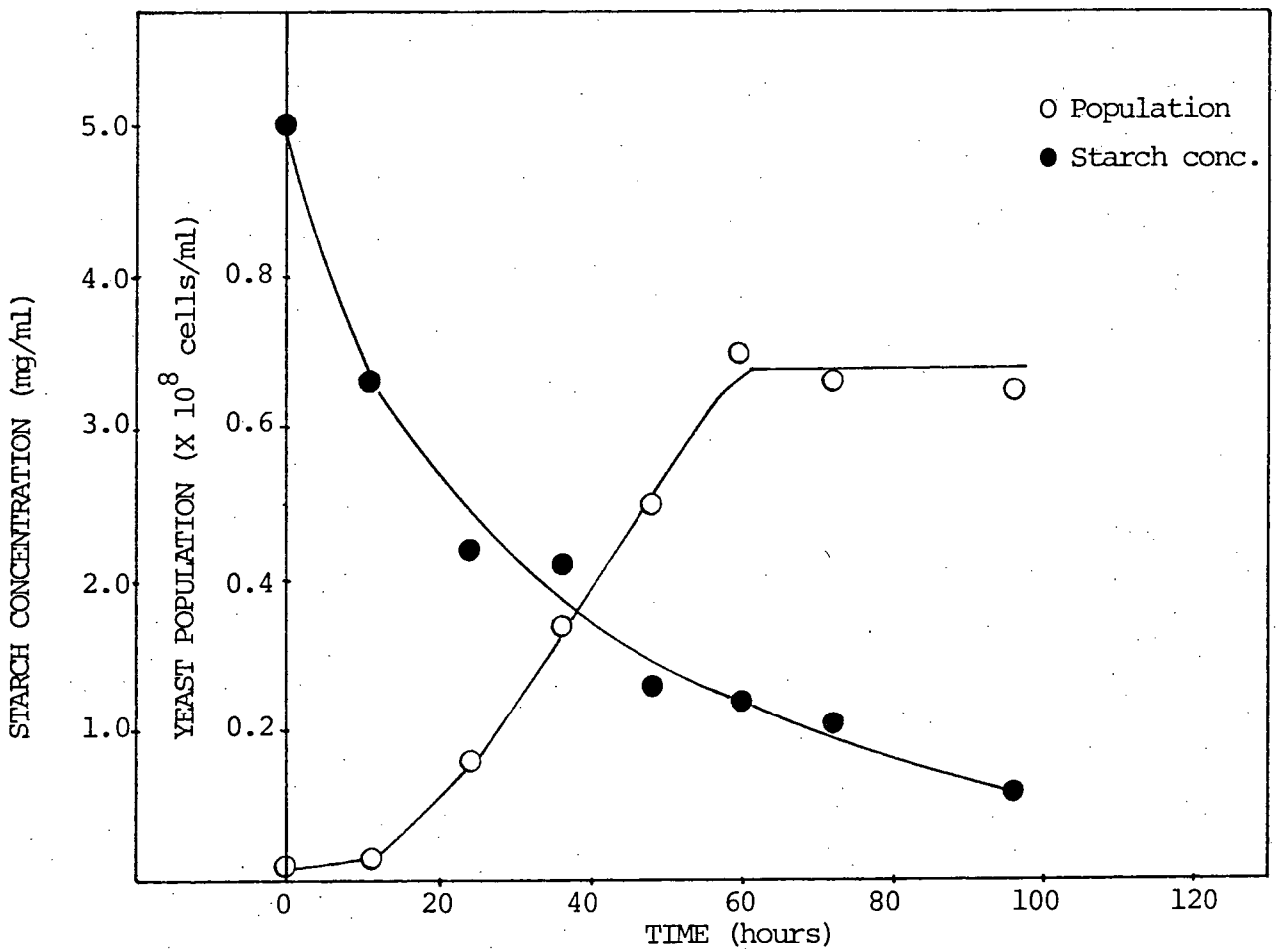

FIGURE 1. Progress of cultivation of Endomycopsis sp. on media containing 0.5% starch and 0.4% $(NH_4)_2 HPO_4$.

TABLE I. Cell density and concentration of residual starch after five days cultivation of Endomycopsis sp. on media containing 0.5% starch, 1.0% urea, and varying amounts of KH_2PO_4.

KH_2PO_4 concentration (%)	Yeast population ($X\ 10^8$ cells/ml)	Starch concentration (mg/ml)
0.1	1.20	0.19
0.2	1.12	0.17
0.5	1.20	0.20
1.0	1.15	0.18

growth and extent of starch disappearance on urea concentration. The results, which are presented in Figure 2, indicate that 1.0% is optimum. Levels of 2.0, 3.0 and 4.0% urea were inhibitory.

Concentration of Potato Slurry

Growth rate, amylolytic activity, and protein content were subsequently evaluated while varying the amount of potato solids supplied in the media from 2.0% to 10.0%. In accordance with the results of the preliminary experiments, 1.0% urea was added with 0.5% KH_2PO_4. Figures 3-6 show the time course of the propagations in 2.0, 4.0, 6.0, and 10.0% potato solids. A summary of the data is presented in Table II.

Six-liter Propagations

The 100 ml shaken flask experiments were then

scaled up to six liters using media composition observed to be optimal for protein accumulation and enzyme activity. The time course of propagation in six liters of medium containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 is shown in Figure 1. With oxygen tension maintained at levels much higher than in the shaken flasks, a significant rise in protein values was observed.

Mixed Propagations

Two non-proteolytic, non-amylolytic yeasts, Candida utilis and Saccharomyces cerevisiae (Ahearn, 1968; Sylven, 1958) were grown symbiotically with Endomycopsis sp. Comparisons were made of yeast growths, amylase activity in the culture fluids, and protein concentration in the cell crops in Endomycopsis - Candida and Endomycopsis - Saccharomyces cultures.

The results of experiments with varying Candida: Endomycopsis ratios (1:2, 1:1, 2:1) gave no indication that amylolytic activity, growth rate, and protein accumulation could be correlated with the proportion of the two yeasts in the inoculum. Figures 8-10 and Table III summarize these observations.

As expected, the lag phase of yeast growth was shortened considerably, the amylase activity increased significantly, and the protein level rose sharply when the propagation was carried out under sufficient aeration and vigorous agitation

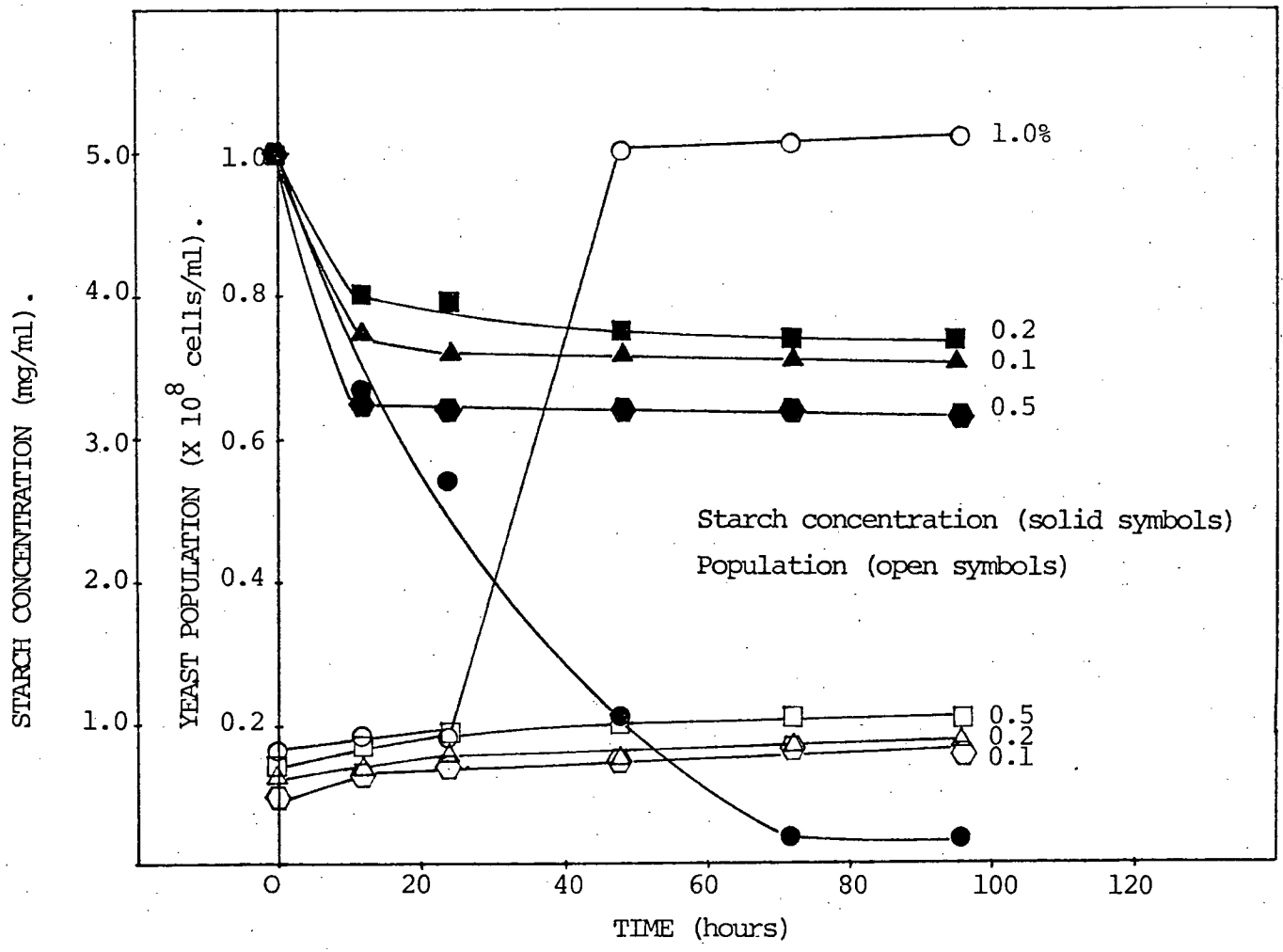

FIGURE 2. Progress of cultivation of Endomycopsis sp. on media containing 0.5% starch media with varying levels of urea.

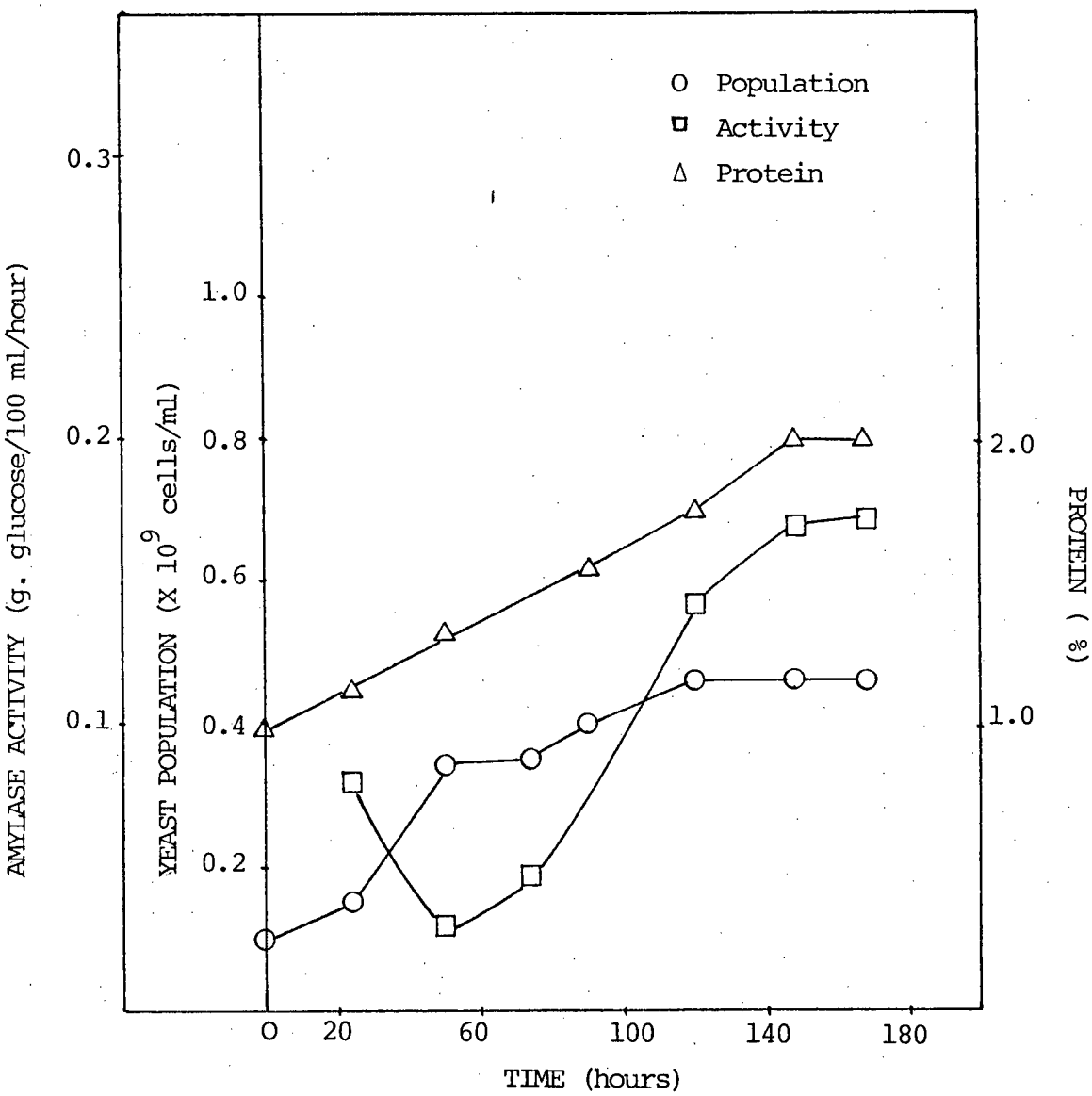

FIGURE 3. Progress of cultivation of Endomycopsis sp. on media containing 2.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

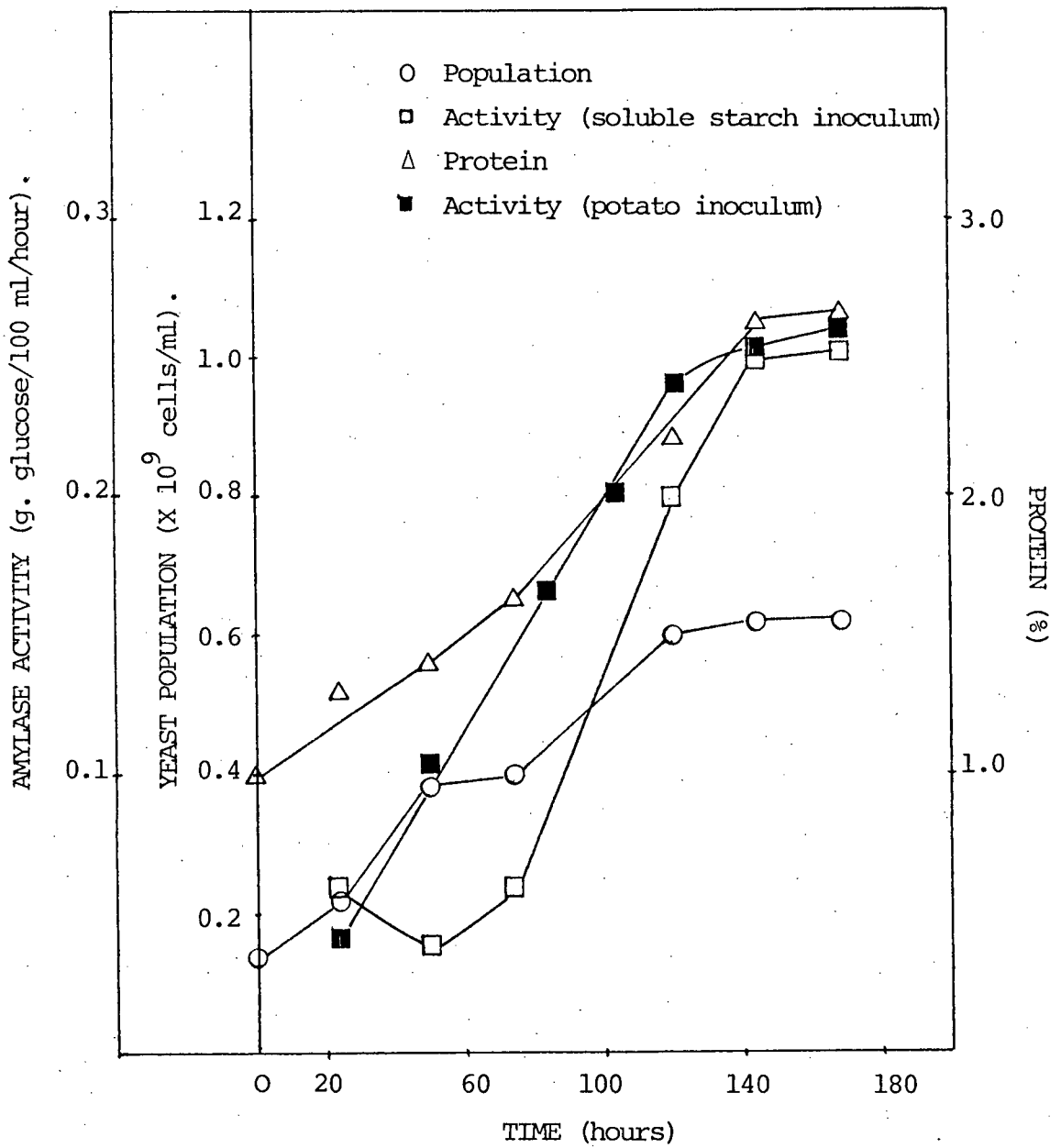

FIGURE 4. Progress of cultivation of Endomycopsis sp. on media containing 4.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

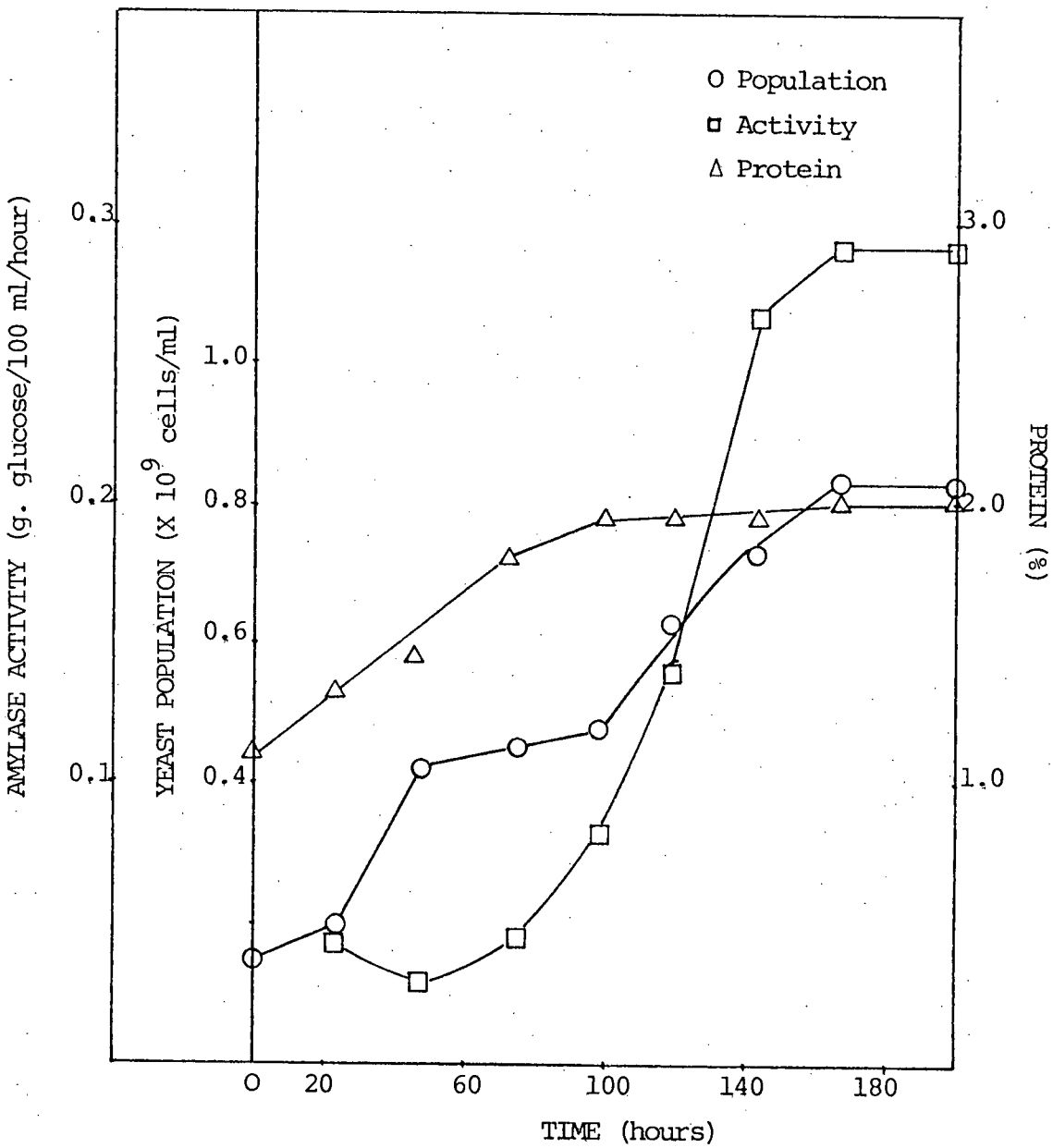

FIGURE 5. Progress of cultivation of Endomycopsis sp. on media containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

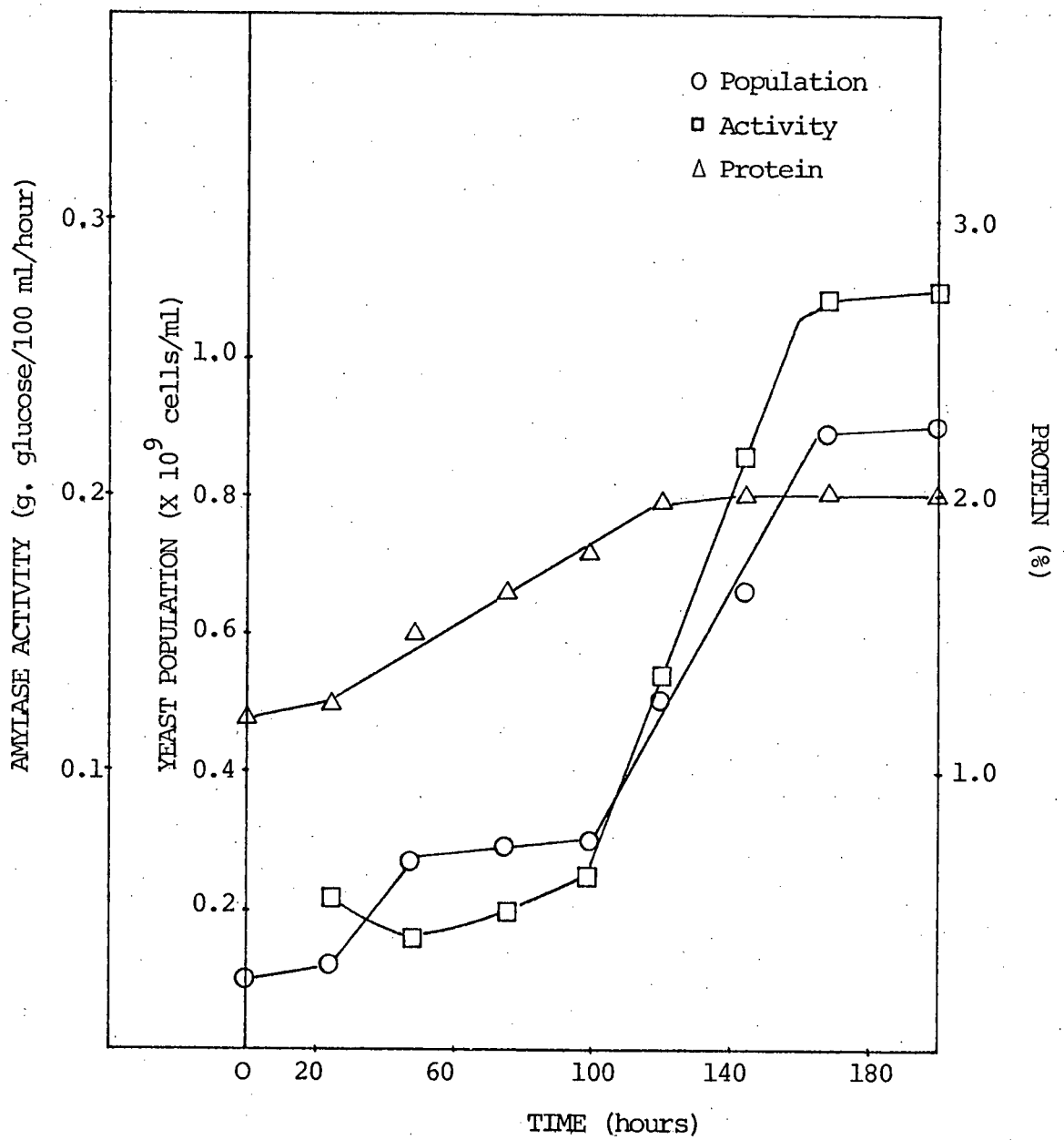

FIGURE 6. Progress of cultivation of Endomycopsis sp. on media containing 10.0% potato solids, 1.0% urea and 0.5% KH_2PO_4 (shaken flasks).

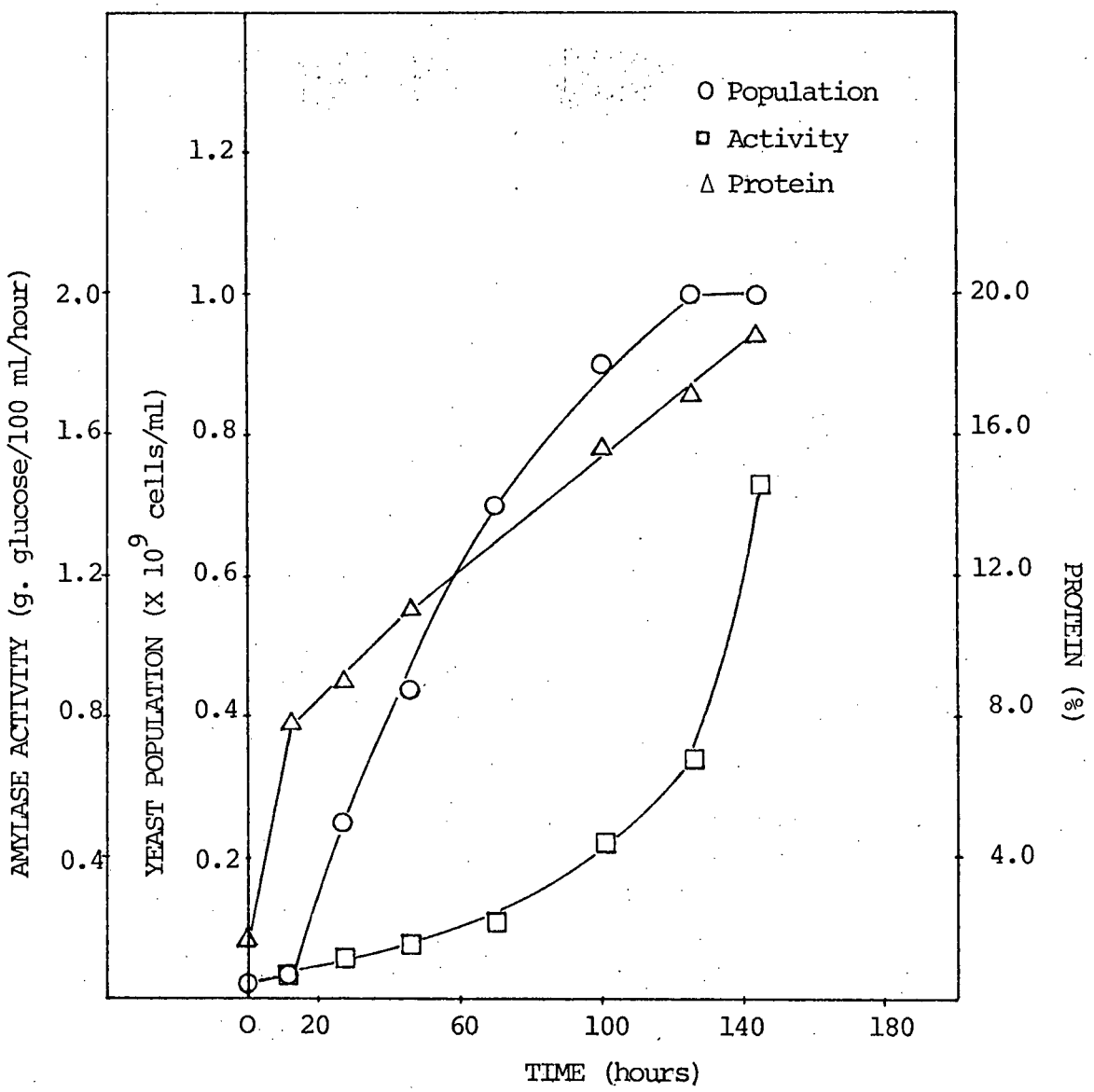

FIGURE 7. Progress of cultivation of Endomycopsis sp. on media containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (Vir Tis fermenter).

TABLE II. Amylolytic activity, and protein content after five days propagation, of Endomycopsis sp. on media containing 1.0% urea, 0.5% KH_2PO_4 and varying amounts of potato solids.

Potato Solids (%)	Amylase Activity (gm glucose/100 ml/hr)	Protein in Solids (%)
2.0	0.175	2.0
4.0	0.251	2.6
6.0	0.292	2.0
10.0	0.273	2.0

in a 12-liter fermenter. The time course of propagation of Endomycopsis sp. with Candida utilis (2:1) in 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 is presented in Figure 11.

Using various ratios of Saccharomyces cerevisiae to Endomycopsis sp. (1:2, 1:1, 2:1), as inocula, growth rates and protein levels obtained did not differ significantly from those observed in cultures of Candida utilis with Endomycopsis sp. However, amylase activities were considerably lower. Figures 12-14 show the results of these experiments. These observations are difficult to interpret in view of reports in the literature indicating the absence of appreciable proteolytic activity in Saccharomyces cerevisiae cultures, (Ahearn, 1968; Sylven, 1958).

A further attempt was made to assess the possibility of obtaining a protein-rich product with an enzymatically-active liquor from sequential propagations of Endomycopsis sp.

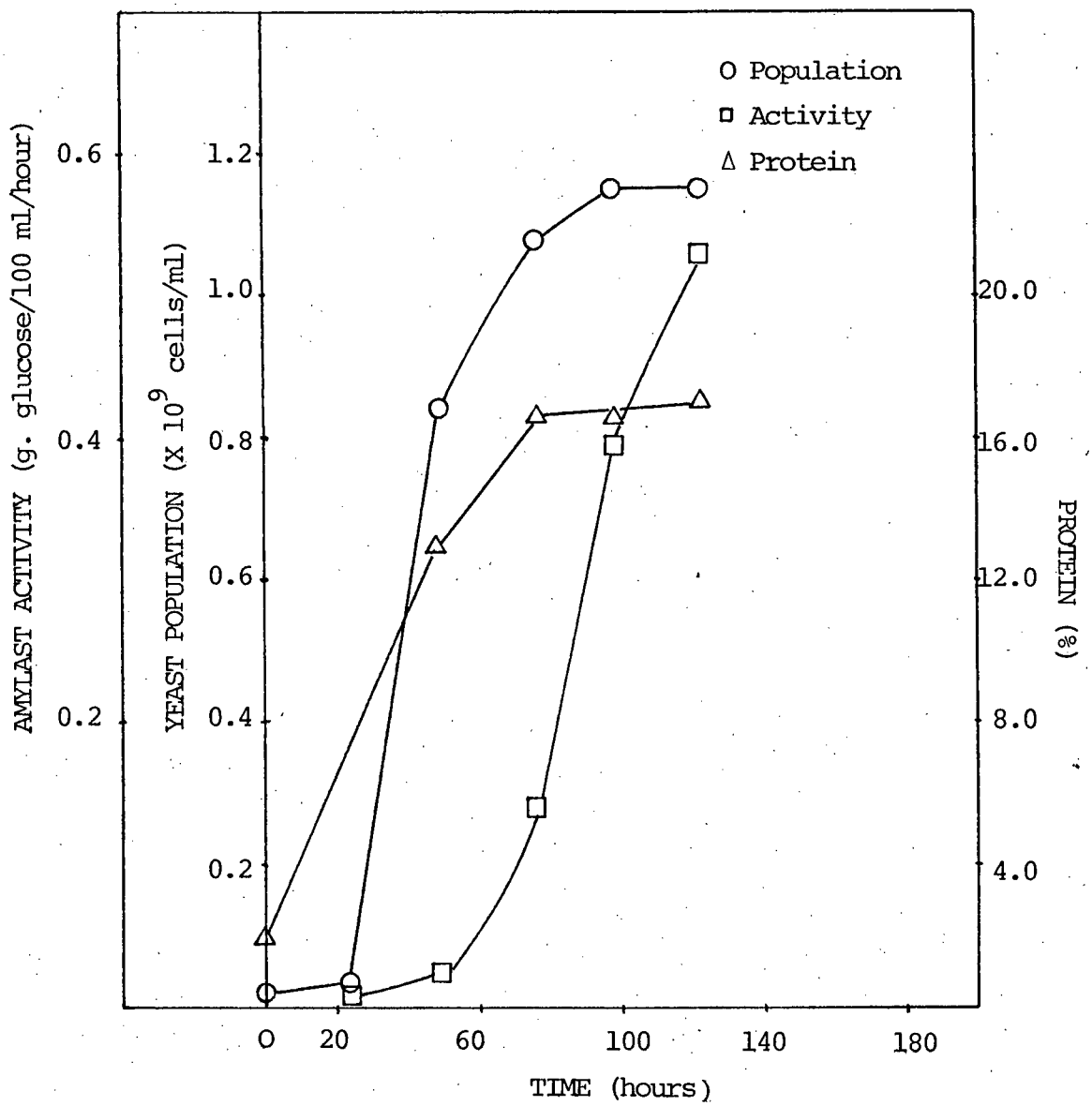

FIGURE 8. Progress of mixed cultivation of 2 Endomycopsis sp.: 1 Candida utilis on media containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

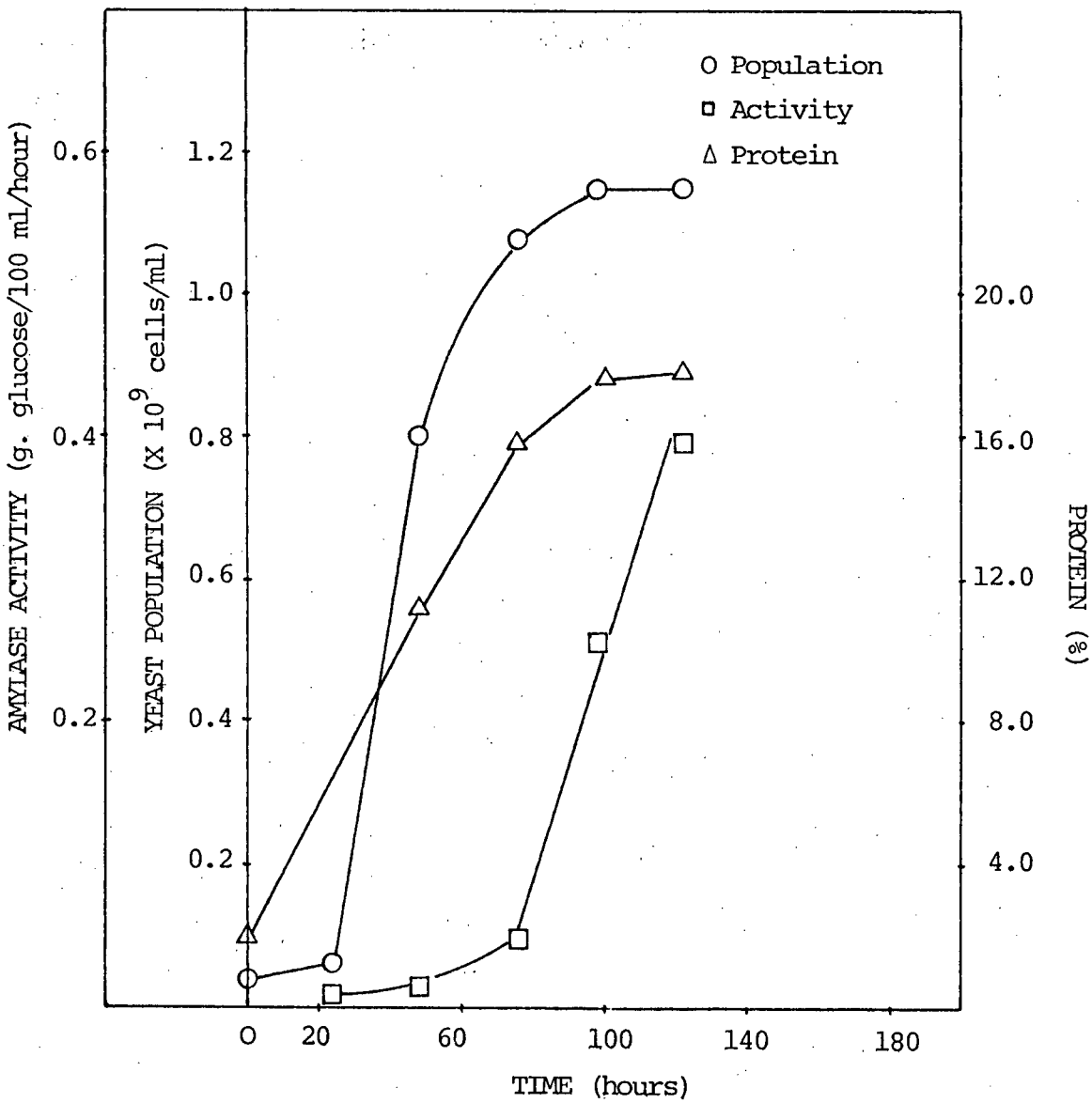

FIGURE 9. Progress of mixed cultivation of 1 Endomycopsis sp.: 1 Candida utilis on media containing 6.0% potato solids, 1.0% urea and 0.5% KH_2PO_4 (shaken flasks).

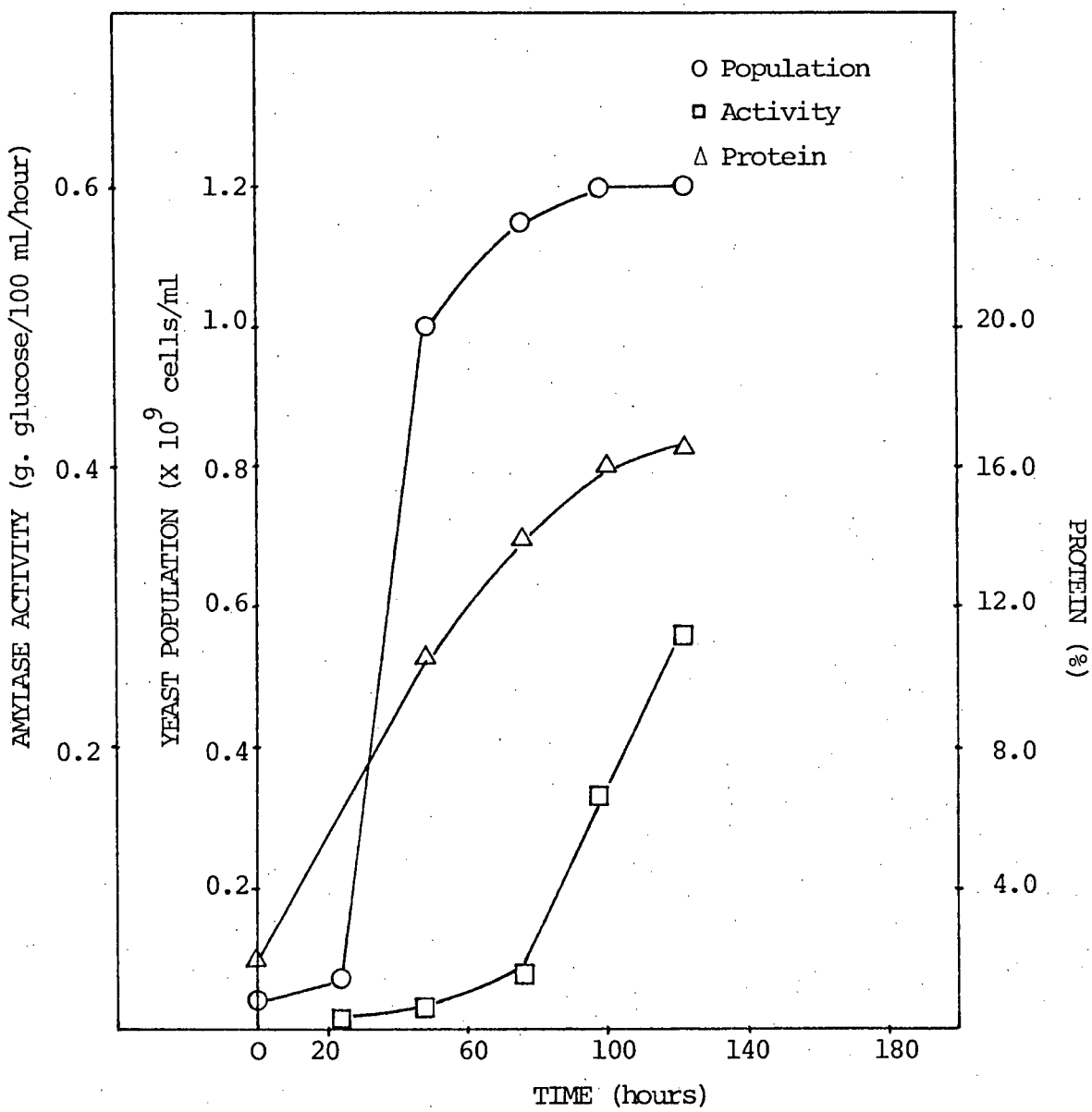

FIGURE 10. Progress of mixed cultivation of 1 Endomycopsis sp.: 2 Candida utilis on media containing 6.0% potato solids, 1.0% urea and 0.5% KH_2PO_4 (shaken flasks).

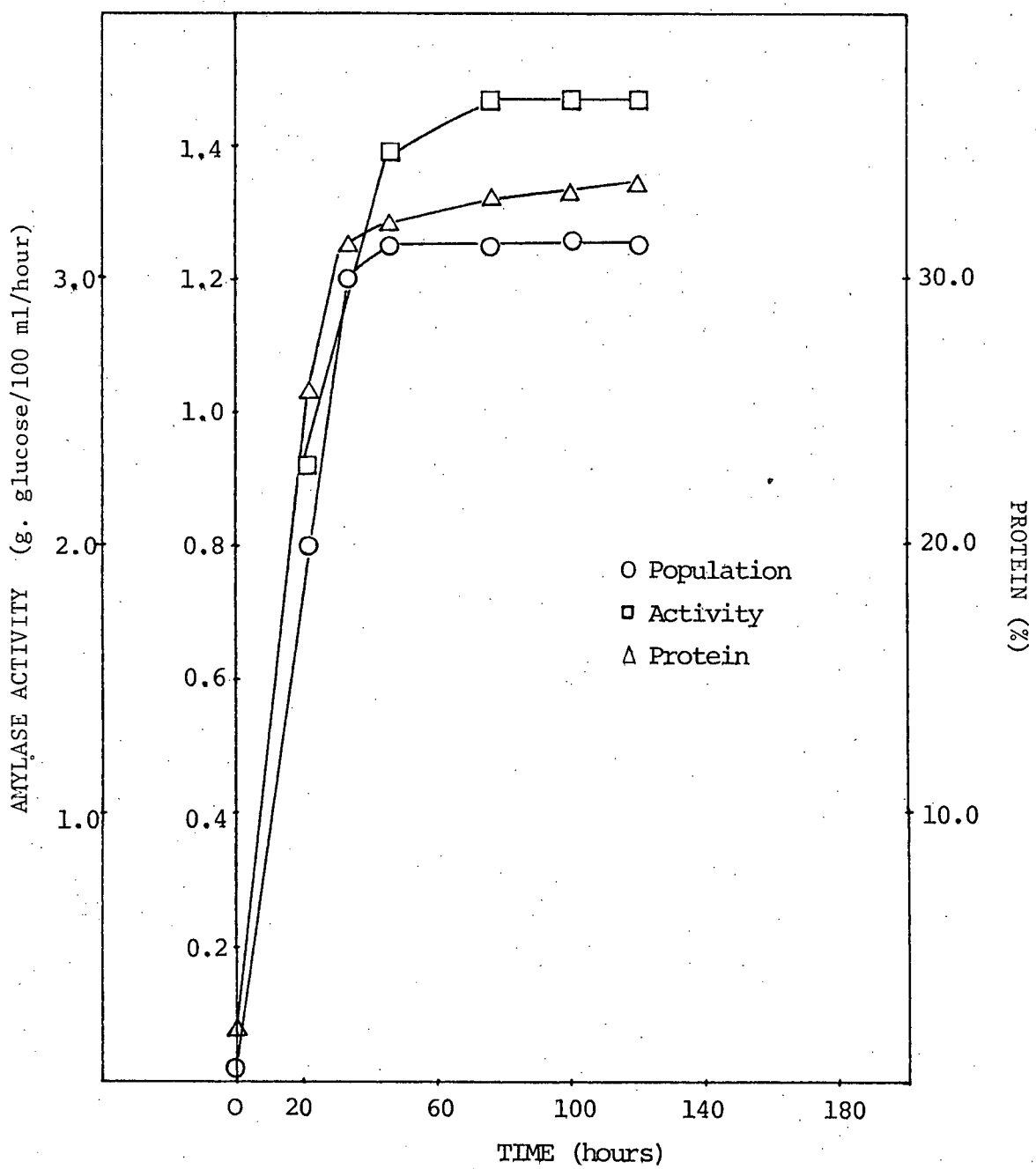

FIGURE 11. Progress of cultivation of 2 Endomycopsis sp.: 1 Candida utilis in media containing 6.0% potato solids, 1.0% urea, 0.5% KH_2PO_4 (Vir Tis fermenters).

TABLE III. Amylolytic activity and protein content after five days propagation of Candida utilis with Endomycopsis sp. on media containing 6.0% potato solids, 1.0% urea and 0.5% KH_2PO_4.

Candida:Endomycopsis Ratio	Amylase Activity (gm glucose/100 ml/hr)	Protein in Solids (%)
1:2	0.560	17.0
1:1	0.394	17.8
2:1	0.278	16.6

and Saccharomyces cerevisiae. This possibility was demonstrated by introducing the ancillary yeast after the Endomycopsis amylases had built up. Although no attempt was made to ascertain the best time for Saccharomyces cerevisiae inoculation, the results shown in Figure 15 indicated that the introduction of the ancillary yeast at the point of maximum Endomycopsis population, was accompanied by further increase in yield, amylase activity and protein content. However, a different pattern was observed when a six liter propagation was carried out. The introduction of Saccharmyces cerevisiae reduced the amylase activity considerably although it increased the cell density and the protein content slightly.

Comparison of Various Starchy Substrates

On the basis of yeast growth, amylase activity and protein yield, an attempt was made to compare with potato,

two starchy substrates, grown widely in the Philippines, namely purple yam and cassava tubers. The progress of mixed cultivation of two parts Endomycopsis to one part Candida utilis in a medium consisting of 6.0% purple yam, 1.0% urea, and 0.5% KH_2PO_4 is given in Figure 17. Whereas maximum cell density was reached in less than two days in a medium containing 6.0% potato solids, it took about three days in medium containing an equivalent concentration of the yam. The yam medium also gave lower protein yield and amylase activity.

It can be seen from Figure 18 that protein accumulation in mixed culture of Endomycopsis sp. and Candida utilis (2:1) on 6.0% cassava medium was comparable to that in 6.0% purple yam. However, amylase activity in the cassava medium was extremely low.

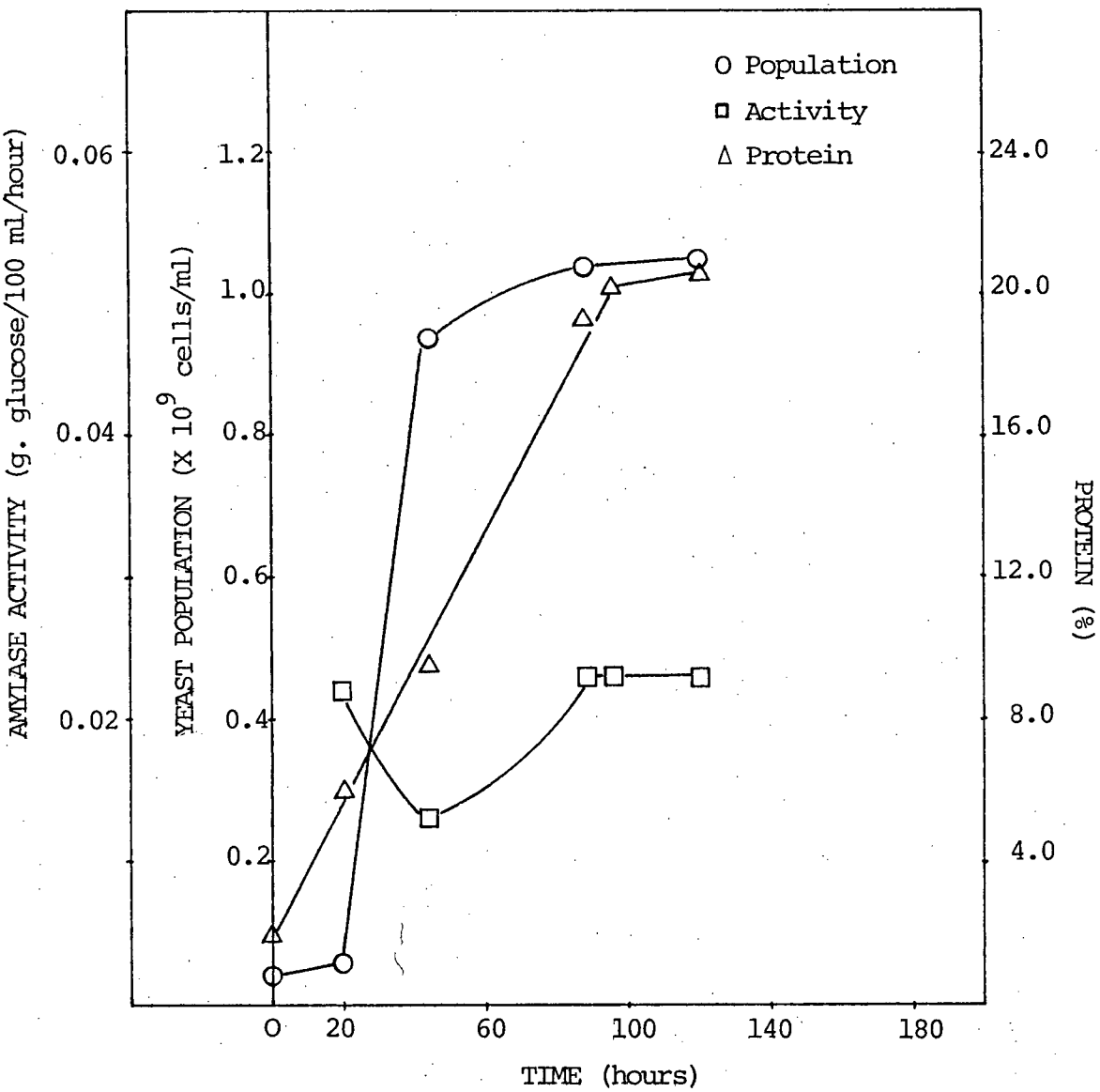

FIGURE 12. Progress of mixed cultivation of 2 Endomycopsis sp.: 1 Saccharomyces cerevisiae on media containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

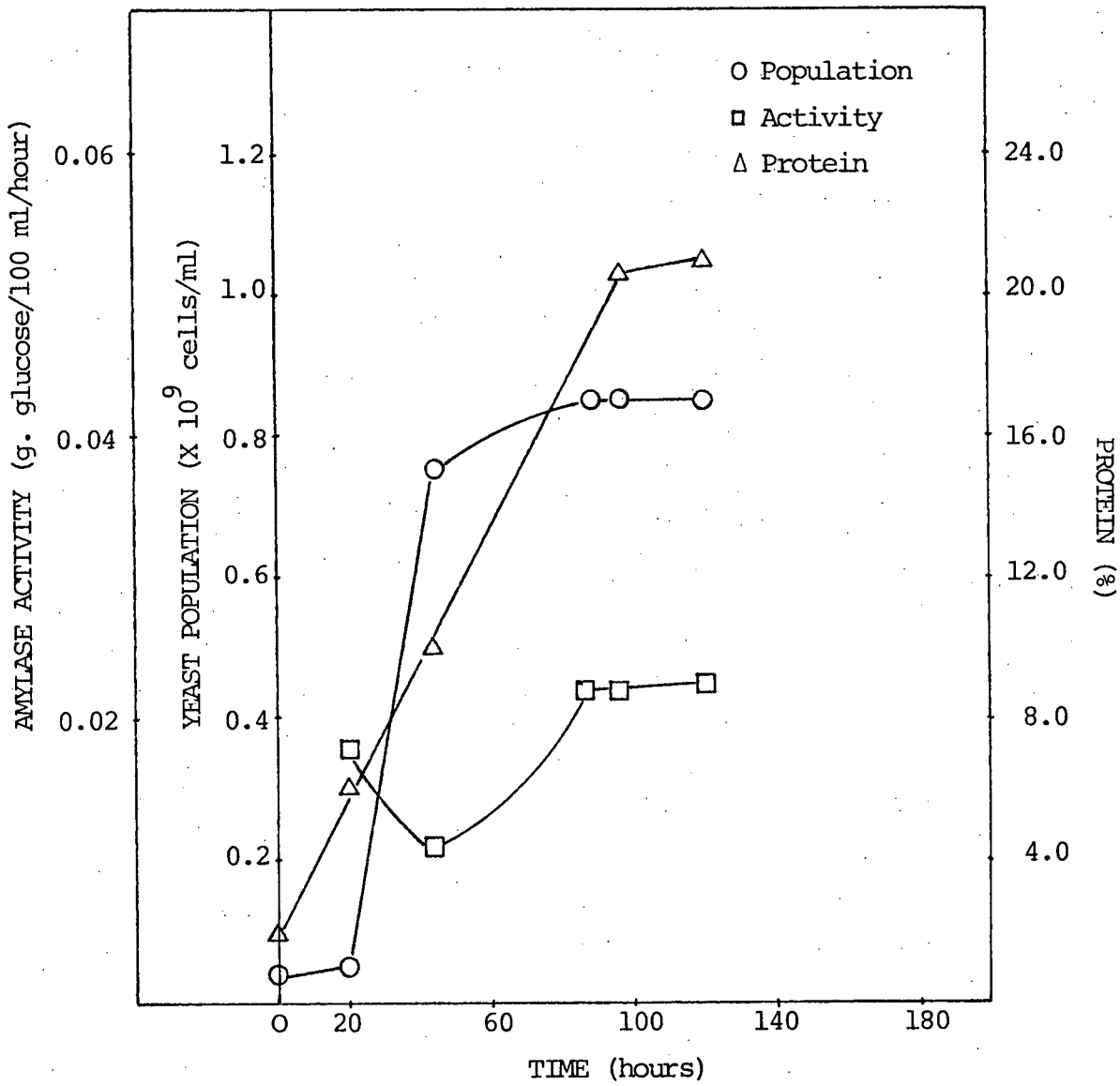

FIGURE 13. Progress of mixed cultivation of 1 Endomycopsis sp.: 1 Saccharomyces cerevisiae on media containing 6.0% potato solids, 1.0% urea, and 0.5% KH_2PO_4 (shaken flasks).

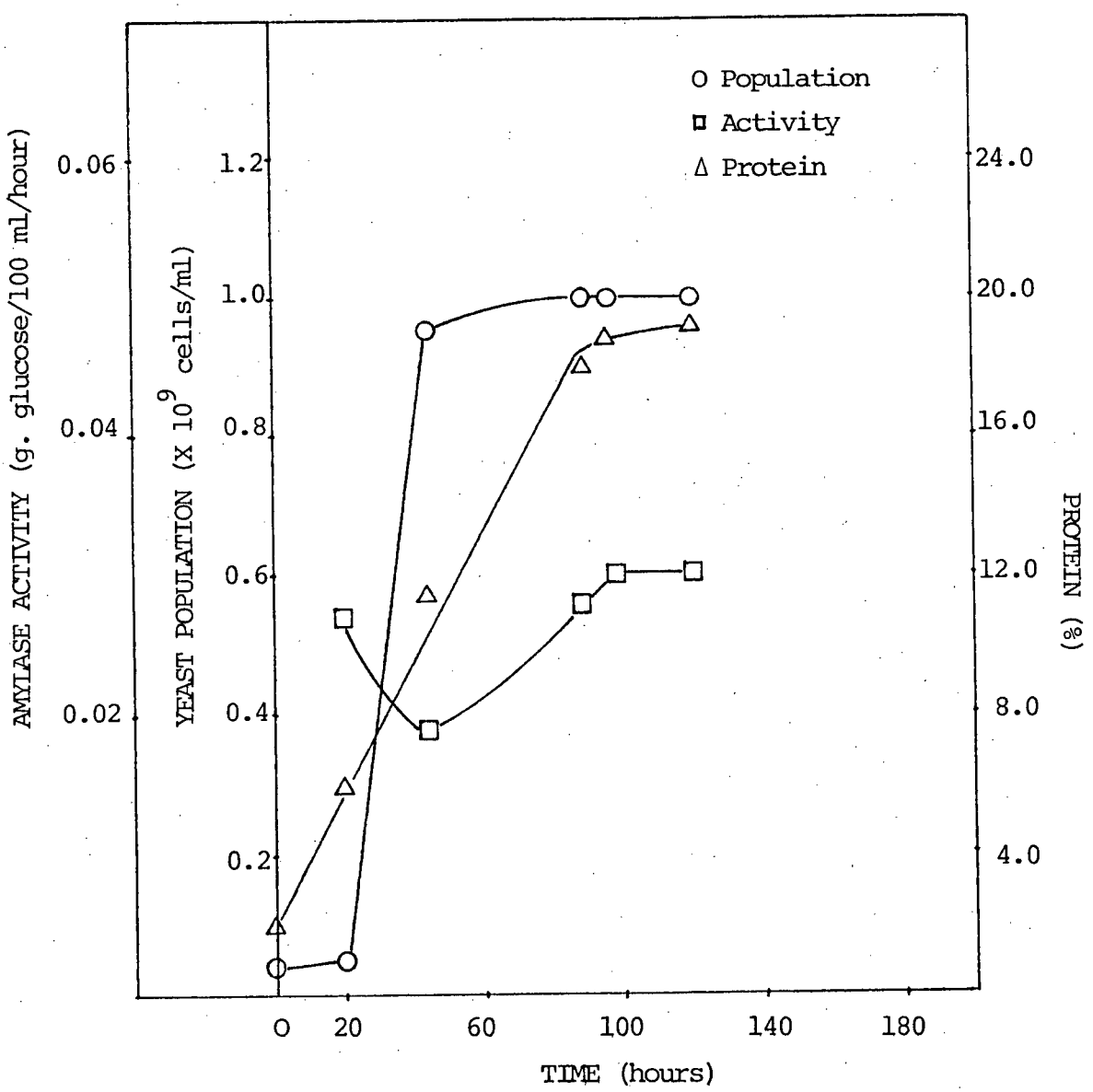

FIGURE 14. Progress of mixed cultivation of 1 Endomycopsis sp.: 2 Saccharomyces cerevisiae on media containing 6.0% potato solids, 1.0% urea, 0.5% KH_2PO_4 (shaken flasks).

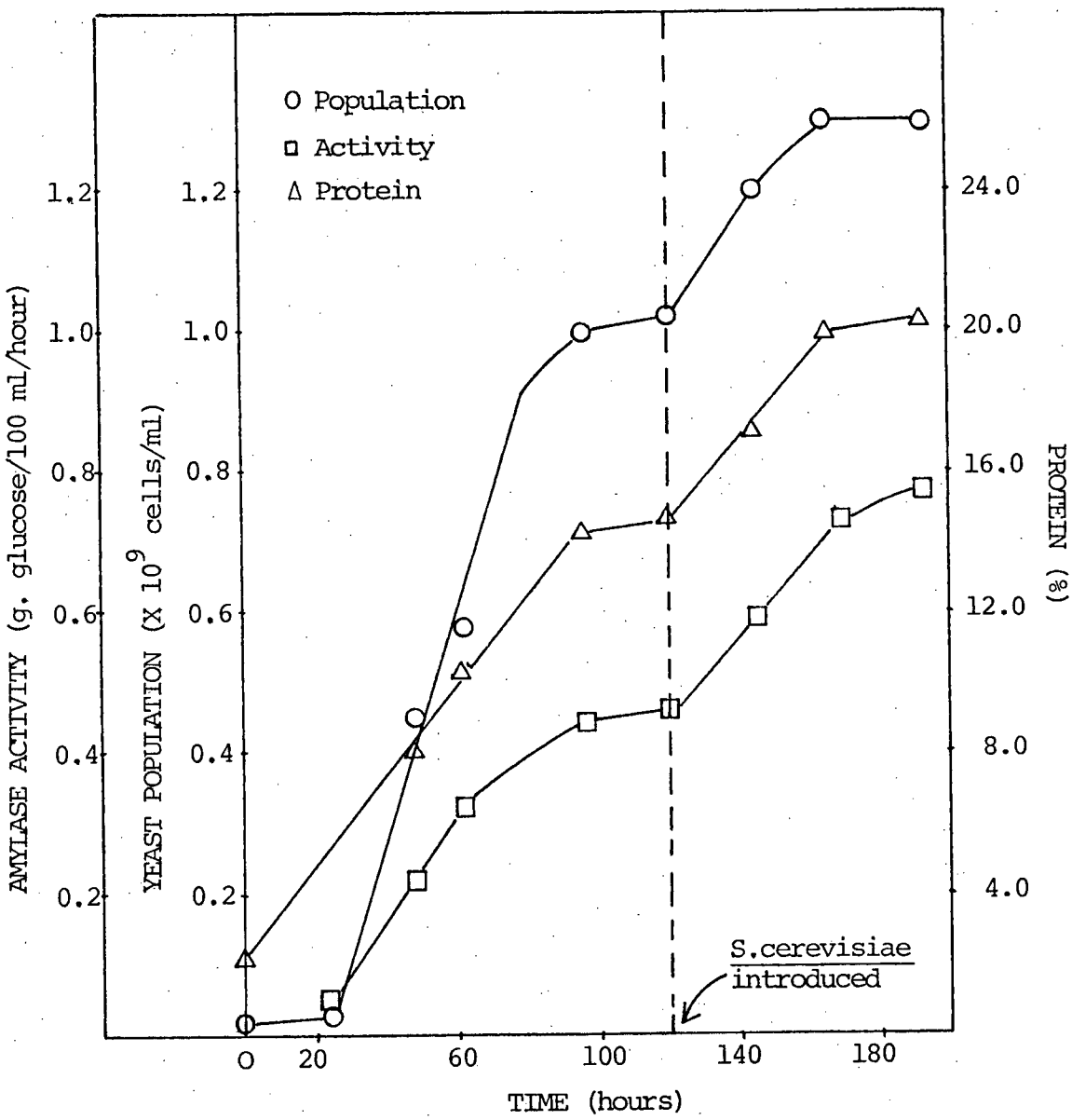

FIGURE 15. Progress of sequential cultivation of Endomycopsis sp. and Saccharomyces cerevisiae on media containing 6.0% potato solids, 1.0% urea, 0.5% KH_2PO_4 (shaken flasks).

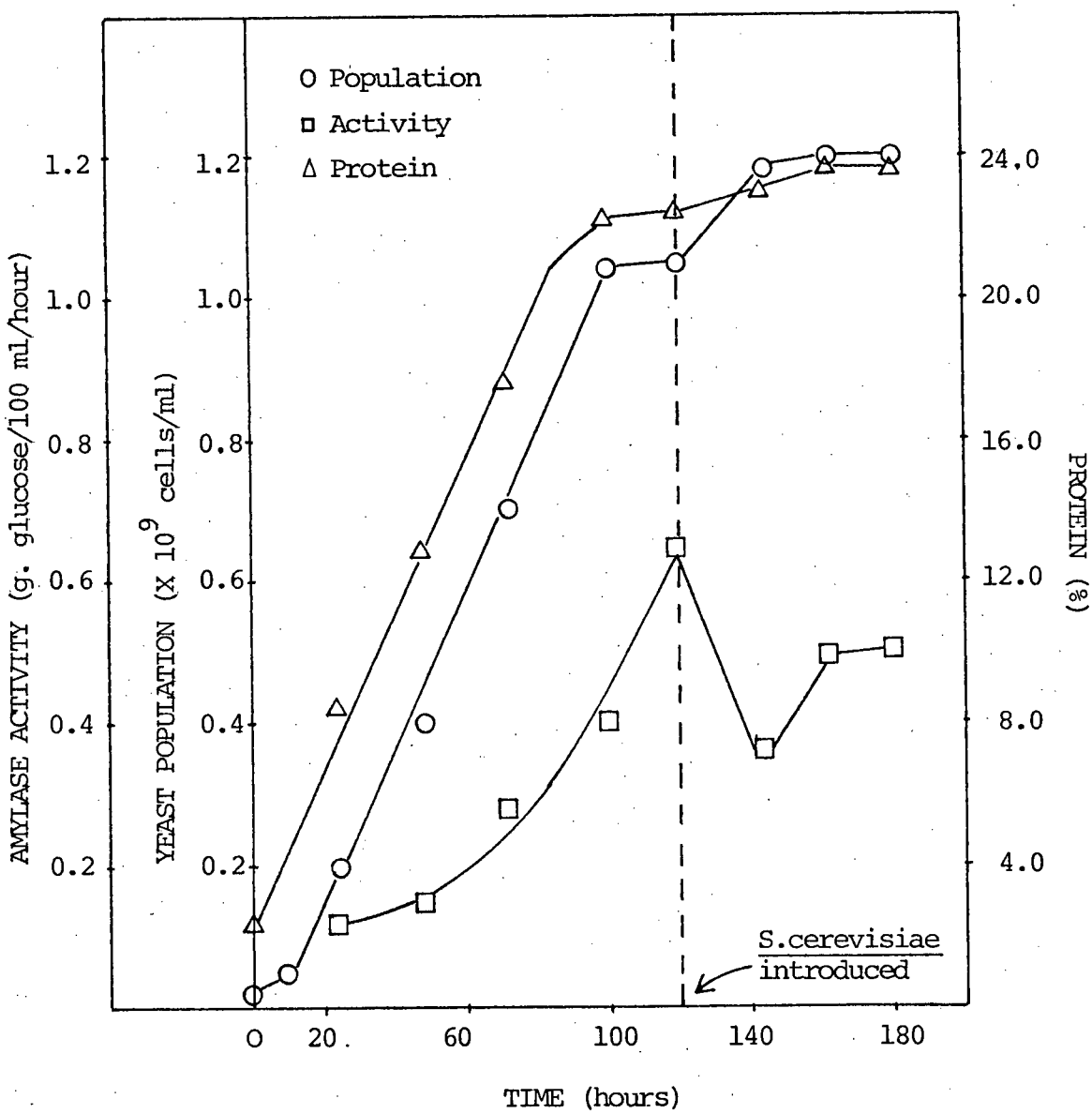

FIGURE 16. Progress of sequential cultivation of Endomycopsis sp. and Saccharomyces cerevisiae on media containing 6.0% potato solids, 1.0% urea, 0.5% KH_2PO_4 (Vir Tis fermenter).

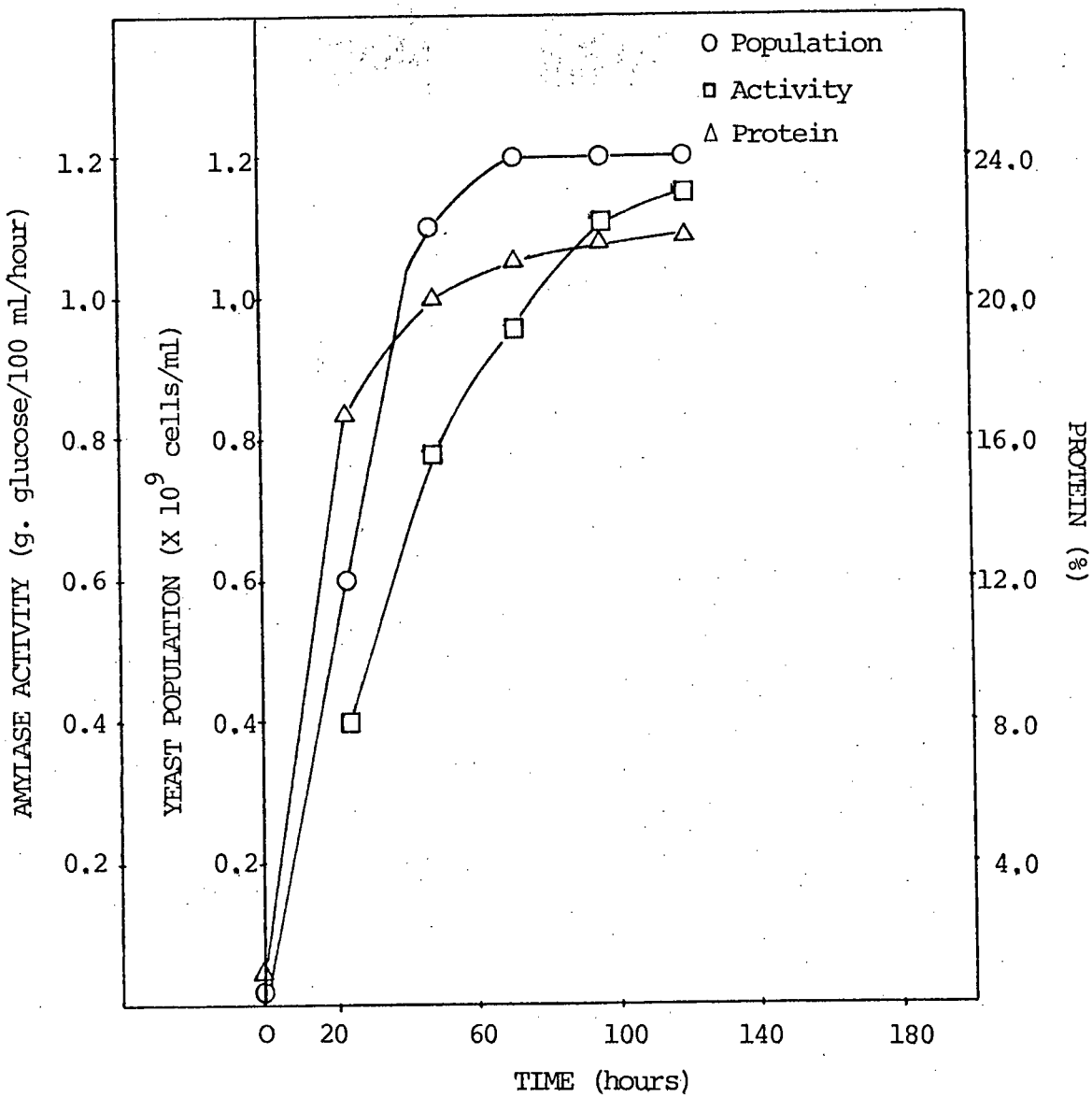

FIGURE 17. Progress of mixed cultivation of 2 Endomycopsis sp.: 1 Candida utilis on media containing 6.0% purple yam, 1.0% urea, 0.5% KH_2PO_4 (Vir Tis fermenter).

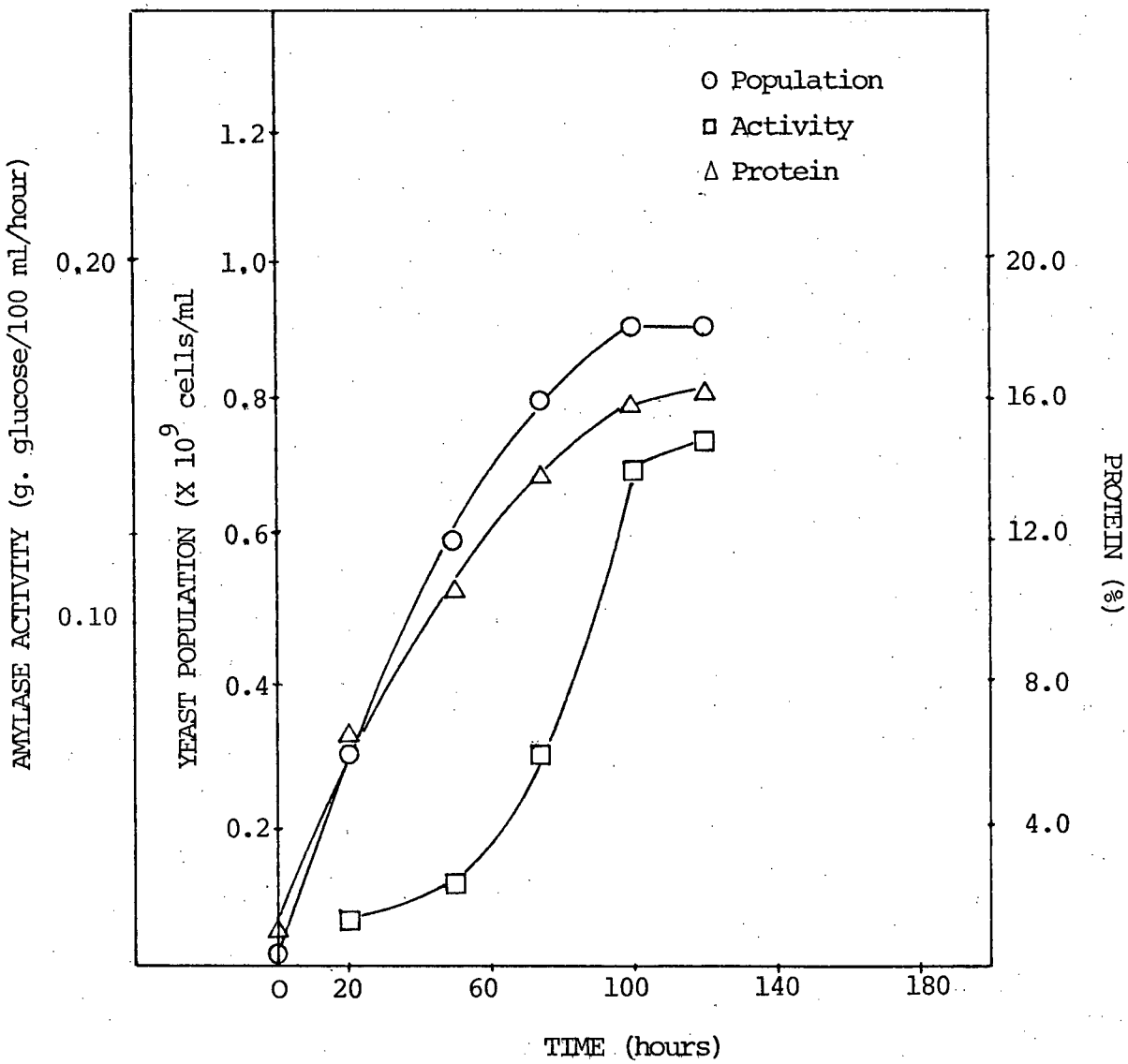

FIGURE 18. Progress of mixed cultivation of 2 Endomycopsis sp.: 1 Candida utilis on media containing 6.0% cassava, 1.0% urea, 0.5% KH_2PO_4 (Vir Tis fermenter).

DISCUSSION

The principle underlying the utilization of starchy substrates is shown diagrammatically in Figure 19. Starch serves as the carbon and energy source for the growth of two organisms. By means of the amylolytic activity of the Endomycopsis yeast, starch is degraded into lower saccharides, predominantly glucose, which in turn are assimilated by an ancillary organism. Though unable to utilize starch directly, the ancillary organism will utilize the sugars for the synthesis of cell substance and the production of vitamins, specific amino acids, or other compounds of nutritional or medical value. As Candida utilis and Saccharomyces cerevisiae have been known in terms of nutritional, organoleptic and pathological properties, these yeasts were employed as ancillary organisms for the production of yeast proteins and vitamins.

Preliminary experiments with the Endomycopsis yeast indicated that variations in the quantity of assimilable phosphate added to the starchy substrate (i.e. KH_2PO_4) did not significantly affect the propagation. This seems reasonable in view of the phosphorus content of potato which ranges from 6.83% to 27.14% P_2O_5 in potato ash (Talburt, 1967). The water soluble phosphorus compounds in potato could conceivably exist in assimilable form especially in acid media. Pasternak, (1951) has shown that the phosphorus in

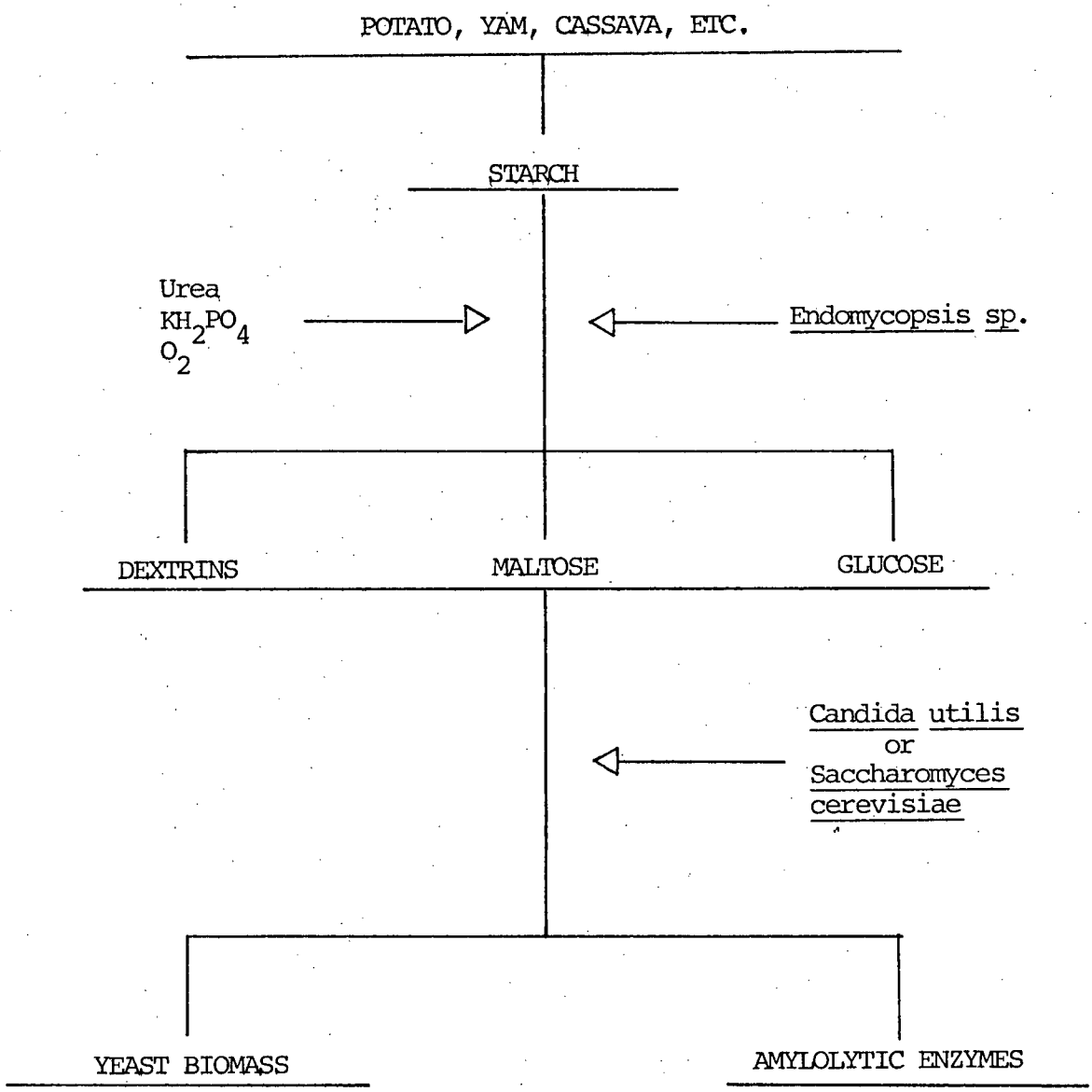

FIGURE 19. Diagram of the principle underlying the utilization of starchy substrates by yeast cultivation.

potato is present as orthophosphate esterified with the C_6-OH of a glucosyl residue of the amylopectin moiety of the potato starch.

The Endomycopsis propagations exhibited sensitivity to variations in the amount of urea incorporated in the starchy media. One percent urea was optimal; levels above 1.0% were inhibitory and could be due in part to the possible production of toxic substances or creation of hyperosmotic environments.

It is evident, from the data shown in Table III, that the yields of protein were very low for all potato concentrations used. These results could be traceable to low oxygen tension and high glucose concentration in the propagation media. Current views correlate oxygen and glucose concentrations to regulation of yeast respiratory structures during growth. Several reports which have been excellently reviewed by Moss, et al. (1969) indicate increased cytochrome concentrations in microorganisms grown in limited oxygen. "Glucose effect", i.e. the observation that high glucose levels bring about repression of certain enzymes, has been demonstrated with the yeast respiratory enzymes and mitochondria. It has been suggested (Moss, et al. 1969) that a substance which stems from glucose inhibits both protein and lipoprotein synthesis and that inhibition of lipoprotein synthesis leads to diminished activity of lipoprotein-bound

enzymes. It seems conceivable that both low oxygen tension and high glucose concentrations may have been responsible for the low yields of protein in this experiment. In fact, the shaken flasks developed strong alcoholic and mild ester-like odors by the second day of propagation. However, the results of propagation carried out in the Virtis fermenter in which higher oxygen tensions could be maintained, indicated increased protein production. However, it should be noted that the latter propagations were also subjected to more vigorous mechanical agitation.

The importance of intimate contact between micro-organisms and their substrates for metabolic activity is well established. Gaden (1952) has shown that mechanical agitation fragments mycelial organisms. Thus, at high agitation rates, fragmentation may occur, increasing the surface exposed to the media, whereas at low agitation rates, clumping may occur, decreasing the contact surface between the organisms and the substrates. And at extremely low agitation rates, autolysis may occur as cells settle to the bottom of the propagator. It is believed that Gaden's observations could be operative in this sytem as Endomycopsis yeasts are mycelial or pseudo-mycelial, (Keuhner, 1953; Lodder, 1952).

Enzymatic assays of the centrifuged culture fluid indicated that amylolytic activity of the Endomycopsis' enzyme system was related to its growth pattern. The growth curves for different concentrations of potato substrate,

shown in Figures 3-6, indicate two exponential phases separated by a phase of little or no growth. As the concentration of potato solids supplied in the media was increased from 2.0% to 10.0%, the magnitude of the second exponential phase increased. Rapid increases in amylase activity occurred during the second exponential phase. The dips in the corresponding amylase activity curves coincided with the first exponential growth phase and are thus suggestive of an adaptive period. Evidence for this is the absence of a dip in the activity curve when the inoculum was grown on potato slurry instead of soluble starch as was done previously. The activity curve showing the absence of adaptation is given in Figure 4.

It is believed that the complexity of the chemical composition of both the culture filtrate as well as that of the enzyme system itself adversely affected the accuracy of the enzymatic assays. Hence, the activities obtained must be regarded as only approximate. By carrying out analyses of highly purified extracts, such estimations could be rendered more meaningful. Nevetheless, the results of this experiment show that activity rose steeply as the yeast cells divided rapidly.

During mixed cultivations of Endomycopsis sp. and Candida utilis, glucose in the media did not accumulate as it was consumed by the Candida utilis for metabolic activity as soon as it was produced from starch breakdown by the

Endomycopsis sp. It is perhaps due to this product exhaustion that the symbiotic cultues exhibited amylase activities markedly higher than single cultures on media of similar composition (Figures 8-10). The activity of the culture filtrate was higher in media inoculated with a larger proportion of Endomycopsis sp., as shown in Table III. In the absence of "glucose effect", mixed cultures also gave higher protein yields. Growth of Candida utilis predominated and as Candida cells are known to have higher protein content than Endomycopsis cells, protein yields from mixed cultures were necessarily higher.

In agreement with these results, it was observed that under conditions of sufficient aeration and vigorous agitation, Vir Tis propagations of mixed cultures, consisting of two parts Endomycopsis to one part Candida, gave very high protein yields and highly active liquors, as shown in Figure 11. In contrast, in Vir Tis propagations of single cultures of Endomycopsis sp., the protein level was about 15% lower and amylase activity was about two units lower, as shown in Figure 7.

Mixed cultures of Endomycopsis sp. and Saccharomyces cerevisiae gave somewhat higher protein yields but surprisingly lower amylase activities in comparison to mixed cultures of Endomycopsis sp. and Candida utilis, as indicated in Figures 12-14. Among other factors such as rate

of enzyme synthesis by the organism and enzyme excretion from the cells, the concentration of enzymes in the culture will depend on the enzyme's stability in the medium. It is likely that the drop in pH of the media from 5.5 to 2.5 exerted an unfavorable influence on the stability of the amylases and consequently on the concentration of enzymes in the media. Tsuchiya, et al. (1950) indicated the importance of avoiding growth pH values below 4.5 during amylase production by Aspergillus niger.

The possibility that Saccharomyces cerevisiae might have considerable proteolytic activity was eliminated in view of the results of sequential propagations with Endomycopsis sp. In these experiments, Endomycopsis sp. was cultivated by itself and amylase activity was allowed to increase until the end of the exponential growth phase. At this point Saccharomyces cerevisiae was introduced into the culture. No decrease of amylase activity was observed. In fact, further increases in activity, cell density and protein content were obtained, as indicated in Figure 15. The pH of the culture changed from 5.0 to 6.4.

However, an entirely different pattern, shown in Figure 16, was observed when a parallel six-liter propagation was carried out. An extremely large volume of silicone antifoam was required due to persistent, excessive foaming. This could have inhibited cell growth and enzyme synthesis.

Clearly, the discrepancies in the two propagations require further study. It may be of interest to determine whether the reduction in growth and amylase activity could be related to oxygen tension, degree of agitation, etc.

Compared to potato substrates, purple yam (Dioscorea alata) and cassava (Manihot esculenta) gave low protein yields and amylase activities. These differences could be due to intrinsic properties of the tubers themselves. It is known that a substantial number of the members of the genus Dioscorea contain varying amounts of alkaloids, tannins, and saponins, (Coursey, 1966; Martin, 1970). Cassava cultivars contain varying amounts of toxic cyanogenic glucosides mainly linamarin. The superiority of potato as a substrate for production of amylolytic enzymes and yeast protein could also be associated with the presence of natural enzyme stimulators and growth factors and the absence of natural inhibitors. It is well known that the activity of some extracellular amylases is inhibited by specific inhibitors probably proteins or mucoproteins of undetermined composition. The α-amylase of Bacillus subtilis, for example, is inhibited by water-soluble inhibitors extracted from wheat endosperm, rye, and sorghum which also inhibit salivary and pancreatic amylases, (Davies, 1963). Trace metals are essential for growth, some of them being essential for the activity and stability of many extracellular enzymes.

Many cases of failure to synthesize active enzymes in growth factor-deficient media are known and the effect of growth factors on extracellular enzyme formation as distinct from effects of growth as a whole, has been demonstrated, (Davies, 1963).

CONCLUSIONS

The results of this study demonstrate the applicability of Endomycopsis sp. for utilization of potato waste and for protein enrichment of some root crops that are widely cultivated in the Philippines, such as yam and cassava. In experiments performed with purple yam, which per se contains only 2% protein, a yeast product was obtained containing about 22% protein, representing about tenfold protein enrichment. The other product consisted of a crude enzyme preparation of the amylolytic complex recovered from the culture after harvesting the cell crops. The amylase activity of the enzyme preparation was about 1.1 units.

The propagations were carried out in the presence of 1.0% urea and 0.5% KH_2PO_4, at pH 5.0 and 28°C, where synthesis and activity of the amylolytic enzyme complex were known to be greatest.

Mixed and dual propagations of Endomycopsis sp. with Candida utilis and Saccharomyces cerevisiae resulted in increases in amylase activities and protein yields. Variations were observed, depending on the starch substrate used, the concentration of urea added and apparently the amount of oxygen supplied.

As food industrial wastes hold considerable reserves of starch, they may serve as substrates for the simultaneous production of amylolytic enzymes and yeast protein.

By selective methods, a variety of cultures can be tried in combination with the starch-hydrolyzing yeast specie, in favor of some specific product, such as amino acids, vitamins flavor enhancers, and other compounds of nutritional or medical value.

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